emant ta zabal zazu

Universidad del País Vasco | Euskal Herriko Unibertsitatea

NAZIOARTEKO BIKAINTASUN CAMPUSA
CAMPUS DE EXCELENCIA INTERNACIONAL

Bioinspired three-dimensional multifunctional systems to enhance the therapeutic potential of mesenchymal stromal cells

Zelula estromal mesenkimalen eragin immunomodulatzai-lea sustatzeko bioinspiraturiko hiru dimentsiotako sistema funtzio anizdunak

NanoBioCel group

Laboratory of Pharmaceutics, School of Pharmacy

University of the Basque Country (UPV/EHU)

Ainhoa González Pujana

Vitoria-Gasteiz, 2019

(cc)2019 AINHOA GONZALEZ PUJANA (cc by-nc-sa 4.0)

Agradecimientos

Una gran etapa llega a su fin y con ello quisiera agradecer todo el apoyo que he recibido durante estos cuatro años de tesis doctoral.

Quisiera comenzar dando las gracias a todas las personas que conforman NanoBioCel. Gracias a mis directores de tesis, Rosa Hernández y Edorta Santos, por el tiempo dedicado a este proyecto y por todo lo que me habéis enseñado sobre esta profesión tan especial. Gracias Rosa por mostrarte siempre disponible para escuchar mis preocupaciones y buscarles una solución. Edorta, gracias por la ilusión que transmites, por emocionarte con cada resultado positivo y por aconsejarme ante cualquier problema. Al resto de profesores, por todos los ánimos y por estar siempre dispuestos a resolver cualquier duda. A Blanca y Andrés, por hacer la burocracia un poquito más fácil, y sobre todo, más divertida. Gracias a todos y cada uno de mis compañeros, por el millón de anécdotas que han alegrado el día a día durante estos años. Esto no habría sido lo mismo sin vosotros. En especial, gracias Tania por ayudarme tanto en mis comienzos. Y cómo no, por ser la organizadora oficial de eventos del laboratorio y amenizar los experimentos con música y café. Gracias Clau, Ilia y Aiala por todos los buenos momentos de risas y cafés que han sido tan necesarios a lo largo de este tiempo. Y por supuesto, gracias Mari, Sara e Itxaso, por haber sido un apoyo fundamental. Por dar los mejores consejos y por quitar hierro a las preocupaciones. Y por los grandes momentos de fantasía, que son innumerables.

Del mismo modo, quisiera dar las gracias a los demás grupos de investigación con los que he podido colaborar a lo largo de esta tesis. Por un lado, al Cluster de Microfluídica del grupo BIOMICs de la UPV/EHU por haberme dado la oportunidad de aprender sobre un campo diferente al mío. Por otro lado, al MooneyLab por hacer posible una experiencia increíble durante mi estancia de 6 meses en la universidad de Harvard y el Wyss Institute. Thank you so much Professor David Mooney for giving me the opportunity to be part of your research group during my stay. I am truly grateful for your warm welcome and your invaluable advice. Thanks to all the Mooneylab members, it was great to learn so much from you. En especial, gracias Irene, por enseñarme tanto en el laboratorio y ayudarme siempre que lo he necesitado. Pero sobre todo, gracias por ser una amiga. Por tu acogida, los planes improvisados y tantísimos Tatte que hicieron aún más especiales mis meses en Boston. Y como no, infinitas gracias al espectacular grupo de amigos con el que viví esta experiencia americana. Cualquier cosa que diga se queda corta para explicar lo muchísimo que me divertí con vosotros.

Por otro lado, no podría estar más agradecida por el resto de personas que me he ido topando en este camino. En, gracias por ser tan especial, por cuidarme tanto y por hacerme reír hasta que se me cae la lágrima. Porque sí, recordaré estos años tal y como queríamos hacerlo, gracias a todos los grandes momentos que hemos vivido. Caba, Sergio, la vida de doctoranda no habría sido igual sin la sal que le ponéis.

Gracias a todos los que sin terminar de entender qué es una célula madre o una microcápsula me habéis acompañado durante estos años. Tap, Eider, gracias por hacerme sentir siempre arropada, por vuestras respuestas inmediatas, y simplemente, por ser las mejores amigas que se pueden tener. Ane, Maitane, creo que no hubiese decidido seguir en la universidad si no fuera por los grandes años que pasamos juntas durante la carrera. Gracias por tantísimos momentos de risas y diversión, pero también por vuestros ánimos y por seguir estando ahí para lo que sea aunque pase el tiempo.

Gracias a mi familia por su apoyo incondicional. Isa, Lucía, Sara, Ibai, Juancar, Óscar, Nico, Bego, Antonia, osaba, todos, gracias por el interés que mostráis por mi trabajo y por ayudarme a desconectar cuando más lo he necesitado. Vuestra capacidad para hacerme reír hasta que no puedo más me ha hecho centrarme y poner en balanza lo realmente importante. Gracias Mentxu y Ernesto, por mostraros siempre atentos y disponibles para ayudar en lo que sea, incluso en el diseño gráfico de este trabajo.

Finalmente, gracias a los verdaderos artífices de que haya podido llegar hasta aquí. Ama, Aita, Mikel, infinitas gracias por creer siempre en mí. Por entender mis inquietudes y alentarme a seguirlas. Por ayudarme tanto en todo, sin vosotros no lo habría conseguido. Sergio, gracias por haberme acompañado en todas las decisiones que me han conducido hasta aquí. Por vivir mis alegrías como si fueran tuyas y por ser mi sostén en los peores momentos. Gracias porque contigo estos años han sido mucho más fáciles y mucho más bonitos.

Gracias a todos, de corazón.

ACKNOWLEDGEMENTS TO THE FINANCIAL SUPPORT

Ainhoa González Pujana gratefully acknowledges the predoctoral grant provided by the Basque Government. This doctoral thesis has been funded by the Spanish Ministry of Economy and Competitiveness (SAF2017-82292-R). Additionally, this project has been partially supported by the Basque Government (Consolidated Groups IT907-16).

ACKNOWLEDGEMENTS TO THE EDITORIALS

Authors thank the editorials for granting permission to reuse the published articles in this thesis. The published versions can be accessed at the following links:

Gonzalez-Pujana et al. Sens. Actuator B-Chem. 299, 126954 (2019)
https://doi.org/10.1016/j.snb.2019.126954

Gonzalez-Pujana et al. Drug Deliv. 24, 1654-1666 (2017)
https://doi.org/10.1080/10717544.2017.1391894

Gonzalez-Pujana et al. J. Drug Deliv. Sci. Technol. 42, 49-62 (2017)
https://doi.org/10.1016/j.jddst.2017.03.028

Gonzalez-Pujana et al. Alginates and Their Biomedical Applications, Springer Nature Series in Biomaterials Science and Engineering, Vol 11, Ch 3, 67-100 (2018).
https://doi.org/10.1007/978-981-10-6910-9_3

The following chapters have already been submitted to international journals:

Mesenchymal stromal cell based therapies for the treatment of immune disorders: recent milestones and future challenges (Introduction part 1) has been submitted to *Expert Opinion On Drug Delivery* (Manuscript ID EODD-2019-0203).

Multifunctional biomimetic hydrogel systems to boost the immunomodulatory potential of mesenchymal stromal cells (Chapter 3) has been submitted to *Biomaterials* (Manuscript ID jbmt50283).

The manuscript *Three-dimensional encapsulation and inflammatory licensing alter mesenchymal stromal cell reference gene expression in real time RT-qPCR* (Chapter 4) is ready for submission but will be sent to the target journal once Chapter 3 is accepted.

Success is not final,

failure is not fatal,

it is the courage to continue that counts.

Glossary

2D: two-dimensional

3D: three-dimensional

AD: Alzheimer's disease

ADM: acellular dermal matrix

aECM: artificial extracellular matrix

Alg-Nb: norborene modified very low viscosity sodium alginate

Alg-Tz: tetrazine modified very low viscosity sodium alginate

ALS: amyotrophic lateral sclerosis

AMD: age macular degeneration

APA: alginate-poly-L-lysine-alginate

APCs: antigen presenting cells

ASCs: adipose-derived mesenchymal stromal cells

BBB: blood brain barrier

BDNF: brain-derived neurotrophic factor

BHK: baby hamster kidney

BK: Bestkeeper algorithm

BMP: bone morphogenetic protein

BMSCs: bone marrow mesenchymal stromal cells

BrdU: bromodeoxyuridine

BSA: bovine serum albumin

CCK-8: cell counting kit-8

CNS: central nervous system

CPC: choroidal plexus cell

CT: computed tomography

CV: coefficient of variance

D1-MSCs-EPO: erythropoietin producing D1 mesenchymal stromal cells

DAMP: danger-associated molecular pattern

DAO: dot array occupancy

ddH2O: double-distilled water

DME: diabetic macular edema

DMEM: Dulbecco's modified Eagle medium

DMSO: dimethyl sulfoxide

DPBS: Dulbecco's Phosphate-Buffered Saline

DS: degree of substitution

DSS: dextran sodium sulfate

DXM: dexamethasone

E: polymerase chain reaction efficiency (E)

EAE: experimental autoimmune encephalomyelitis

ECM: extracellular matrix

EDC: 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimidehydrochloride

EDTA: ethylenediaminetetraacetic acid

ELISA: enzyme-linked immunosorbent assay

EPO: erythropoietin

EVs: extracellular vesicles

FBS: fetal bovine serum

FC: fold change

FITC: fluorescein isothiocyanate

FN: fibronectin

GAL-1: galectin-1

GAL-9: galectin-9

GC: galactosylated chitosan

GCV: ganciclovir

GDL: glucono-delta-lactone acid

GDNF: glial cell-line derived neurotrophic factor

GFP: green fluorescent protein

GLP-1: glucagon-like peptide 1

GMSC: gingival mesenchymal stromal cells

GN: geNorm algorithm

GvHD: graft-versus-host disease

HBSS: Hank's balanced salt solution

hDF: human dermal fibroblast

HEPES: N-2-hydroxyethylpiperazine- N-2-ethane sulfonic acid

HGF: hepatocyte growth factor

HLA-DR: human Leukocyte Antigen - DR isotype

HMGB1: high mobility group box 1 protein

hMSCa: adipose-derived human mesenchymal stromal cells

hMSCs: human mesenchymal stromal cells

HSCT: hematopoietic stem cell transplantation

IBD: inflammatory bowel disease

ICH: intracerebral hemorrhage

IDO: indoleamine 2,3-dioxygenase

iECM: immunomodulatory extracellular matrix

IFN-γ: interferon γ

IKVAV: isoleucine-lysine-valine-alanine-valine

IL-1: interleukin-1

IL-10: interleukin-10

IL-1R: interleukin-1 receptor

IL-1Ra: interleukin-1 receptor antagonist

IL-1α: interleukin-1α

IL-1β: interleukin-1β

IL-2: interleukin-2

iPSC: induced pluripotent stem cells

ISCT: Internal Society for Cellular Therapy

LCT: Living Cell Technologies

MFI: mean fluorescence intensity

MIQUE: minimum information for publication of quantitative real-time PCR experiments

MMP: metalloproteinase

MRI: magnetic resonance imaging

MS: multiple sclerosis

MSCs: mesenchymal stromal cells

MWCO: molecular weight cut-off

Nb: norborene or 5-(aminomethyl)bicyclo[2.2.1]hept-2-ene

NCDs: noncommunicable diseases

NF: NormFinder algorithm

NGF: nerve growth factor

NHS: N-hydroxysuccinimide

NK cells: natural killer cells

NOD/SCID mice: non-obese diabetic/severe combined immunodeficient mice

NRT: non-reverse transcription control

NTC: non-template control

PAMPS: pathogen-associated molecular patterns

PBS: phosphate buffered saline

PD: Parkinson's disease

PDMS: polydimethylsiloxane

PEG: polyethylene glycol

PEX: hemopexin

pFAK: phosphorylated focal adhesion kinase

PFO: pericapsular fibrotic overgrowth

PGE2: prostaglandin E2

PLGA: poly(lactic-co-glycolic) acid

PLL: poly-L-lysine

PLO: poly-L-ornithine

PRR: pattern recognition receptor

PTX: pentoxyfilline

RGD: arginine-glycine-aspartic acid

RIN: RNA integrity number

ROS: reactive oxygen species

RT-qPCR: real-time, reverse transcription, quantitative polymerase chain reaction

SD: standard deviation

SPIO: superparamagnetic iron oxide

STZ: streptozotocin

T1D: type 1 diabetes

TAMRA: carboxytetramethylrhodamine

TCP: tissue culture plates

TGF-β: transforming growth factor beta

TLR: toll-like receptor

TMTD: triazole-thiomorpholine dioxide

TNBS: trinitrobenzene sulfonic acid

TNF-α: tumor necrosis factor α

Treg: regulatory T cells

Tz: tetrazine or (4-(1,2,4,5-Tetrazin-3-yl)phenyl)methanamine - trifluoroacetic acid

UCMSC: umbilical cord mesenchymal stromal cell

UPLVG: ultra-pure low-viscosity high guluronic acid alginate

UP-VLVG: ultra-pure very low viscosity sodium alginate

VEGF: vascular endothelial growth factor

YIGSR: tyrosine-isoleucine-glycine-serine-arginine

α-**MEM**: minimum essential medium α

Index

ENGLISH VERSION **25**

Introduction **27**

Mesenchymal stromal cell based therapies for the treatment of immune disorders: recent milestones and future challenges 29

Cell microencapsulation technology: current vision of its therapeutic potential through the administration routes 55

Hypothesis & Objectives **91**

Experimental section **97**

The role of osmolarity adjusting agents in the regulation of encapsulated cell behavior to provide a safer and more predictable delivery of therapeutics 99

Extracellular matrix protein microarray-based biosensor with single cell resolution: integrin profiling and characterization of cell-biomaterial interactions 131

Multifunctional biomimetic hydrogel systems to boost the immunomodulatory potential of mesenchymal stromal cells 159

Three-dimensional encapsulation and inflammatory licensing alter mesenchymal stromal cell reference gene expression in real-time RT-qPCR 187

Discussion **221**

Conclusions **251**

EUSKARAZKO BERTSIOA **255**

Sarrera **257**

Zelula estromal mesenkimaletan oinarritutako terapiak immunitate gaitzen tratamenduan: aurrerapen berriak eta etorkizuneko erronkak 259

Zelula-mikrokapsularatze teknologia: potentzial terapeutikoaren egungo ikuspegia inplantazio bidearen arabera 281

Hipotesia eta helburuak **311**

Lan esperimentala **317**

Osmolaritate-doitzaileen eragina mikrokapsularatutako zelulen portaeran: farmakoen askapen seguruagoen eta aurresangarriagoen bila 319

Matrize extrazelularraren proteinetan oinarritutako mikroarray-biosentsorea: integrinen profilaketa eta zelula-biomaterial interakzioen karakterizazioa 323

Zelula estromal mesenkimalen ekintza immunomodulatzailea sustatzeko bioinspiraturiko hidrogel funtzio anizdunak 327

Hiru dimentsiotako kultiboak eta hanturazko baldintzapenak zelula estromal mesenkimalen erreferentzia geneen espresioa aldatzen dute RT-qPCRan 343

Eztabaida **347**

Ondorioak **377**

APPENDIX / IRUZKINA **381**

ENGLISH VERSION

Introduction

Introduction

Mesenchymal stromal cell based therapies for the treatment of immune disorders: recent milestones and future challenges

This chapter has been sumbitted to *Expert Opinion On Drug Delivery*
(Manuscript ID EODD-2019-0203)

Mesenchymal stromal cell based therapies for the treatment of immune disorders: recent milestones and future challenges

Ainhoa Gonzalez-Pujana[a,b], Manoli Igartua[a,b], Edorta Santos-Vizcaino[a,b]*, Rosa Maria Hernandez[a,b,*]

[a] NanoBioCel Group, Laboratory of Pharmaceutics, School of Pharmacy, University of the Basque Country (UPV/EHU), Spain.
[b] Biomedical Research Networking Centre in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Spain.
*Corresponding authors: Edorta Santos-Vizcaino, Rosa Maria Hernandez.

ABSTRACT

Introduction: Mesenchymal stromal cells (MSCs) present unique immunomodulatory properties that make them promising candidates for the treatment of inflammatory and immune disorders. The MSC-mediated immunomodulation is a complex combination of mechanisms, in which the secretome plays a fundamental role. The plethora of bioactive molecules MSCs produce, such as indoleamine 2,3-dioxygenase (IDO) or prostaglandin E2 (PGE2), efficiently regulates innate and adaptive immunity. As a result, MSCs have been extensively employed in preclinical studies, leading to the conduction of multiple clinical trials.

Areas covered: This review summarizes the effects of some of the key biomolecules in the MSC secretome and the advances in preclinical studies exploring the treatment of disorders including graft-versus-host disease (GvHD) or inflammatory bowel disease (IBD). Further, a relevant insight on the state of the field is provided by discussing late-stage clinical trials and the first MSC-based therapies that recently obtained regulatory approval.

Expert opinion: Extensive research evidences the potential of MSC-based immunomodulatory therapies. However, to establish the bases for clinical translation, the future of study lies in the standardization of protocols and in the development of strategies that boost the therapeutic potential of MSCs.

---

1. Introduction

Mesenchymal stromal cells (MSCs) are non-hematopoietic precursor cells present in all mammalian supportive stromal tissues. Among them, bone marrow, umbilical cord and adipose tissue are the most used cell sources [1]. When clinical interest in MSCs importantly increased, approximately three decades ago [2], the defining characteristics of these cells were inconsistent among researchers. This led the Internal Society for Cellular Therapy (ISCT) to propose in 2006 a minimal phenotypic and functional criteria for human MSC definition, based on three main aspects [2]. First, MSCs must be plastic-adherent and proliferate forming populations of fibroblast-like cells. Second, the MSC phenotype is characterized by the expression of CD73, CD90 and CD105 surface antigens and the lack of expression of the leukocyte marker CD45, hematopoietic progenitor and endothelial cell marker CD34, monocyte/macrophage markers CD11b or CD14, B cells markers CD19 or CD79α, and human leukocyte antigen HLA-DR [2]. Finally, MSCs must present the ability to differentiate into cells of mesodermal lineages: adipocytes, chondrocytes and osteoblasts [2]. Despite self-renewing progenitors have been identified among human bone marrow MSCs (BMMSCs), it remains unclear whether this characteristic is present in MSCs from different origins. Consequently, the ISCT recommends designing MSCs as *multipotent mesenchymal stromal cells*, and reserving the term *mesenchymal stem cells* for cells that meet such criteria [3].

The capacity of MSCs for trilineage mesenchymal differentiation confers them regenerative and healing properties. Hence, their application in tissue diseases has been extensively studied, especially for bone and cartilage repair [4-6]. Indeed, as reported by Ferreira *et al.*, up to 2015 most of the studies using MSCs were focused on treating musculoskeletal disorders [7]. However, besides this regenerative ability, MSCs also present immunomodulatory properties. Several studies have reported MSCs to regulate both innate and adaptive immunity by modulating basic functions such as activation, maturation, proliferation or cytolytic activity of multiple immune cells [8] including T, B and natural killer (NK) lymphocytes [9-11], dendritic cells [12], monocytes and macrophages [13,14].

To perform this immunomodulatory function, MSCs switch from a pro-inflammatory to an anti-inflammatory phenotype in response to their local microenvironment. This switch in polarization is mediated through toll-like receptors (TLR) expressed on the cell surface, which

recognize cytokines present in the surrounding media [15]. During the onset of inflammation, the low presence of inflammatory mediators, such as tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ), results in a pro-inflammatory MSC phenotype. Consequently, MSCs produce factors that promote the activation of the immune response and the migration of immune cells to the site of injury [16]. However, in the late phase of inflammation, the high levels of inflammatory cytokines polarize MSCs towards an immunosuppressive phenotype. MSCs then suppress the immune response, protecting tissues from oxidative injury and promoting repair and homeostasis [16,17].

MSCs present a hypoimmunogenic phenotype since they do not express HLA-DR molecules (MHC class II surface receptors) unless stimulated by molecules such as IFN-γ [2], and lack of expression of co-stimulatory molecules including CD40, CD80, CD83,CD86 and CD154 [18] (Fig. 1A). Therefore, their immunosuppressive potential together with their low immunogenicity renders MSCs promising candidates for the treatment of inflammatory and immune disorders including graft versus host disease (GvHD), inflammatory bowel disease (IBD) or multiple sclerosis (MS) [19-21].

This review seeks to appraise the recent advances in the use of MSCs in anti-inflammatory and immunomodulatory therapies. First, the MSC secretome and its effects on the immune system will be revised, attending to some of the most relevant soluble factors MSCs produce. Subsequently, recent preclinical studies and late-stage clinical trials will be summarized, providing an insight on the current state-of-the-art in the field. Finally, the expert opinion section will address the future challenges to translate MSC-based therapies into the clinical practice, including the standardization of protocols and the development of strategies that boost the therapeutic potential of MSCs.

2. MSCs secretome

MSC-mediated immunomodulation is the result of a complex combination of different mechanisms. On the one hand, direct cell-cell interactions induce important immunosuppressive effects by means of different surface ligands present in MSCs (Fig. 1B). Among them, PD-L1 ligands interact with PD-1 inhibitory receptors on T cells, resulting in the suppression of T cell

proliferation and cytotoxic degranulation [22]. Similarly, MSCs express FAS ligand (FASL), which binds to FAS present in T cells inducing apoptosis [23]. Additionally, such association induces the secretion of monocyte chemotactic protein 1 (MCP-1) by MSCs, recruiting T cells for FASL-mediated apoptosis [24]. Further, apoptotic T cells induce the secretion of transforming growth factor β (TGF-β) by macrophages, which in turn upregulates $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T cells [24].

However, the paracrine effects of bioactive factors released by MSCs are considered to be the major responsible for their therapeutic activity [7,25] (Fig. 1C). The MSC-derived secretome includes a plethora of bioactive molecules, including interleukins, metabolic enzymes, growth factors and chemokines. Moreover, MSCs are able to transfer bioactive molecules by the release of extracellular vesicles (EVs) [20]. MSC-derived EVs have been demonstrated to exert their function directly on a wide variety of immune cells involved in innate and adaptive immune responses, including T and B cells, monocytes and dendritic cells [20].

One of the main effectors in MSC-mediated immunosuppression is indoleamine 2,3-dioxygenase (IDO), a metabolic enzyme that is secreted by MSCs upon inflammatory conditions [26]. IDO catalyzes the initial step in tryptophan metabolism via the kynurenine degradation pathway [27]. As a result, cells that express IDO exert two important effects on their microenvironment: (1) tryptophan depletion and (2) the production of a series of toxic kynurenine metabolites [10,28]. Tryptophan starvation results in T cell cycle arrest and anergy [29]. Moreover, kynurenine metabolites inhibit T cell proliferation by exerting a cytotoxic action on effector T cells [10] and favor the differentiation of Tregs [28]. The toxic effect of the kynurenine metabolites acts mainly on T cells, but has been reported to also affect B and NK cells [10]. Regarding the latter, the overall IDO immunosuppressive function plays an important role in MSC-induced inhibition of NK cells [9]. Moreover, IDO enhances the differentiation of monocytes into interleukin-10 (IL-10) producing $CD206^{+}$ M2 macrophages, which in turn, promote T cell suppression [13].

Prostaglandin-E2 (PGE2) is an anti-inflammatory factor [30] synthesized by cyclooxygenases COX1, COX2 and prostaglandin synthetase from arachidonic acid [31]. It is constitutively released by MSCs, but its secretion is dramatically enhanced upon exposure to inflammatory

cytokines such as IFN-γ, TNF-α [32] or interleukin-1β (IL-1β) [33]. PGE2 has been reported to inhibit T cell proliferation [33] and to promote Tregs generation [34], as well as to inhibit NK proliferation, cytotoxicity and cytokine production [9,35]. Further, PGE2 promotes M2 macrophage reprograming, increasing their IL-10 production [36]. Overall, the inhibition of inflammatory responses together with the promotion of Tregs renders PGE2 a key factor in MSC-mediated immunomodulation.

MSCs constitutively express hepatocyte growth factor (HGF), a pleiotropic factor that enhances cell growth, migration and angiogenesis and presents important anti-fibrotic effects [37,38]. Moreover, HGF also presents potent immunomodulatory effects. Regarding T cells, HGF has been demonstrated to suppress their proliferation [39], down-regulate the expression of activation surface markers, induce IL-10 producing Tregs and decrease IL-17 producing T

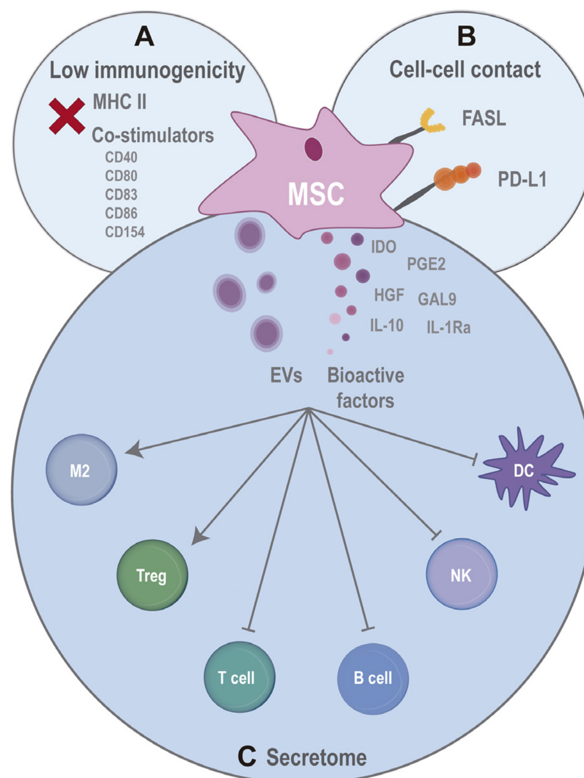


Fig.1 Immunomodulatory properties of MSCs. (A) MSCs present low immunogenicity and mediate their immunosuppressive effects by **(B)** ligand-receptor cell-cell interactions and **(C)** the secretion of soluble bioactive factors or extracellular vesicles (EVs). MSCs: mesenchymal stromal cells.

cells [12]. HGF is also capable of inhibiting dendritic cell functions [12]. Interestingly, cell-cell contact has been reported important for MSCs to produce HGF, as demonstrated when comparing MSCs and mononuclear cells in contact dependent or independent conditions [40].

Galectins are soluble proteins that when secreted by cells, bind different ligands interfering in many physiological processes such as inflammation or immune responses [41,42]. Multiple of the 15 galectin subtypes contribute to MSC-mediated immunomodulation. Galectin-1 (Gal-1) is strongly expressed by MSCs and has been reported to mediate immunoregulatory effects by suppressing T cell proliferation [43]. Further, Gal1 enhances the generation of tolerogeneic dendritic cells [44]. In contrast to Gal1, which is constitutively expressed by MSCs, galectin 9 (Gal-9) is importantly induced by inflammatory stimuli. When released by MSCs, Gal-9 demonstrated anti-proliferative effects on T [45] and B cells [46]. Further, the binding of Gal-9 to its receptor, TIM-3, on activated lymphocytes leads to their apoptosis [47].

IL-10 is a cytokine with potent anti-inflammatory properties, which has been shown to suppress T cell proliferation [48] and to contribute to stabilize the Treg phenotype [49]. Dendritic cells are also important targets of action of IL-10, by inhibiting expression of surface molecules and inflammatory molecules related to T cell activation [50]. IL-10 expression by MSCs remains controversial, since in the literature, some publications maintain that MSCs have not been demonstrated to produce the cytokine [51,52], while some others claim the capacity of MSCs to release IL-10 [17,53]. As previously discussed by other authors [54], it is logical to support the hypothesis that MSCs release IL-10 only under certain local conditions, such as the presence of inflammatory cytokines, including TNF-α or IFN-γ. Despite this controversy, the role of MSCs is irrefutable in the induction of IL-10 production by other cells such as T and B cells or macrophages [12,36,55,56].

Interleukin-1 receptor antagonist (IL-1Ra) is a naturally occurring cytokine that acts as a competitive inhibitor of interleukin-1 (IL-1). IL1Ra binds to IL-1 receptors and does not induce any intracellular response. Consequently, it blocks IL-1 binding, avoiding its pro-inflammatory signaling [57]. As a result, IL1Ra limits the production and secretion of chemokines that attract neutrophils, macrophages, and lymphocytes [58]. Moreover, it has been demonstrated that IL1Ra produced by MSCs controls the polarization of macrophages towards a M2

phenotype and inhibits B cell differentiation [59], as well as Th17 polarization [60]. Recently, a well-characterized subpopulation of IL-1Ra expressing MSCs was reported to block the production and activity of IL-1α and TNF-α [61].

3. Preclinical studies with MSCs in immune and inflammatory diseases

Considering the potent immunomodulatory effects of MSCs, they hold great promise for the treatment of multiple disorders. Since preclinical studies with MSCs are a pre-requisite to establish the principles of human clinical trials, MSC-based therapies are extensively being explored in animal models of prevalent immune-mediated diseases.

3.1 Graft-versus-host-disease (GvHD)

Allogeneic hematopoietic stem cell transplantation (HSCT) is a potentially curative treatment for multiple high-risk hematological malignancies. The major complication of HSCT is GvHD, which occurs when donor T cells respond to genetically defined proteins on host cells, being the most relevant the human leucocyte antigens (HLAs). This results in inflammatory responses and immune system disorders that can be life-threatening [62,63]. High incidence rates and the lack of standard therapeutic strategies urge the development of new therapeutic alternatives. In this context, MSC-based therapies are promising candidates. Humanized mice models have been widely employed to study the effects of MSCs in GvHD, since they simulate the human immunobiology more closely than conventional murine models [64].

Despite preclinical studies support the potential of MSCs in the management of GvHD, some conditions have been reported to limit the efficacy of the therapy [65,66]. In this regard, Tisato *et al.* reported that multiple doses of umbilical cord-derived MSCs (UCMSCs) were useful as prophylaxis to prevent GvHD development, but not effective when administered at the onset of the disease [65]. Posterior studies reported that effectivity was dependent upon the timing of MSCs therapy, showing positive outcomes only after delayed administration [66]. The positive effects included reduction of liver and gut pathology and increased mice survival, which were attributed to direct inhibition of $CD4^+$ T cell proliferation and diminished levels of inflammatory cytokines such as TNF-α in serum [66]. However, other authors have

published conflicting data, suggesting that MSCs did not succeed in preventing GvHD [67-69] or delaying mortality [69]. Disparities between studies are probably due to heterogeneity in protocols including the disease stage, MSC source and dosage, cell administration or the host's susceptibility to the treatment. In response to this issue, Zanotti *et al*. proposed the

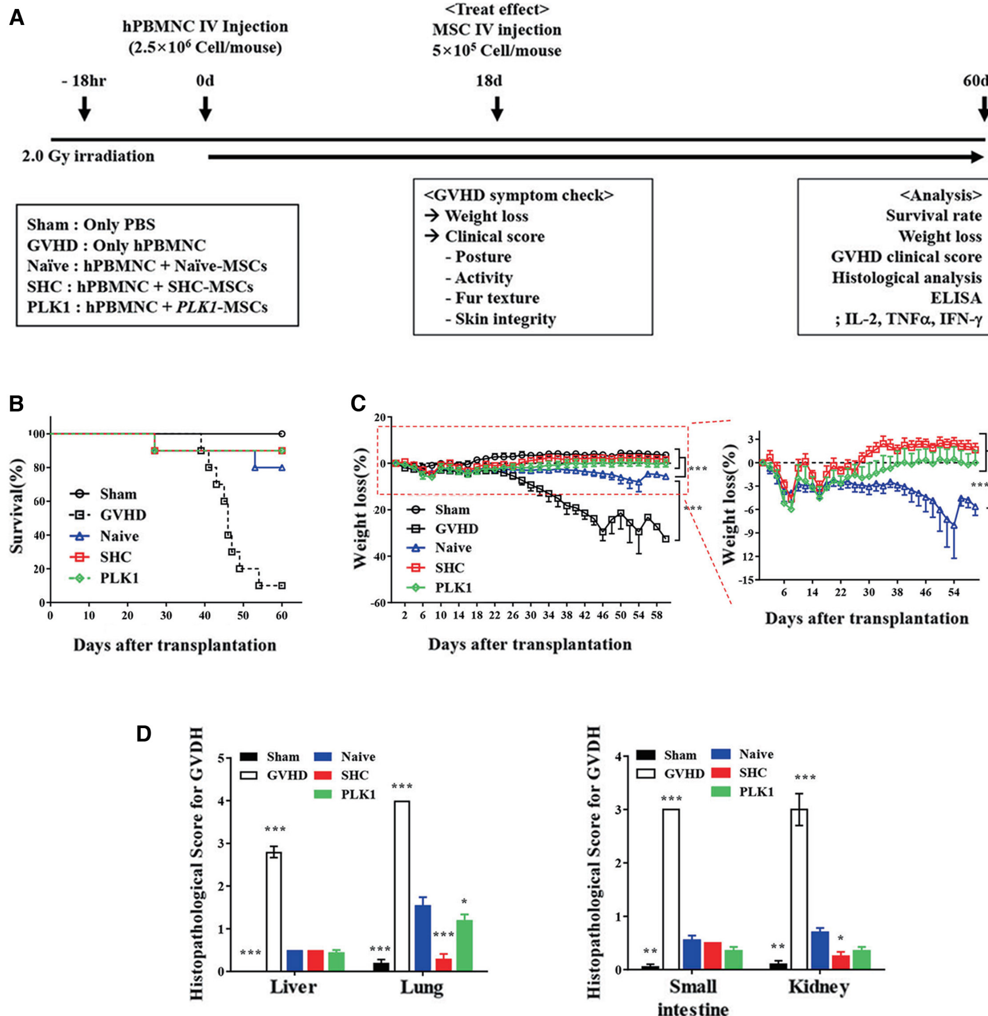


Fig. 2. MSCs primed with Hypoxia and Calcium ions (SHC-MSCs) and PLK1-overexpressing MSCs demonstrated an increased efficacy for treating GvHD. (A) Experimental scheme of MSC administration in a humanized GvHD mouse model. Mice were injected with 2.5 × 10^6 human PBMNCs followed by 5 × 10^5 naive MSCs (Naive group), SHC-MSCs (SHC group), or PLK1-overexpressing MSCs (PLK1 group). The Sham group was injected with PBS instead of PBMNCs. **(B)** Survival rate (n = 10) and **(C)** body weight (n = 10). **(D)** Histopathological score for GvHD target organs (n = 10). Data are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, one-way or two way ANOVA with the Bonferroni post-hoc test. MSCs: mesenchymal stromal cells. PLK-1: polo-like kinase-1. GvHD: graft-versus-host disease. PBMNCs: peripheral blood mononuclear cells. Adapted from ref [21],® 2018, Springer Nature.

encapsulation of MSCs in alginate microcapsules, suggesting this procedure enables the design of precise protocols to standardize cell dosage, and additionally, improves the biosafety of the therapy. Successfully, the subcutaneous implantation of these alginate microcapsules in GvHD mice resulted in an increased survival rate and clinical score at day 8 [70].

In the attempt to potentiate the immonumodulatory activity of MSCs in GvHD, Kim *et al.* recently proposed preconditioning the cells with hypoxia and calcium ions. Administration of these preconditioned MSCs importantly ameliorated symptomatology in a humanized GvHD mouse model. As a result, survival increased, while weight loss and histopathologic injuries in GvHD target organs significantly reduced, in comparison to mice infused with non-preconditioned MSCs [21] (Fig. 2). In a different strategy, intravenous infusion of UCMSC-derived EVs resulted in alleviated *in vivo* symptomatology and associated histological changes while reduced mortality in a mouse model of allogeneic HSCT. These effects were attributed to a reduction in T cell absolute numbers, together with diminished levels of inflammatory cytokines including interleukin-2 (IL-2), TNF-α or IFN-γ and increased levels of IL-10 [71].

3.2 Inflammatory bowel disease (IBD)

IBD, comprising Crohn's disease and ulcerative colitis, is a chronic, immunologically mediated disease that results in inflammatory processes along the gastrointestinal tract [72]. Immunological imbalances have been well-documented in patients suffering from IBD, including the increase in T cell activation or the reduced levels of Tregs in circulation [73]. Because of their anti-inflammatory and immunoregulatory properties, MSC-based therapies have been widely explored in preclinical studies of IBD.

MSC based therapy in dextran sodium sulfate (DSS) or trinitrobenzene sulfonic acid (TNBS) induced IBD rodent models has demonstrated to ameliorate the clinical severity of colitis, reducing body weight loss, diarrhea and inflammation, while increasing survival [74-76]. Therapeutic effects have been attributed to MSC induction of Tregs, which ultimately downregulate Th1 and Th17 effector responses and promote Th2 differentiation *in vivo* [74-76]. These successful outcomes have been reported with MSCs from different origins [74,76,77]. The timing of administration has been demonstrated to importantly affect the outcomes of the therapy. Indeed, early injection significantly ameliorated DSS-induced colitis in terms of

both, disease activity index and histological score, in comparison to delayed injection. The mechanisms responsible for these positive outcomes were the induction of M2 macrophages and Tregs [75]. Interestingly, the infusion of preconditioned MSCs has been demonstrated to reduce the disease activity more effectively than control MSCs. *In vitro* co-culture studies revealed that preconditioned MSCs produced significantly higher levels of PGE2, which blocked the induction of anti-inflammatory cytokines including TNF-α and IL-2, while promoting IL-10 secreting T cells [78].

Alternatively, it is possible to take advantage of the therapeutic benefits of MSCs without directly administrating the cells. In one of these strategies, murine bone marrow-derived macrophages were cultured with adipose-derived MSCs (ASCs) or with ASC-conditioned media. The co-culture switched macrophage polarization towards an anti-inflammatory M2 phenotype, increasing IL-10 production and enhancing their capability to inhibit T cells. Systemic infusion of the resulting M2 polarized macrophages inhibited colitis in mice, reducing mortality and weight loss [79]. In a different approach, MSC-derived EVs were intravenously administered in a TNBS rat colitis model. EVs significantly reduced colonic damage by diminishing the production of proinflammatory cytokines and increasing IL-10 levels [80].

3.3 Multiple sclerosis (MS)

MSC-mediated immunomodulation has been studied in the treatment of autoimmune diseases such as MS, systemic lupus erythematosus or amyotrophic lateral sclerosis [19]. MS is a T-cell mediated autoimmune disorder characterized by chronic inflammation of the central nervous system leading to demyelination and neurodegeneration [81]. Multiple studies have demonstrated the capacity of MSCs to improve the outcomes of the disease in preclinical models [82-84]. Milder disease and fewer relapses have been reported to be the result of decreased inflammatory infiltrates and reduced demyelination or axonal loss [82].

However, important concerns have arisen regarding the autologous MSC use for MS therapies. MSCs from mice presenting early phases of MS were reported to present no differences to naive MSCs from healthy donors [85]. Nevertheless, MSCs from mice presenting later stage MS, which is accompanied by more severe symptoms such as tail paralysis or hind limb weakness, showed no therapeutic efficacy in comparison to naive MSCs [86]. Therefore,

MSC functionality was demonstrated to depend on the severity and stage of the disease [86]. The diminished potential of MSCs in late stage MS mice was attributed to reductions in the cell secretome [87]. Similarly, the antioxidant capacity of MSCs negatively correlates with the progressive phase of MS [88].

Interestingly, combinations of drugs such as methylprednisolone or rapamycin with MSCs have been reported to significantly ameliorate the clinical symptomatology in experimental autoimmune encephalomyelitis (EAE) mice MS models by attenuating inflammatory infiltration and demyelination [83,84]. These effects have been associated to a reduced cytolytic activity of $CD8^+$ T cells and to an increase in Tregs number [83,84]. Additionally, the reduced secretion of pro-inflammatory cytokines (IFN-γ, TNF-α or IL-17) and the increased release of anti-inflammatory cytokines (IL-4, IL-10) played an important role in disease amelioration [83,84]. In another approach to enhance MSC-mediated immunomodulation, cells licensed with IFN-γ improved MS symptomatology more effectively than control cells. This was attributed to increased numbers of Tregs and reduced secretion of inflammatory cytokines in mice treated with licensed cells [89].

Moreover, strategies using the MSC-conditioned medium, instead of cell-based therapies, have demonstrated the capacity of soluble factors to improve the outcome of MS. In particular, it was reported that conditioned medium derived from human MSCs reduced functional deficits in EAE mice by enhancing the development of oligodendrocytes and neurons [90].

4. Clinical trials for the treatment of inflammatory and immune disorders

Over the last years, the advances in preclinical studies have led to a significant increase in the number of clinical trials conducted with MSCs. Indeed, in 2012, Shi *et al.* reported that over 100 clinical trials with MSCs had been registered on the international database clinicaltrials.gov [51], whereas in 2016 Gao *et al.* [8] claimed 400 registered studies. As of August 2019, the number has increased over 900.

Amongst them, the analysis of phase III clinical trials provides key insight of the current state of the field (Table 1). Despite still the majority of clinical trials with MSCs are in early

Table 1. Phase III clinical trials using MSCs in the treatment of immune disorders. GvHD: graft-versus-host-disease. ALS: amyotrophic lateral sclerosis.

Indication	Sponsor & Product name	Status	Source	Findings	Identifier
Industry sponsored					
GvHD	Osiris therapeutics (*Prochymal*)	Completed May 2009	Allogeneic BMSCs	Primary endpoint did not significantly improve in comparison to the placebo group [91]	NCT00366145
Pediatric GvHD	Mesoblast Ltd. (*Remestemcel-L*)	Completed April 2018	Allogeneic BMSCs	Successfully met the primary endpoint. Day 28 overall response significantly improved.	NCT02336230
Crohn's fistular disease	TiGenix S.A.U (*Cx601*)	Completed July 2015	Allogeneic ASCs	Successfully met primary outcomes demonstrating efficacy and safety. Secondary outcomes were also met in the one year follow-up. In March 2018, European approval was announced (Alofisel) [95-97]	NCT01541579
Crohn's disease	Mesoblast Ltd. (*Prochymal*)	Active March 2009	Allogeneic BMSCs	Estimated primary completion date February 2019, no results published	NCT01233960
ALS	Brainstorm cell therapeutics (*NurOwn*)	Recruiting September 2017	Autologous BMSCs	Ongoing	NCT03280056
Osteoarthritis	R-Bio (*JointStem*)	Recruiting June 2019	Autologous ASCs	Ongoing	NCT03990805
Institution sponsored					
Rheumatoid arthritis	Royan Institute	Completed December 2013	Not specified	Safety and efficacy were demonstrated [104].	NCT01873625
Osteoarthritis	Stanford University	Recruiting April 2018	ASCs	Ongoing	NCT03467919
Osteoarthritis	Emory University	Recruiting March 2019	Autologous ASCs	Ongoing	NCT03818737
GvHD	Guangdong General Hospital	Recruiting October 2014	Not specified	Ongoing	NCT02291770
GvHD	Fujian Medical University	Recruiting December 2017	Not specified	Ongoing	NCT03631589

phases [18], late stage studies are expected to stablish the bases of clinical MSC-based therapies. In 2009, Osiris Therapeutics conducted the first major phase III clinical trial with MSCs (NCT00366145). Their product, Prochymal®, consisted of allogeneic BMMSCs and was intended for the treatment of steroid-refractory GvHD. Despite the primary endpoint of the study did not significantly improve in comparison to the placebo group [91], this study represented an important starting point for future trials. In 2013, Prochymal® assets were divested to Mesoblast Inc., which conducted the adaptive clinical trial for pediatric steroid-refractory GvHD (NCT02336230), naming the product Remestemcel-L®. In 2018, a press release announced that the study met the primary endpoint and improved the overall response (with a 69% of subjects responding to Remestemcel-L in comparison to the protocol-defined control rate of 45%). Some authors claim that these successful results might lead to the first FDA approved MSC product in the USA [92]. Further, on September 2015, Mesoblast's licensee JCR Pharmaceutical Co. Ltd. received the approval from the Japanese Pharmaceuticals and Medical Devices Agency (PMDA) for their product TemCell® for severe GvHD treatment [93].

In 2015, TiGenix S.A.U. completed a phase III clinical trial will allogeneic ASCs (Cx601), for the treatment of perianal fistula in patients with Crohn's disease (NCT01541579). This study was the adaptive trial of a previous one (NCT00475410) sponsored by Cellerix S.A for the treatment of complex fistulas in patients without IBD. After its completion in August 2009, it reported no significant differences between the use of autologous ASCs, with and without fibrin glue, and the control treatment based on fibrin glue alone [94]. In 2011, when Tigenix S.A.U. adquired Cellerix, they implemented important changes for the new NCT01541579 protocol: autologous ASCs were substituted by allogeneic ASCs, the dose increased from 60 to 120 million cells and participants suffered from Crohn's disease (these patients were excluded in the previous trial). This adaptive study successfully met primary outcomes demonstrating efficacy and safety [95]. Results were maintained for at least one year, when the follow-up study reported that 56.3% of Cx601 treated patients experienced fistula closure in comparison to a 38.6% of the placebo group [96]. This study was defined by Galipeau *et al*. as the first unambiguously successful use of MSCs in an advanced clinical trial [92]. As a result, in March 2018, the European Medicines Agency (EMA) announced the approval for Cx601, under the commercial name Alofisel®, as the first allogeneic MSC-based therapy approved in Europe [97].

The conduction of these late stage clinical trials has led to milestones that pave the way towards commercialization of MSC-based therapeutic products. However, there is still much work to be done in the development of MSC therapies for multiple immune disorders that currently lack of effective treatments. An in depth understanding of the mechanisms governing the therapeutic effects of MSCs and their interaction with the local microenvironment should help guide the future clinical studies towards the development of successful pharmaceutical biotechnologies.

5. Conclusion

The ability to produce and release a plethora of paracrine bioactive factors confers MSCs important immunomodulatory properties. Consequently, MSCs show great potential in the treatment of many immune and inflammatory disorders, as demonstrated in preclinical studies. The advances with animal models have led to an important increase in the number of clinical trials conducted. Although many of them did not manage to meet the primary outcomes, they represent important references for future clinical studies [91, 94]. Indeed, adaptive late-stage trials have obtained positive results, giving rise to the pioneering approval of MSC-based therapies in Europe or Japan [93, 97].

6. Expert opinion

Over the last decades, significant progress has been made in MSC-based immunomodulatory therapies. However, the publication of mixed and contradictory results is preventing research from advancing in a faster pace. The disparities are probably due to the large variability in key factors such as cell origin (tissue, donor), dosage or administration protocols. Inconsistencies among these parameters importantly limit the therapeutic benefit of MSCs. Therefore, the standardization of MSC-therapies is a crucial step towards the clinical development. In this vein, standardized cell isolation and culture methods, expansion techniques and robust quality control systems are still required. Similarly, the optimization of *in vivo* administration protocols is fundamental. Intravenously infused MSCs are undetectable shortly after administration [98] and their clearance by tissue resident perivascular macrophages importantly limits cell persistence [92]. Additionally, disease indications requiring tissue

targeting demand new strategies adapted to administration of MSCs in extravascular compartments. In such regard, administration routes such as the intraperitoneal or subcutaneous may be of special interest [92].

Another crucial variable that directly influences the outcome of MSC-based therapies is the immunomodulatory capacity of the cells. This potential is strongly influenced by multiple factors including the tissue source of MSCs, the health condition and age of the donor or the number of cell passages prior to the implantation. In addition, in the particular disease intended to be treated, the local conditions were cells are implanted may not provide an adequate microenvironment to polarize MSCs towards an immunosuppressive phenotype. Consequently, the future of study lies in the development of effective preconditioning strategies that boost the immunomodulatory potential of MSCs in such scenarios.

Currently, great effort is being made in this area (Fig. 3). Among the alternatives, licensing the cells with inflammatory cytokines such as IFN-γ or TNF-α has been demonstrated to upregulate the production of key immunomodulatory factors by MSCs, including IDO and PGE2 [8]. However, the effects of inflammatory licensing have been reported transient and further research is necessary to develop strategies that maintain its effects [99]. Alternatively, hypoxic preconditioning consists of culturing cells with an oxygen supply ranging from 0 to 10% [100]. Since *in vivo* environments are often hypoxic (varying from 1 to 12%), MSC culture in these conditions enhances their viability once implanted in the harsh damaged tissue. Moreover, hypoxic preconditioning has been demonstrated to promote the secretion of cytoprotective molecules and to enhance proliferation, while maintaining MSCs in an undifferentiated state [7]. However, MSCs present a high sensitivity to oxygen tension and slight variations may importantly influence the differentiation and paracrine activities of the cells. For that reason, the protocol variability that exists nowadays has led to conflicting results [7], and standardization of the technique is still required. Moreover, MSC culture in a three-dimensional (3D) environment has also been proposed to enhance their therapeutic potential, for recreating the natural niche of cells. The most widely employed 3D culture strategy for this purpose is the formation of cell spheroids, which has been demonstrated to promote MSCs' immunomodulatory capacity [101]. Alternatively, encapsulation of MSCs in a thin conformal hydrogel coating was recently proposed to enhance MSCs immunomodulatory properties in

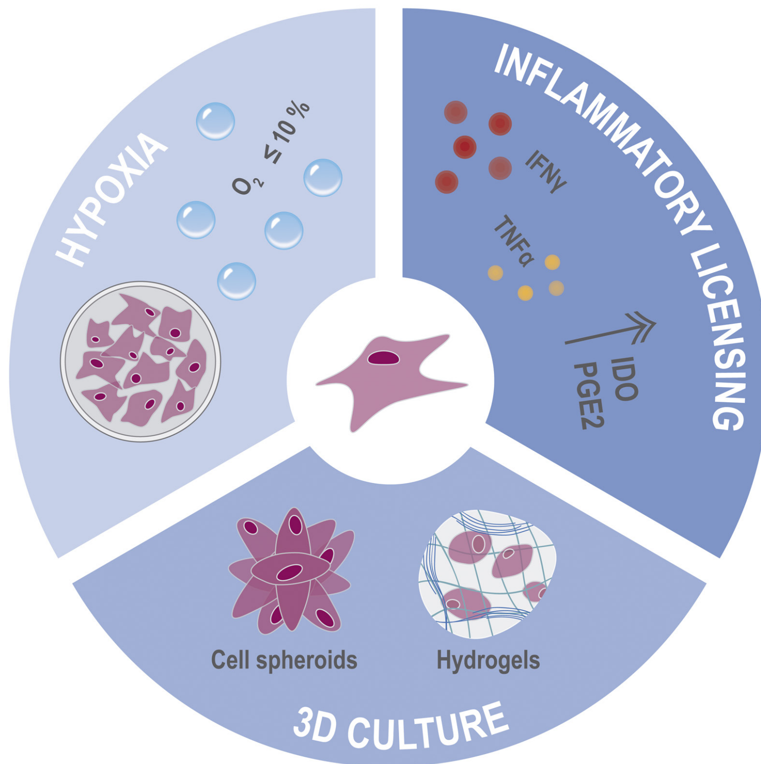


Fig. 3. MSCs preconditioning strategies. Hypoxic culture, cell licensing with inflammatory cytokines including interferon γ (IFN-γ) or tumor necrosis factor α (TNF-α) and different 3D culture strategies have been explored to boost the immunomodulatory potential of MSCs.

a model of allogeneic bone marrow transplantation [102]. Other hydrogel systems, such as alginate-poly-L-lysine-alginate microcapsules have also shown to improve MSCs metabolic activity and the secretion of vascular endothelial growth factor (VEGF) [103]. Although extensive evidence supports the validity of MSCs preconditioning strategies, understanding the underlying mechanisms is still required for their clinical translation.

The important preclinical advances together with the pioneering clinical approval of the first MSC-based treatments is only the start of a new era in cell therapy. The current efforts conducted to develop strategies that boost the therapeutic properties of MSCs, to standardize protocols and to create specific guidelines will definitely establish the bases for the leap towards the final clinical translation.

Funding

Authors thank for the project SAF2017-82292-R (MINECO) and the support from the Basque Country Government (Grupos Consolidados, No ref: IT907-16). A. Gonzalez-Pujana thanks the Basque Government (Department of Education, Universities and Research) for the PhD grant (PRE_2018_2_0133).

References

[1] K. Le Blanc and D. Mougiakakos. Multipotent Mesenchymal Stromal Cells and the Innate Immune System. Nat.Rev.Immunol. 2012;12:383-396. https://doi.org/10.1038/nri3209 .

[2] M. Dominici, K. Le Blanc, I. Mueller, et al. Minimal Criteria for Defining Multipotent Mesenchymal Stromal Cells. the International Society for Cellular Therapy Position Statement. Cytotherapy 2006;8:315-317. https://doi.org/10.1080/14653240600855905.

[3] E.M. Horwitz, K. Le Blanc, M. Dominici, et al. Clarification of the Nomenclature for MSC: The International Society for Cellular Therapy Position Statement. Cytotherapy 2005;7:393-395. https://doi.org/10.1080/14653240500319234.

[4] Y. Zhang, M. Chen, J. Tian, et al. In Situ Bone Regeneration Enabled by a Biodegradable Hybrid Double-Network Hydrogel. Biomater.Sci. 2019;7:3266-3276. https://doi.org/10.1039/c9bm00561g .

[5] L. Jiang, D. Su, S. Ding, et al. Salt-Assisted Toughening of Protein Hydrogel with Controlled Degradation for Bone Regeneration. Adv.Funct.Mater. 2019;29:1901314. . Available at https://doi.org/10.1002/adfm.201901314https://doi.org/10.1002/adfm.201901314.

[6] V.P. Ribeiro, A. da Silva Morais, F.R. Maia, et al. Combinatory Approach for Developing Silk Fibroin Scaffolds for Cartilage Regeneration. Acta Biomater. 2018;72:167-181. https://doi.org/10.1016/j.actbio.2018.03.047.

[7] J.R. Ferreira, G.Q. Teixeira, S.G. Santos, et al. Mesenchymal Stromal Cell Secretome: Influencing Therapeutic Potential by Cellular Pre-Conditioning. Front.Immunol. 2018;9:2837. https://doi.org/10.3389/fimmu.2018.02837.

[8] F. Gao, S.M. Chiu, D.A. Motan, et al. Mesenchymal Stem Cells and Immunomodulation: Current Status and Future Prospects. Cell. Death Dis. 2016;7:e2062. https://doi.org/10.1038/cddis.2015.327.

[9] G.M. Spaggiari, A. Capobianco, H. Abdelrazik, et al. Mesenchymal Stem Cells Inhibit Natural Killer-Cell Proliferation, Cytotoxicity, and Cytokine Production: Role of Indoleamine 2,3-Dioxygenase and Prostaglandin E2. Blood 2008;111:1327-1333. https://doi.org/10.1182/blood-2007-02-074997.

[10] P. Terness, T.M. Bauer, L. Rose, et al. Inhibition of Allogeneic T Cell Proliferation by Indoleamine 2,3-Dioxygenase-Expressing Dendritic Cells: Mediation of Suppression by Tryptophan Metabolites. J.Exp.Med. 2002;196:447-457. https://doi.org/10.1084/jem.20020052.

[11] A. Corcione, F. Benvenuto, E. Ferretti, et al. Human Mesenchymal Stem Cells Modulate B-Cell Functions. Blood 2006;107:367-372. https://doi.org/10.1182/blood-2005-07-2657.

[12] M. Benkhoucha, M.L. Santiago-Raber, G. Schneiter, et al. Hepatocyte Growth Factor Inhibits CNS Autoimmunity by Inducing Tolerogenic Dendritic Cells and CD25+Foxp3+ Regulatory T Cells. Proc.Natl.Acad.Sci.U.S.A. 2010;107:6424-6429. https://doi.org/10.1073/pnas.0912437107.

[13] M. Francois, R. Romieu-Mourez, M. Li, et al. Human MSC Suppression Correlates with Cytokine Induction of Indoleamine 2,3-Dioxygenase and Bystander M2 Macrophage Differentiation. Mol.Ther. 2012;20:187-195. https://doi.org/10.1038/mt.2011.189.

[14] J.H. Ylostalo, T.J. Bartosh, K. Coble, et al. Human Mesenchymal Stem/Stromal Cells Cultured as Spheroids are Self-Activated to Produce Prostaglandin E2 that Directs Stimulated Macrophages into an Anti-Inflammatory Phenotype. Stem Cells 2012;30:2283-2296. https://doi.org/10.1002/stem.1191.

[15] R.S. Waterman, S.L. Tomchuck, S.L. Henkle, et al. A New Mesenchymal Stem Cell (MSC) Paradigm: Polarization into a Pro-Inflammatory MSC1 Or an Immunosuppressive MSC2 Phenotype. PLoS One 2010;5:e10088. https://doi.org/10.1371/journal.pone.0010088.

[16] M. Gazdic, V. Volarevic, N. Arsenijevic, et al. Mesenchymal Stem Cells: A Friend Or Foe in Immune-Mediated Diseases. Stem Cell. Rev. 2015;11:280-287. https://doi.org/10.1007/s12015-014-9583-3.

[17] M.E. Bernardo and W.E. Fibbe. Mesenchymal Stromal Cells: Sensors and Switchers of Inflammation. Cell.Stem Cell. 2013;13:392-402. https://doi.org/10.1016/j.stem.2013.09.006.

[18] A. Naji, M. Eitoku, B. Favier, et al. Biological Functions of Mesenchymal Stem Cells and Clinical Implications. Cell Mol.Life Sci. 2019;https://doi.org/10.1007/s00018-019-03125-1.

[19] X. Chen, S. Wang and W. Cao. Mesenchymal Stem Cell-Mediated Immunomodulation in Cell Therapy of Neurodegenerative Diseases. Cell.Immunol. 2018;326:8-14. https://doi.org/10.1016/j.cellimm.2017.06.006.

[20] F. Mao, Q. Tu, L. Wang, et al. Mesenchymal Stem Cells and their Therapeutic Applications in Inflammatory Bowel Disease. Oncotarget 2017;8:38008-38021. https://doi.org/10.18632/oncotarget.16682.

[21] Y. Kim, H.J. Jin, J. Heo, et al. Small Hypoxia-Primed Mesenchymal Stem Cells Attenuate Graft-Versus-Host Disease. Leukemia 2018;32:2672-2684. https://doi.org/10.1038/s41375-018-0151-8.

[22] E.L. de Mare-Bredemeijer, S. Mancham, M.M. Verstegen, et al. Human Graft-Derived Mesenchymal Stromal Cells Potently Suppress Alloreactive T-Cell Responses. Stem Cells Dev. 2015;24:1436-1447. https://doi.org/10.1089/scd.2014.0485.

[23] Y. Yu, L. Liao, B. Shao, et al. Knockdown of MicroRNA Let-7a Improves the Functionality of Bone Marrow-Derived Mesenchymal Stem Cells in Immunotherapy. Mol.Ther. 2017;25:480-493. https://doi.org/10.1016/j.ymthe.2016.11.015.

[24] K. Akiyama, C. Chen, D. Wang, et al. Mesenchymal-Stem-Cell-Induced Immunoregulation Involves FAS-Ligand-/FAS-Mediated T Cell Apoptosis. Cell.Stem Cell. 2012;10:544-555. https://doi.org/10.1016/j.stem.2012.03.007.

[25] F.J. Vizoso, N. Eiro, S. Cid, et al. Mesenchymal Stem Cell Secretome: Toward Cell-Free Therapeutic Strategies in Regenerative Medicine. Int.J.Mol.Sci. 2017;18:10.3390/ijms18091852. https://doi.org/10.3390/ijms18091852.

[26] M. Krampera, L. Cosmi, R. Angeli, et al. Role for Interferon-Gamma in the Immunomodulatory Activity of Human Bone Marrow Mesenchymal Stem Cells. Stem Cells 2006;24:386-398. https://doi.org/10.1634/stemcells.2005-0008.

[27] H. Sugimoto, S. Oda, T. Otsuki, et al. Crystal Structure of Human Indoleamine 2,3-Dioxygenase: Catalytic Mechanism of O2 Incorporation by a Heme-Containing Dioxygenase. Proc.Natl.Acad.Sci.U.S.A. 2006;103:2611-2616. https://doi.org/10.1073/pnas.0508996103.

[28] F. Fallarino, U. Grohmann, S. You, et al. The Combined Effects of Tryptophan Starvation and Tryptophan Catabolites Down-Regulate T Cell Receptor Zeta-Chain and Induce a Regulatory Phenotype in Naive T Cells. J.Immunol. 2006;176:6752-6761. https://doi.org/10.4049/jimmunol.176.11.6752.

[29] F. Li, R. Zhang, S. Li, et al. IDO1: An Important Immunotherapy Target in Cancer Treatment. Int.Immunopharmacol. 2017;47:70-77. https://doi.org/10.1016/j.intimp.2017.03.024.

[30] R. Duffin, R.A. O'Connor, S. Crittenden, et al. Prostaglandin E(2) Constrains Systemic Inflammation through an Innate Lymphoid Cell-IL-22 Axis. Science 2016;351:1333-1338. https://doi.org/10.1126/science.aad9903.

[31] P. Kalinski. Regulation of Immune Responses by Prostaglandin E2. J.Immunol. 2012;188:21-28. https://doi.org/10.4049/jimmunol.1101029.

[32] K. English, F.P. Barry, C.P. Field-Corbett, et al. IFN-Gamma and TNF-Alpha Differentially Regulate Immunomodulation by Murine Mesenchymal Stem Cells. Immunol.Lett. 2007;110:91-100. https://doi.org/10.1016/j.imlet.2007.04.001.

[33] L. Jarvinen, L. Badri, S. Wettlaufer, et al. Lung Resident Mesenchymal Stem Cells Isolated from Human Lung Allografts Inhibit T Cell Proliferation Via a Soluble Mediator. J.Immunol. 2008;181:4389-4396. https://doi.org/10.4049/jimmunol.181.6.4389.

[34] R. Tatara, K. Ozaki, Y. Kikuchi, et al. Mesenchymal Stromal Cells Inhibit Th17 but Not Regulatory T-Cell Differentiation. Cytotherapy 2011;13:686-694. https://doi.org/10.3109/14653249.2010.542456.

[35] S. Galland, J. Vuille, P. Martin, et al. Tumor-Derived Mesenchymal Stem Cells use Distinct Mechanisms to Block the Activity of Natural Killer Cell Subsets. Cell.Rep. 2017;20:2891-2905. https://doi.org/10.1016/j.celrep.2017.08.089.

[36] A.B. Vasandan, S. Jahnavi, C. Shashank, et al. Human Mesenchymal Stem Cells Program Macrophage Plasticity by Altering their Metabolic Status Via a PGE2-Dependent Mechanism. Sci.Rep. 2016;6:38308. https://doi.org/10.1038/srep38308.

[37] J.S. Choi, H.A. Ryu, S.H. Cheon, et al. Human Adipose Derived Stem Cells Exhibit Enhanced Liver Regeneration in Acute Liver Injury by Controlled Releasing Hepatocyte Growth Factor. Cell.Physiol.Biochem. 2019;52:935-950. https://doi.org/10.33594/000000065.

[38] E.J. Lee, I. Hwang, J.Y. Lee, et al. Hepatocyte Growth Factor Improves the Therapeutic Efficacy of Human Bone Marrow Mesenchymal Stem Cells Via RAD51. Mol.Ther. 2018;26:845-859. https://doi.org/10.1016/j.ymthe.2017.12.015.

[39] M. Di Nicola, C. Carlo-Stella, M. Magni, et al. Human Bone Marrow Stromal Cells Suppress T-Lymphocyte Proliferation Induced by Cellular Or Nonspecific Mitogenic Stimuli. Blood 2002;99:3838-3843. https://doi.org/10.1182/blood.v99.10.3838.

[40] A. Nasef, A. Chapel, C. Mazurier, et al. Identification of IL-10 and TGF-Beta Transcripts Involved in the Inhibition of T-Lymphocyte Proliferation during Cell Contact with Human Mesenchymal Stem Cells. Gene Expr. 2007;13:217-226.

[41] J.M. Ilarregui, G.A. Bianco, M.A. Toscano, et al. The Coming of Age of Galectins as Immunomodulatory Agents: Impact of these Carbohydrate Binding Proteins in T Cell Physiology and Chronic Inflammatory Disorders. Ann.Rheum.Dis. 2005;64 Suppl 4:iv96-103. https://doi.org/10.1136/ard.2005.044347.

[42] L. Johannes, R. Jacob and H. Leffler. Galectins at a Glance. J.Cell.Sci. 2018;131:10.1242/jcs.208884. https://doi.org/10.1242/jcs.208884.

[43] F. Gieseke, J. Bohringer, R. Bussolari, et al. Human Multipotent Mesenchymal Stromal Cells use Galectin-1 to Inhibit Immune Effector Cells. Blood 2010;116:3770-3779. https://doi.org/10.1182/blood-2010-02-270777.

[44] J.M. Ilarregui, D.O. Croci, G.A. Bianco, et al. Tolerogenic Signals Delivered by Dendritic Cells to T Cells through a Galectin-1-Driven Immunoregulatory Circuit Involving Interleukin 27 and Interleukin 10. Nat.Immunol. 2009;10:981-991. https://doi.org/10.1038/ni.1772.

[45] F. Gieseke, A. Kruchen, N. Tzaribachev, et al. Proinflammatory Stimuli Induce Galectin-9 in Human Mesenchymal Stromal Cells to Suppress T-Cell Proliferation. Eur.J.Immunol. 2013;43:2741-2749. https://doi.org/10.1002/eji.201343335.

[46] C. Ungerer, P. Quade-Lyssy, H.H. Radeke, et al. Galectin-9 is a Suppressor of T and B Cells and Predicts the Immune Modulatory Potential of Mesenchymal Stromal Cell Preparations. Stem Cells Dev. 2014;23:755-766. https://doi.org/10.1089/scd.2013.0335.

[47] S.N. Kim, H.J. Lee, M.S. Jeon, et al. Galectin-9 is Involved in Immunosuppression Mediated by Human Bone Marrow-Derived Clonal Mesenchymal Stem Cells. Immune Netw. 2015;15:241-251. https://doi.org/10.4110/in.2015.15.5.241.

[48] P.M. Chen, K.J. Liu, P.J. Hsu, et al. Induction of Immunomodulatory Monocytes by Human Mesenchymal Stem Cell-Derived Hepatocyte Growth Factor through ERK1/2. J.Leukoc.Biol. 2014;96:295-303. https://doi.org/10.1189/jlb.3A0513-242R.

[49] M. Murai, O. Turovskaya, G. Kim, et al. Interleukin 10 Acts on Regulatory T Cells to Maintain Expression of the Transcription Factor Foxp3 and Suppressive Function in Mice with Colitis. Nat.Immunol. 2009;10:1178-1184. https://doi.org/10.1038/ni.1791.

[50] J.E. de Vries. Immunosuppressive and Anti-Inflammatory Properties of Interleukin 10. Ann.Med. 1995;27:537-541. https://doi.org/10.3109/07853899509002465.

[51] Y. Shi, J. Su, A.I. Roberts, et al. How Mesenchymal Stem Cells Interact with Tissue Immune Responses. Trends Immunol. 2012;33:136-143. https://doi.org/10.1016/j.it.2011.11.004.

[52] E. Ivanova-Todorova, I. Bochev, R. Dimitrov, et al. Conditioned Medium from Adipose Tissue-Derived Mesenchymal Stem Cells Induces CD4+FOXP3+ Cells and Increases IL-10 Secretion. J.Biomed.Biotechnol. 2012;2012:295167. https://doi.org/10.1155/2012/295167.

[53] A. Putra, F.B. Ridwan, A.I. Putridewi, et al. The Role of TNF-Alpha Induced MSCs on Suppressive Inflammation by Increasing TGF-Beta and IL-10. Open Access Maced J.Med.Sci. 2018;6:1779-1783. https://doi.org/10.3889/oamjms.2018.404.

[54] D. Kyurkchiev, I. Bochev, E. Ivanova-Todorova, et al. Secretion of Immunoregulatory Cytokines by Mesenchymal Stem Cells. World J.Stem Cells 2014;6:552-570. https://doi.org/10.4252/wjsc.v6.i5.552 .

[55] D. Lu, T. Ma, X. Zhou, et al. B Lymphocytes are the Target of Mesenchymal Stem Cells Immunoregulatory Effect in a Murine Graft-Versus-Host Disease Model. Cell Transplant. 2019;:963689719860127. https://doi.org/10.1177/0963689719860127.

[56] M. Najar, G. Raicevic, H. Fayyad-Kazan, et al. Bone Marrow Mesenchymal Stromal Cells Induce Proliferative, Cytokinic and Molecular Changes during the T Cell Response: The Importance of the IL-10/CD210 Axis. Stem Cell.Rev. 2015;11:442-452. https://doi.org/10.1007/s12015-014-9567-3.

[57] W.P. Arend, M. Malyak, C.J. Guthridge, et al. Interleukin-1 Receptor Antagonist: Role in Biology. Annu.Rev.Immunol. 1998;16:27-55. https://doi.org/10.1146/annurev.immunol.16.1.27.

[58] V. Volarevic, A. Al-Qahtani, N. Arsenijevic, et al. Interleukin-1 Receptor Antagonist (IL-1Ra) and IL-1Ra Producing Mesenchymal Stem Cells as Modulators of Diabetogenesis. Autoimmunity 2010;43:255-263. https://doi.org/10.3109/08916930903305641.

[59] P. Luz-Crawford, F. Djouad, K. Toupet, et al. Mesenchymal Stem Cell-Derived Interleukin 1 Receptor Antagonist Promotes Macrophage Polarization and Inhibits B Cell Differentiation. Stem Cells 2016;34:483-492. https://doi.org/10.1002/stem.2254.

[60] K. Lee, N. Park, H. Jung, et al. Mesenchymal Stem Cells Ameliorate Experimental Arthritis Via Expression of Interleukin-1 Receptor Antagonist. PLoS One 2018;13:e0193086. https://doi.org/10.1371/journal.pone.0193086.

[61] L.A. Ortiz, M. Dutreil, C. Fattman, et al. Interleukin 1 Receptor Antagonist Mediates the Antiinflammatory and Antifibrotic Effect of Mesenchymal Stem Cells during Lung Injury. Proc.Natl.Acad.Sci.U.S.A. 2007;104:11002-11007. https://doi.org/10.1073/pnas.0704421104.

[62] J.L. Ferrara, J.E. Levine, P. Reddy, et al. Graft-Versus-Host Disease. Lancet 2009;373:1550-1561. https://doi.org/10.1016/S0140-6736(09)60237-3.

[63] L. Zhang, J. Chu, J. Yu, et al. Cellular and Molecular Mechanisms in Graft-Versus-Host Disease. J.Leukoc.Biol. 2016;99:279-287. https://doi.org/10.1189/jlb.4RU0615-254RR.

[64] V.J. Mehler, C. Burns and M.L. Moore. Concise Review: Exploring Immunomodulatory Features of Mesenchymal Stromal Cells in Humanized Mouse Models. Stem Cells 2019;37:298-305. https://doi.org/10.1002/stem.2948.

[65] V. Tisato, K. Naresh, J. Girdlestone, et al. Mesenchymal Stem Cells of Cord Blood Origin are Effective at Preventing but Not Treating Graft-Versus-Host Disease. Leukemia 2007;21:1992-1999. https://doi.org/10.1038/sj.leu.2404847.

[66] L.M. Tobin, M.E. Healy, K. English, et al. Human Mesenchymal Stem Cells Suppress Donor CD4(+) T Cell Proliferation and Reduce Pathology in a Humanized Mouse Model of Acute Graft-Versus-Host Disease. Clin.Exp.Immunol. 2013;172:333-348. https://doi.org/10.1111/cei.12056.

[67] F. Bruck, L. Belle, C. Lechanteur, et al. Impact of Bone Marrow-Derived Mesenchymal Stromal Cells on Experimental Xenogeneic Graft-Versus-Host Disease. Cytotherapy 2013;15:267-279. https://doi.org/10.1016/j.jcyt.2012.09.003.

[68] A.G. Laing, Y. Riffo-Vasquez, E. Sharif-Paghaleh, et al. Immune Modulation by Apoptotic Dental Pulp Stem Cells in Vivo. Immunotherapy 2018;10:201-211. https://doi.org/10.2217/imt-2017-0117.

[69] C. Gregoire, C. Ritacco, M. Hannon, et al. Comparison of Mesenchymal Stromal Cells from Different Origins for the Treatment of Graft-Vs.-Host-Disease in a Humanized Mouse Model. Front.Immunol. 2019;10:619. https://doi.org/10.3389/fimmu.2019.00619.

[70] L. Zanotti, A. Sarukhan, E. Dander, et al. Encapsulated Mesenchymal Stem Cells for in Vivo Immunomodulation. Leukemia 2013;27:500-503. https://doi.org/10.1038/leu.2012.202.

[71] L. Wang, Z. Gu, X. Zhao, et al. Extracellular Vesicles Released from Human Umbilical Cord-Derived Mesenchymal Stromal Cells Prevent Life-Threatening Acute Graft-Versus-Host Disease in a Mouse Model of Allogeneic Hematopoietic Stem Cell Transplantation. Stem Cells Dev. 2016;25:1874-1883. https://doi.org/10.1089/scd.2016.0107.

[72] A.N. Ananthakrishnan. Epidemiology and Risk Factors for IBD. Nat.Rev.Gastroenterol.Hepatol. 2015;12:205-217. https://doi.org/10.1038/nrgastro.2015.34.

[73] K. Chao, S. Zhang, J. Yao, et al. Imbalances of CD4(+) T-Cell Subgroups in Crohn's Disease and their Relationship with Disease Activity and Prognosis. J.Gastroenterol.Hepatol. 2014;29:1808-1814. https://doi.org/10.1111/jgh.12592.

[74] Q.Q. Chen, L. Yan, C.Z. Wang, et al. Mesenchymal Stem Cells Alleviate TNBS-Induced Colitis by Modulating Inflammatory and Autoimmune Responses. World J.Gastroenterol. 2013;19:4702-4717. https://doi.org/10.3748/wjg.v19.i29.4702.

[75] Y. Kawata, A. Tsuchiya, S. Seino, et al. Early Injection of Human Adipose Tissue-Derived Mesenchymal Stem Cell After Inflammation Ameliorates Dextran Sulfate Sodium-Induced Colitis in Mice through the Induction of M2 Macrophages and Regulatory T Cells. Cell Tissue Res. 2019;376:257-271. https://doi.org/10.1007/s00441-018-02981-w.

[76] Y. Zhang, Y. Jin, Y. Lin, et al. Adipose-Derived Mesenchymal Stem Cells Ameliorate Ulcerative Colitis through miR-1236 Negatively Regulating the Expression of Retinoid-Related Orphan Receptor Gamma. DNA Cell Biol. 2015;34:618-625. https://doi.org/10.1089/dna.2015.2961.

[77] A. Kagia, M. Tzetis, E. Kanavakis, et al. Therapeutic Effects of Mesenchymal Stem Cells Derived from Bone Marrow, Umbilical Cord Blood, and Pluripotent Stem Cells in a Mouse Model of Chemically Induced Inflammatory Bowel Disease. Inflammation 2019;https://doi.org/10.1007/s10753-019-01033-x.

[78] F.Y. Yang, R. Chen, X. Zhang, et al. Preconditioning Enhances the Therapeutic Effects of Mesenchymal Stem Cells on Colitis through PGE2-Mediated T-Cell Modulation. Cell Transplant. 2018;27:1352-1367. https://doi.org/10.1177/0963689718780304.

[79] P. Anderson, L. Souza-Moreira, M. Morell, et al. Adipose-Derived Mesenchymal Stromal Cells Induce Immunomodulatory Macrophages which Protect from Experimental Colitis and Sepsis. Gut 2013;62:1131-1141. https://doi.org/10.1136/gutjnl-2012-302152.

[80] J. Yang, X.X. Liu, H. Fan, et al. Extracellular Vesicles Derived from Bone Marrow Mesenchymal Stem Cells Protect Against Experimental Colitis Via Attenuating Colon Inflammation, Oxidative Stress and Apoptosis. PLoS One 2015;10:e0140551. https://doi.org/10.1371/journal.pone.0140551.

[81] J. Correale, M.I. Gaitan, M.C. Ysrraelit, et al. Progressive Multiple Sclerosis: From Pathogenic Mechanisms to Treatment. Brain 2017;140:527-546. https://doi.org/10.1093/brain/aww258.

[82] E. Gerdoni, B. Gallo, S. Casazza, et al. Mesenchymal Stem Cells Effectively Modulate Pathogenic Immune Response in Experimental Autoimmune Encephalomyelitis. Ann.Neurol. 2007;61:219-227. https://doi.org/10.1002/ana.21076.

[83] M.J. Kim, C.H. Ryu, S.M. Kim, et al. Combined Treatment with Methylprednisolone and Human Bone Marrow-Derived Mesenchymal Stem Cells Ameliorate Experimental Autoimmune Encephalomyelitis. Tissue Eng.Regen.Med. 2018;15:183-194. https://doi.org/10.1007/s13770-017-0101-y.

[84] M. Togha, M. Jahanshahi, L. Alizadeh, et al. Rapamycin Augments Immunomodulatory Properties of Bone Marrow-Derived Mesenchymal Stem Cells in Experimental Autoimmune Encephalomyelitis. Mol.Neurobiol. 2017;54:2445-2457. https://doi.org/10.1007/s12035-016-9840-3.

[85] I. Kassis, P. Petrou, M. Halimi, et al. Mesenchymal Stem Cells (MSC) Derived from Mice with Experimental Autoimmune Encephalomyelitis (EAE) Suppress EAE and have Similar Biological Properties with MSC from Healthy Donors. Immunol.Lett. 2013;154:70-76. https://doi.org/10.1016/j.imlet.2013.06.002.

[86] A. Sargent, L. Bai, G. Shano, et al. CNS Disease Diminishes the Therapeutic Functionality of Bone Marrow Mesenchymal Stem Cells. Exp.Neurol. 2017;295:222-232. https://doi.org/10.1016/j.expneurol.2017.06.013.

[87] P. Sarkar, J. Redondo, K. Kemp, et al. Reduced Neuroprotective Potential of the Mesenchymal Stromal Cell Secretome with Ex Vivo Expansion, Age and Progressive Multiple Sclerosis. Cytotherapy 2018;20:21-28. https://doi.org/10.1016/j.jcyt.2017.08.007.

[88] J. Redondo, P. Sarkar, K. Kemp, et al. Dysregulation of Mesenchymal Stromal Cell Antioxidant Responses in Progressive Multiple Sclerosis. Stem Cells Transl.Med. 2018;7:748-758. https://doi.org/10.1002/sctm.18-0045 .

[89] M. Torkaman, M. Ghollasi, M. Mohammadnia-Afrouzi, et al. The Effect of Transplanted Human Wharton's Jelly Mesenchymal Stem Cells Treated with IFN-Gamma on Experimental Autoimmune Encephalomyelitis Mice. Cell.Immunol. 2017;311:1-12. https://doi.org/10.1016/j.cellimm.2016.09.012.

[90] L. Bai, D.P. Lennon, A.I. Caplan, et al. Hepatocyte Growth Factor Mediates Mesenchymal Stem Cell-Induced Recovery in Multiple Sclerosis Models. Nat.Neurosci. 2012;15:862-870. https://doi.org/10.1038/nn.3109.

[91] P. Martin J., J. Uberti P., R. Soiffer J., et al. Prochymal Improves Response Rates in Patients with Steroid-Refractory Acute Graft Versus Host Disease (SR-GVHD) Involving the Liver and Gut: Results of A Randomized, Placebo-Controlled, Multicenter Phase III Trial in GVHD. Biology of Blood and Marrow Transplantation - BIOL BLOOD MARROW TRANSPLANT 2010;16https://doi.org/10.1016/j.bbmt.2009.12.057.

[92] J. Galipeau and L. Sensebe. Mesenchymal Stromal Cells: Clinical Challenges and Therapeutic Opportunities. Cell.Stem Cell. 2018;22:824-833. https://doi.org/10.1016/j.stem.2018.05.004.

[93] K. Muroi, K. Miyamura, M. Okada, et al. Bone Marrow-Derived Mesenchymal Stem Cells (JR-031) for Steroid-Refractory Grade III Or IV Acute Graft-Versus-Host Disease: A Phase II/III Study. Int.J.Hematol. 2016;103:243-250. https://doi.org/10.1007/s12185-015-1915-9.

[94] M.D. Herreros, M. Garcia-Arranz, H. Guadalajara, et al. Autologous Expanded Adipose-Derived Stem Cells for the Treatment of Complex Cryptoglandular Perianal Fistulas: A Phase III Randomized Clinical Trial (FATT 1: Fistula Advanced Therapy Trial 1) and Long-Term Evaluation. Dis.Colon Rectum 2012;55:762-772. https://doi.org/10.1097/DCR.0b013e318255364a.

[95] J. Panes, D. Garcia-Olmo, G. Van Assche, et al. Expanded Allogeneic Adipose-Derived Mesenchymal Stem Cells (Cx601) for Complex Perianal Fistulas in Crohn's Disease: A Phase 3 Randomised, Double-Blind Controlled Trial. Lancet 2016;388:1281-1290. https://doi.org/10.1016/S0140-6736(16)31203-X.

[96] J. Panes, D. Garcia-Olmo, G. Van Assche, et al. Long-Term Efficacy and Safety of Stem Cell Therapy (Cx601) for Complex Perianal Fistulas in Patients with Crohn's Disease. Gastroenterology 2018;154:1334-1342.e4. https://doi.org/10.1053/j.gastro.2017.12.020.

[97] L.J. Scott. Darvadstrocel: A Review in Treatment-Refractory Complex Perianal Fistulas in Crohn's Disease. BioDrugs 2018;32:627-634. https://doi.org/10.1007/s40259-018-0311-4.

[98] A. Galleu, Y. Riffo-Vasquez, C. Trento, et al. Apoptosis in Mesenchymal Stromal Cells Induced in Vivo Recipient-Mediated Immunomodulation. Sci.Transl.Med. 2017;9:10.1126/scitranslmed.aam7828. https://doi.org/10.1126/scitranslmed.aam7828.

[99] J.A. Zimmermann, M.H. Hettiaratchi and T.C. McDevitt. Enhanced Immunosuppression of T Cells by Sustained Presentation of Bioactive Interferon-Gamma within Three-Dimensional Mesenchymal Stem Cell Constructs. Stem Cells Transl.Med. 2017;6:223-237. https://doi.org/10.5966/sctm.2016-0044.

[100] M. Ejtehadifar, K. Shamsasenjan, A. Movassaghpour, et al. The Effect of Hypoxia on Mesenchymal Stem Cell Biology. Adv. Pharm.Bull. 2015;5:141-149. https://doi.org/10.15171/apb.2015.021.

[101] T.J. Bartosh, J.H. Ylostalo, A. Mohammadipoor, et al. Aggregation of Human Mesenchymal Stromal Cells (MSCs) into 3D Spheroids Enhances their Antiinflammatory Properties. Proc.Natl.Acad.Sci.U.S.A. 2010;107:13724-13729. https://doi.org/10.1073/pnas.1008117107.

[102] A.S. Mao, B. Ã–zkale, N.J. Shah, et al. Programmable Microencapsulation for Enhanced Mesenchymal Stem Cell Persistence and Immunomodulation. Proc.Natl.Acad.Sci.USA 2019;:201819415. https://doi.org/10.1073/pnas.1819415116.

[103] N. Attia, E. Santos, H. Abdelmouty, et al. Behaviour and Ultrastructure of Human Bone Marrow-Derived Mesenchymal Stem Cells Immobilised in Alginate-Poly-L-Lysine-Alginate Microcapsules. J.Microencapsul. 2014;31:579-589. https://doi.org/10.3109/02652048.2014.898706.

[104] S. Shadmanfar, N. Labibzadeh, M. Emadedin, et al. Intra-Articular Knee Implantation of Autologous Bone Marrow-Derived Mesenchymal Stromal Cells in Rheumatoid Arthritis Patients with Knee Involvement: Results of a Randomized, Triple-Blind, Placebo-Controlled Phase 1/2 Clinical Trial. Cytotherapy 2018;20:499-506. https://doi.org/10.1016/j.jcyt.2017.12.009.

Introduction

Cell microencapsulation technology: current vision of its therapeutic potential through the administration routes

Journal of Drug Delivey Science and Technology, 42 (2017), 49-62.

DOI: https://doi.org/10.1016/j.jddst.2017.03.028

Cell microencapsulation technology: current vision of its therapeutic potential through the administration routes

Ainhoa Gonzalez-Pujana[a,b], Edorta Santos-Vizcaino[a,b], Gorka Orive[a,b], Jose Luis Pedraz[a,b], Rosa Maria Hernandez[a,b,*]

[a] NanoBioCel Group, Laboratory of Pharmaceutics, School of Pharmacy, University of the Basque Country (UPV/EHU), Spain.
[b] Biomedical Research Networking Centre in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Spain.
* Corresponding author: Rosa Maria Hernandez.

ABSTRACT

Regarding the current need of new therapeutic strategies for the correct management of myriad health problems, cell microencapsulation emerges as a promising alternative. Traditionally, the goal of cell microencapsulation has been drug delivery. For this purpose, cells are entrapped within a biocompatible matrix, usually surrounded by a semipermeable membrane. This barrier allows the diffusion of nutrients, oxygen and therapeutic factors secreted by the entrapped cells while it immunoprotects the implant. Thus, a sustained and *de novo* release of therapeutics is achieved. Considering the great potential this application holds, intensive research has taken place in the field, which has led to multiple clinical trials with the aim of moving this strategy towards clinical translation. Moreover, in an emerging alternative, modifications in the design of classic microcapsules have given rise to systems that deliver cells with regenerative purposes. Since numerous body locations have been explored as valuable options for the administration of cell microcapsules, this review offers a current vision of the possibilities this technology offers attending to the administration routes.

1. Introduction

Over the last decades, the field of pharmaceutical technology has undergone major changes. The exciting development has given rise to numerous improvements in areas such as the pharmacokinetics or the administration of many drugs. Nonetheless, multiple diseases demand a tight regulation of therapeutic factors that cannot be achieved with the use of currently available drug delivery systems. Consequently, intensive research is being carried out with the aim of exploring new avenues.

One of the approaches that has shown an enormous potential is cell therapy. Primary cells, stem cells or bioengineered cells have been considered as potentially therapeutic for multiple applications including diabetes, neurodegenerative diseases or regenerative medicine [1]. Nonetheless, the progress in this field has been hampered by the difficulties in maintaining cell viability and avoiding the graft rejection by host immune response [2]. For this reason, much effort has been focused on the development of immunoisolating technologies for the long-term cell transplantation. This success would protect the implanted cells and therefore, would reduce the use of immunosuppressive therapies, having an important impact not only from a therapeutic but also from an economic standpoint [3].

In this regard, nowadays, medical technology stands at the brink of another milestone: the clinical utility of cell encapsulation. In this strategy, cells are physically isolated from the outside environment by their enclosure in a polymeric hydrogel matrix that is surrounded by a semipermeable membrane. When implanted in the body, this barrier allows the diffusion of therapeutic molecules produced by the encapsulated cells, nutrients and oxygen, while it prevents the passage of immune cells and antibodies, immunoprotecting the graft. This concept was pioneered in 1933 when Bisceglie demonstrated the maintenance of tumor cell viability after encapsulated and transplanted in the abdominal cavity of pigs [4].Three decades later, in 1964, Chang and colleagues proposed the use of semipermeable membranes as immunoisolating barriers [5]. Later, in 1980, Lim and Sun demonstrated the therapeutic application of cell encapsulation technology by returning diabetic rats to normoglycemia [6]. Since then, the transplantation of encapsulated cells has been thoroughly studied as a valuable approach to enable the sustained delivery of therapeutics.

Despite such has been the major application of cell microencapsulation, modifications on capsule design have led to other alternatives. Such is the case of non-coated and biodegradable microspheres employed to immobilize cells with regenerative purposes. To date, promising preclinical results have led to the conduction of multiple clinical trials that bring to light the real potential of this technology. In the present review, we discussed the state of the art in the field, highlighting the benefits this technology offers in therapeutics.

2. Therapeutic potential of cell encapsulation

2.1. Long-term drug delivery

The encapsulation of therapeutically active cells represents a valuable alternative for sustained drug delivery. In this strategy, cells act as customized factories producing bioactive molecules *de novo*. Thus, the system enables the delivery of unstable therapeutic factors that must conserve their physicochemical and biological properties to perform their activity. Furthermore, the long-term function of the system is permitted. In this regard, several studies have shown prolonged cell viability with the subsequent continuous production of biomolecules [7]. Consequently, the necessity of frequent administrations is eliminated. In the medical practice, this advantage would significantly improve the quality of life of patients and it would promote patient compliance by reducing the problem of bad adherence to pharmacological treatments.

Moreover, it is important to note that the long-term implantation of cells is possible because of the immunoprotection conferred by the biosystem. In order to achieve this protection, the biomaterials of choice are non-degradable and stable over time to ensure a prolonged function. Cells are entrapped in capsules that constitute a passive barrier between the cells and their immediate environment. The permselective properties allow the ingress of essential nutrients and oxygen to the core of the capsules. Conversely, it restricts the entrance of immune cells and high molecular weight effector immune molecules, such as complement factors or immunoglobulins (Fig. 1A). In any case, the host immune response will be governed by the source of the implanted cells. Indeed, allografts and xenografts evoke different responses and may require disparate capsule configurations.

For allografts, implants in which cell donor and recipient belong to the same species, the impeded contact between the graft and the host immune cells might be sufficient to prevent rejection [8]. Consequently, the sole polymer core may protect the cells. On the contrary, xenografts (transplants in which cell donor and recipient derive from different species) are known to elicit more aggressive responses. This fact is in part due to the release of xenogeneic epitopes that induce the secretion of specific antibodies towards the encapsulated tissue [9] or proinflammatory cytokines [10], compromising graft survival. Relevant immunoreactive epitopes are galactosyl residues (Gal), which may react with naturally pre-existing anti-Gal and non-Gal IgM antibodies triggering strong innate responses [11]. Therefore, xenogeneic cells demand stricter encapsulation requirements, since the polymer core that envelops the cells is too porous to provide their immunoprotection. For this reason, capsules should incorporate additional coatings in order to reduce permeability and establish a suitable molecular weight cut off (MWCO) around 70 kDa, preferably low enough to retain hyperinflammatory xenogeneic epitopes [8]. The most widely used biomaterials to perform these coatings are the polycations poly-L-lysine (PLL) [12,13] and poly-L-ornithine (PLO) [14,15]. Nevertheless, when implanted in the body, these positively charged ions may trigger strong immune responses [16-19]. To palliate this effect, a second coating of diluted alginate is often added in order to mask the positive charges. However, there has been intense debate around this topic, since the interaction between both coatings may not be sufficient to

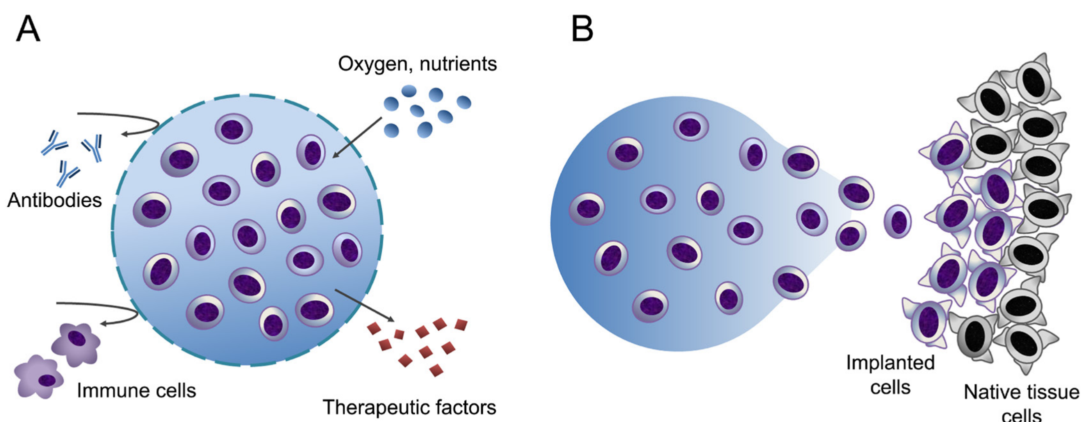


Fig. 1. Therapeutic potential of cell encapsulation. (A) Drug delivery from encapsulated cells allows a sustained release of therapeutics. **(B)** Degradable capsules act as platforms for cell delivery with regenerative purposes.

prevent the exposure of unbound polycation. Hence, the importance of the alginate layer has been questioned [20,21] and coatings remain one of the most limiting factors in the technology [22]. Alternative coatings such as chitosan [23,24], poly(methylene-co-guanidine) [19,25] or the application of diblock copolymers [26,27] are currently under the spotlight, and further, different modifications of capsules have been proposed to overcome this limitation [28,29]. Despite the biocompatibility issues of xenograft encapsulation, it has nowadays gained importance considering that it represents an attractive alternative to allografts, which are notably limited by donor shortage. Furthermore, it is important to note that recently, the use of alternative sources to allo- and xenografts has arisen as an interesting option. Such is the case of induced pluripotent stem cells (iPSCs), which have already been suggested for cell microencapsulation purposes [30]. Their extensive differentiative capacity provides a renewable source for multiple cell types. Moreover, they also overcome other current limitations since they may not origin immune or ethical problems [30].

Ideally, the optimization of immunoprotective capsules may lead to the reduction of immunosuppressive protocols. Fulfilling this goal may be tremendously beneficial for patients, as the severe side effects of these drugs, such as the high risk of infection or the predisposition for cancer development, would decrease significantly. Indeed, it has been stated that the use of immunosuppression for certain applications, such as encapsulation of pancreatic islets, has reached the not acceptable level for clinical therapy consideration [9]. Therefore, achieving this objective seems to be a prerequisite before considering the clinical application.

2.2 Regenerative medicine

Cell encapsulation holds great therapeutic potential not only regarding drug delivery, but also in the field of regenerative medicine. For this application, the goal is to deliver the immobilized cells in the damaged area so that they promote tissue regeneration by direct engraftment and secretion of multiple molecules involved in the injury healing such as cytokines and growth factors (Fig. 1B).

A valuable option for tissue engineering purposes is the use of mesenchymal stromal cells (MSCs). As multipotent progenitor cells, MSCs are able to differentiate into osteogenic, adipogenic and chondrogenic lineages [31]. An additional benefit that MSCs offer is their

hypoimmunogenic and immunomodulatory properties that stimulate endogenous regeneration mechanisms while maintaining immune homeostasis [32,33]. Indeed, multiple studies have proven the activation and recruitment of MSCs to sites of injury, where they enhance the tissue repair [33]. Furthermore, MSCs also contribute to the tissue vascularization, improving on the one hand the healing process itself, and on the other, a better adaptation of the implant because of the improved oxygen and nutrient supply.

Considering the objective of this therapy, matrix design varies since biodegradable properties are required to enable the release of the immobilized cells. Therefore, the capsule does not provide an immunoisolating barrier but acts as a support scaffold that enhances the adequate grafting of the implanted cells [22]. The breakdown rate of the capsule is a critical parameter, since it ought to be adjusted to the time needed by the implanted cells to adequately replace the scaffold. Thus, this rate should be controllable and result in a suitable balance between retention and release of the cells, so that they are able to restore the tissue and deposit their own extracellular matrix (ECM).

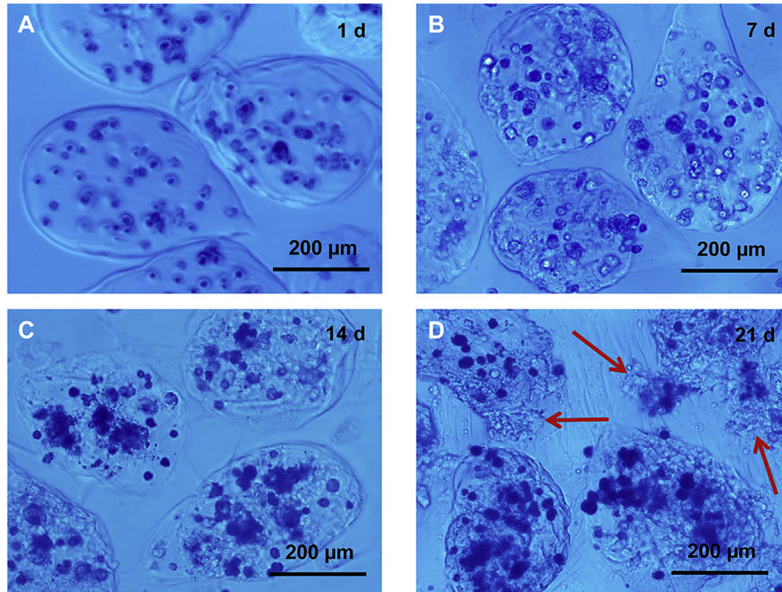


Fig. 2. Biodegradable microspheres. Optical micrographs of hUCMSC-encapsulating oxidized alginate microbeads at **(A)** 1 day, **(B)** 7 days, **(C)** 14 days and **(D)** 21 days. The microbeads started to degrade at day 14. At day 21, some microbeads started to fall apart (arrows in D). Adapted from ref. [39], © 2011, with permission from Elsevier. hUCMSC: human umbilical cord mesenchymal stromal cells.

Consequently, the selection of the biomaterial is crucial for the correct functionality of the system. Both biodegradable biomaterials and inherently non-biodegradable materials which have been subject to modifications to become degradable are suitable choices. An example of the latter is alginate, the most widely employed polymer in cell encapsulation technology. Alginate is a natural biomaterial that is intrinsically non-degradable in mammals since they do not contain the required enzyme to cleave the polymer chains. Although it is possible to dissolve ionically cross-linked alginate hydrogels in the presence of monovalent ions, the average molecular weights of commercially available alginates that are suitable for cell encapsulation, are too elevated to allow a complete renal clearance [34]. Thus, their modification is mandatory for regenerative purposes.

Among the multiple processes employed with such aim [35-38], an attractive option is the partial oxidation of the chains, which forms functional groups susceptible to hydrolysis [34]. This formula presents the advantage to control the degradation rate by only modifying the oxidation degree of the backbone. For instance, a study employing oxidized alginate showed partial degradation at day 14 and by day 21, some microbeads had already started to fall apart (Fig. 2) [39]. A different and advantageous alternative is based on the immobilized cells-driven matrix degradation. This is achieved by the modification of alginates with specific sequences that are cleaved by metalloproteinases (MMPs) secreted by the implanted cells. Thus, the entrapped cells are able to dynamically interact with both matrix and neighboring cells to reproduce more realistically the natural healing process [40,41]. In a recent study, the possibility to tune capsule breakdown by acting on the composition of the beads was demonstrated. Adjusting the alginate and divalent cation concentrations and mixing them with other polymers was shown to represent a simple solution for capsule degradation. In particular, the addition of poloxamer was found to accelerate the breakdown significantly, reaching the almost complete capsule degradation by two weeks and ensuring the cell release for tissue repair [42].

3. Administration routes

Cell microcapsules can be administered in multiple body locations (Fig. 3). This fact represents a clinical advantage, since the therapy can reach even difficult targets such as the brain or the eye. Thus, the following section is intended to show the potential cell microencapsulation technology holds attending to the administration routes.

3.1. Intraperitoneal administration

The peritoneal cavity represents an optimal site for the implantation of cell microcapsules since it can harbor large volumes [10]. Moreover, it is an easily accessible and relatively safe area. Indeed, it has been stated that intraperitoneally implanted empty microcapsules composed of purified alginate in rodents did not elicit a significant foreign body reaction [43].

Undoubtedly, the major application of the intraperitoneal implantation of cell microcapsules is diabetes. Since type 1 diabetes (T1D) requires a strict exogenous supplementation of insulin, this technology seems a suitable alternative for the sustained delivery of the protein [44,45]. An interesting avenue is the encapsulation of islets of Langerhans [45]. There are different sources from which pancreatic β-cells can be extracted. On the one hand, pancreatic

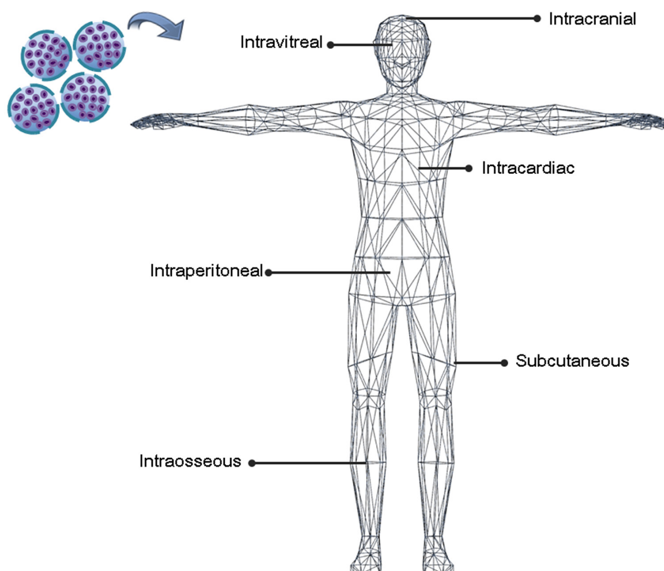


Fig. 3. Main administration routes in cell encapsulation technology.

islets can be obtained from human deceased donors [46,47]. A recent study in NOD/SCID mice demonstrated that the implantation of human islet cells led to an immediate correction of hyperglycemia that was maintained for the 5 weeks of the experimental process [48]. However, the shortage of human pancreatic donors has led researchers to explore different sources including pancreatic xenografts from species such as pigs [24,49].

On the other hand, the encapsulation of cells genetically engineered to produce insulin represents an interesting alternative [50]. Another option is the use of stem cells, which are able to differentiate into insulin-producing β-cells. In particular, the encapsulation of human stem cells can provide a chemically defined, xeno-free and easily scalable alternative for the culture of pancreatic progenitors [51]. Moreover, it was demonstrated that the 3D configuration of the capsules resulted in a stronger expression of primary maturation markers and enhanced insulin delivery in comparison to 2D culture [52]. Because of all these advantages, the encapsulation of stem cells for the treatment of T1D is gaining special attention [53,54]. In a recent study, glucose responsive mature β-cells derived from human embryonic stem cells provided a long-term glycemic control in diabetic immunocompetent C57BL/6J mice [55]. The glycemic correction was prolonged for the 174 days of study, when implants were retrieved. The analysis of explants revealed viable insulin-producing cells, highlighting the potential of this approach.

3.2 Subcutaneous administration

The subcutaneous route has been widely employed for the implantation of cell microcapsules. Besides the relatively easy and clinically applicable administration procedure, the subcutaneous space represents a rapid, inexpensive and simple method of parenteral administration. Moreover, the absorption rate is slower compared to other parenteral routes, providing a sustained effect, which may be beneficial in certain cases.

Subcutaneous implantation of encapsulated cells that produce erythropoietin (EPO) has been successfully studied for the treatment of chronic anemia since the hormone promotes the erythropoiesis and erythrocyte differentiation [56]. The system provides the continuous release of EPO, which avoids the instability of the drug and eliminates the need of repeated administrations. Interestingly, preclinical models showed *in vivo* EPO delivery during 300 days

without the implementation of immunosuppressive protocols [57]. Subsequent studies proved the maintenance of grafted cell viability and functionality, which led to the increase of the hematocrit levels in mice, certifying the possibilities of the technology [58,59].

The subcutaneous route has also been explored to administer encapsulated cells that deliver therapeutic antibodies. This tactic may serve as an anti-tumor therapy considering that the interaction between antibodies and receptors of immune cells results in immunomodulation. This modulation may be achieved via different strategies such as the over-stimulation of receptors that enhance the immune response or by the tampering of receptors involved in immune inhibition. An interesting strategy is the use of hybridoma cells that produce these antibodies [60]. For instance, the delivery of anti-OX40 and anti-CD137 from encapsulated hybridoma cells was demonstrated to elicit an efficient response because of the enhancement of tumor-specific cellular immunity [61]. In an alternative approach, HEK293 cells were genetically modified to secrete recombinant anti-carcinoembrionic antigen and anti-CD3 bispecific antibodies [62].

3.3 Intravitreal administration

Nowadays, the management of numerous ocular diseases goes through intravitreal injections that negatively impact on the comfort of patients and health care professionals. Furthermore, the hematoretinal barrier together with the unique microenvironment of the retina and special anatomy of the eye, have led to the study of alternative strategies attempting to overcome these issues.

Despite this route has not been extensively studied in cell enncapsulation technology, intravitreous administration of microcapsules might reduce the current drawbacks, while enabling the local treatment of multiple retinal diseases including age macular degeneration (AMD) or diabetic macular edema (DME). In these disorders, vascular endothelial growth factor (VEGF) is present as an angiogenic cytokine that causes pathological processes such as choroidal neovascularization [63]. Therefore, therapeutics that inhibit the action of VEGF hold great potential for the management of these ocular diseases. In particular, the soluble form of the VEGF receptor, the kinase insert domain receptor (Flk-1/KDR), has been recently targeted for its delivery through cell encapsulation. In a successful study, KDR producing encapsulated

C_2C_{12} myoblasts were administered in the intravitreous space [64]. *In vitro* studies confirmed the functionality of the system and yielded to *in vivo* analyses, where the activity of encapsulated cells was monitored over 3 weeks (Fig. 4). Additionally, in this work the challenge of manufacturing small microcapsules was accomplished, fabricating capsules with a size up to 100 μm with the use of a Flow Focusing technique. This represents a milestone in the field since the reduced microparticle size may broaden the possibilities of the technology by enabling the reach of problematic targets such as the eye or the central nervous system. In this sense, it is important to note that new microtechnologies have arisen as an interesting alternative to conventional encapsulation methods, since the latter generally do not permit fine control over the capsule size and thickness, and achieving uniform cell distributions remains a challenge. On the contrary, the microtechnology-based methods precisely control the encapsulated cell number and the size and shape of a cell-laden polymer structure. Indeed, microfluidic techniques have become an indispensable tool to produce conformal coatings [65].

3.4. Intraosseous administration

Cell-based therapies represent a promising tool for tissue regeneration. However, some delivery methods result in the dispersal of cells from target sites and poor cell viability, limiting the clinical utility of this approach. In this regard, encapsulation of cells presents multiple benefits in bone regeneration therapy. First, cells are retained in the target site enabling the correct grafting and consequent healing. Further, the system protects the cells from the mixing

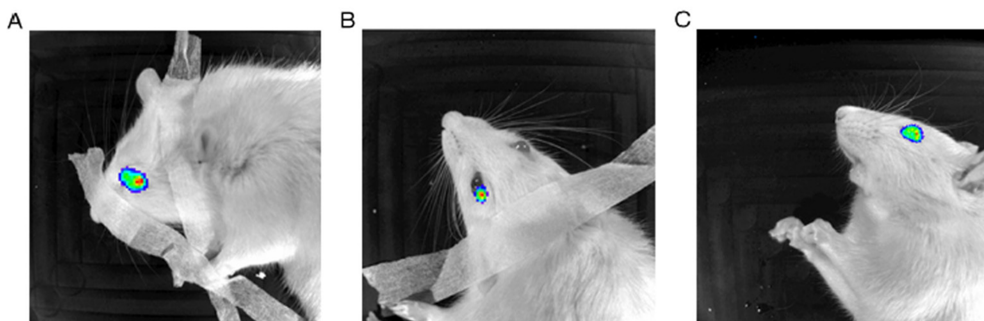


Fig. 4. Intravitreal administration of cell microcapsules. (A-C) Luminometries of encapsulated cells during 3 consecutive weeks. Treated eyes glowed strongly in all the rats. Figure shows a different rat as the representative image of the group (n=3) for each monitorized week, so that all the rats can be displayed. Reprinted from ref. [64], © 2011, with permission from Elsevier.

and injection forces and provides a potential means of injectability, allowing the conformal filling of the defect shape. Moreover, the 3D configuration has been demonstrated to improve key factors as proliferation or calcium and bone protein deposition [66].

To date, cell immobilization has been widely employed in the delivery of cells that support natural tissue regeneration [38,39]. Once implanted, the biodegradability of the system permits the release of the cells with the subsequent regenerative effect. To exert this tissue healing function, cells should promote initial stages of bone regeneration by their differentiation into osteoblasts. Consequently, MSCs are obvious candidates for bone engineering purposes. Indeed, different sources of MSCs have been proposed with such aim, including the extensively employed bone marrow MSCs (BMMSCs) [67] or umbilical cord MSCs (UCMSCs) [39]. Some more unusual others have also resulted in promising outcomes, such as gingival MSCs (GMSCs), which were able to mineralize *in situ* without any *in vitro* premineralization and demonstrated high capacities for osteo-differentiation both *in vitro* and *in vivo* [68].

MSC-mediated bone regeneration is in part controlled by the presence of growth factors that guide specific differentiation into the desired lineage. Thus, recent studies have analyzed the effects of bone morphogenetic protein-2 (BMP-2) [12]. Bone morphogenetic proteins (BMP) are a group of proteins within the transforming growth factor beta (TGFβ) superfamily that play a crucial role in the regulation of bone induction, maintenance and repair [69]. In an interesting approach, induced pluripotent stem cell-derived MSCs (iPSMSCs) were pre-osteoinduced for 2 weeks or transduced with BMP2 and encapsulated in fast-degradable alginate beads [70]. It has been stated that iPSMSCs show a higher proliferative capacity than BMMSCs, constituting a promising alternative, especially for patients with compromised health conditions whose autologous BMMSCs are no longer valid for tissue regeneration. When encapsulated iPSMSCs were implanted into cranial defects of nude rats, cells presented good viability and showed 2-3 fold increases in bone regeneration *in vivo* compared to the control group [70]. Another valuable strategy was focused on the co-encapsulation of human BMMSCs (hBMMSCs) and anti-BMP2 monoclonal antibodies, which can trap BMP ligands providing the necessary signals for osteogenic differentiation. The following five groups of microspheres were transplanted into calvarial defects of mice: (1) co-grafted hBMMSCs and anti-BMP2 antibodies (mAb + hBMMSCs), (2) encapsulated recombinant human BMP2 (rhBMP2),

(3) sole anti-BMP2 antibodies (Anti-BMP2 Ab), (4) sole hBMMSCs and (5) isotype-matched mAb (Iso mAb) with no specificity for BMP2 as the negative control. Results showed improved osteogenesis in response to the inductive signals provided by the combination of hBMMSCs and anti-BMP2 monoclonal antibodies (Fig. 5) [67]. Alternatively, the ability of BMMSCs to differentiate was promoted by the implantation of cells cotrasfected with the BMP-2 and VEGF genes [71]. Interestingly, cotransfection of both genes showed a stronger osteogenic induction than individual BMP-2-transfected microencapsulated cells. This may be explained by the potent angiogenic effect of VEGF, since angiogenesis seems to be a prerequisite for bone regeneration. Furthermore, this growth factor also promotes osteoblast differentiation. These data confirm the importance of presenting inductive signals for differentiation, which results in a promising tool for regenerative purposes in bone defects.

3.5. Intracranial administration

Systemic administration of therapeutic agents is critically hampered by poor delivery to the brain. The blood brain barrier (BBB) regulates the passage of molecules between the blood and the brain parenchyma, ensuring the protection of the brain from noxious factors present in blood. In spite of representing an essential structure to promote brain health, the BBB represents a major obstacle for drug delivery in the diseased brain [72]. With the aim of avoiding the limitations caused by the BBB, the implantation of encapsulated therapeutically active cells directly into the brain renders a promising strategy, since it provides a local long-term delivery of therapeutics produced *de novo* and reduces the side effects caused by systemic delivery [73]. Nevertheless, for administration and extraction procedures, invasive protocols are necessary, which represents a major hurdle in the technology.

Since neurotrophic factors promote the maintenance of normal neuronal survival, function and differentiation, their delivery through cell encapsulation might exert important benefits in neurodegenerative diseases. That is the case of Alzheimer's disease (AD), the most common form of dementia, which is accompanied by neuronal loss. Two thoroughly studied factors in AD are the abnormally phosphorilated tau protein and the deposition of amyloid-β peptide (Aβ) that seem to be related to this degenerative dysfunction. Consequently, the delivery of angiogenic factors such as VEGF has been proposed to exert neuroprotective effects.

The administration of encapsulated VEGF secreting cells in mice resulted in the reduction of hyperphosphorilated tau and in neovascularization, which promoted Aβ clearance. These facts led to a decrease in cell death and improved cognition [74]. Similarly, a posterior study demonstrated enhanced cellular proliferation in the hippocampal dentate gyrus, a successful approach to treat brain amyloidosis [75].

Nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF) secreting cells have been proposed for the treatment of Parkinson's disease (PD). PD is a degenerative

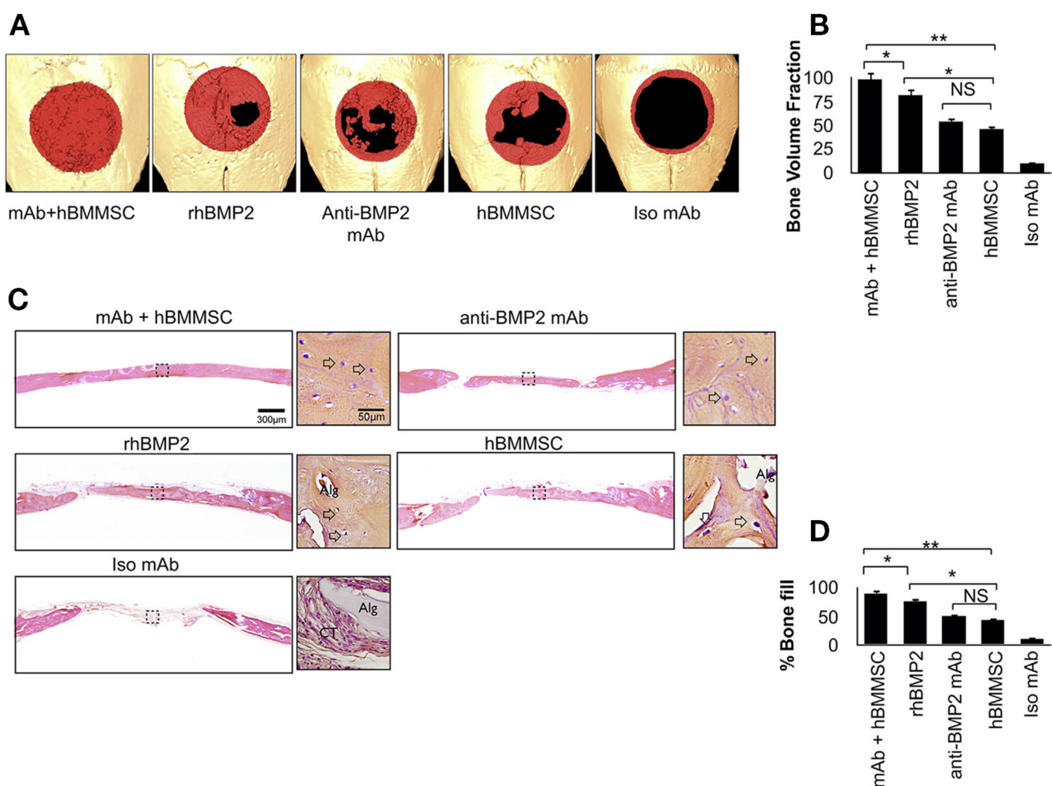


Fig. 5. Bone regeneration. hBMMSCs encapsulated in RGD-alginate microspheres loaded with anti-BMP2 mAb contribute to bone regeneration in a critical-size calvarial defect model. **(A)** Micro-CT results of bone repair in mouse calvarial defects. Regenerated bone is pseudo colored red. **(B)** Semi-quantitative analysis of bone formation based on micro-CT images; **(C)** Microanatomic representation of repair of critical-size defects in the mouse calvaria after 8 weeks of transplantation at high (40x) and low (4x) magnifications stained with H&E. Arrows point to osteocytes in lacunae. **(D)** Histomorphometric analysis of calvarial defects showing the relative amount of bone formation in the critical size calvarial defect model. *$p < 0.05$, **$p < 0.01$, n = 4 for each group. Abbreviations: hBMMSCs: human bone marrow mesenchymal stromal cells. rhBMP2: recombinant human BMP-2. Iso mAb: isotype monoclonal antibodies. NS: no statistically significant differences. Alg: alginate. CT: connective tissue. Reprinted from ref. [67], © 2013, with permission from Elsevier.

central nervous system disease that results in motor impairment due to the gradual loss of dopaminergic neurons in the *substantia nigra*. The local and sustained delivery of these factors may enhance neuron function, correcting the defective imbalance present in the disease. This fact becomes a crucial advantage in comparison to current treatments as it overcomes the problem of temporary efficacy and it represents a strategy that protects neurons, avoiding their dysfunction and loss instead of only elevating the dopamine levels [76]. In particular, it was demonstrated that encapsulated cells were able to deliver GDNF to the striatum of parkinsonian rats over 3 months, which resulted in a significant behavioral improvement [77]. In a different attempt, cell microcapsules were employed as a vehicle for the delivery of NGF in order to support the viability of co-grafted unencapsulated chromaffin cells. Interestingly, encapsulated NGF secreting cells significantly enhanced the survival of co-grafted chromaffin cells, leading to an important improvement in animal rotational behavior [78,79].

Another auspicious avenue is the administration of capsules containing glucagon-like peptide-1 (GLP-1) producing cells. The activation of GLP-1 receptors in brain results in neurotrophic and neuroprotective effects, which can be used to treat disorders such as amyotrophic lateral sclerosis (ALS) [80], AD [81] or brain injury [82-83].

Considering the positive results obtained with the brain delivery of neurotrophic factors, the entrapment of cells that naturally secrete them seems a promising strategy. Such is the case of choroid plexus cells (CPCs), which enable the restoration of brain tissue by the local secretion of biologically active factors [84,85]. In an interesting attempt, CPCs were employed for the treatment of AD [86]. Aβ memory impairment was exogenously induced to rats, and subsequently, capsules containing CPCs were transplanted overlying the cerebral cortex. Results showed an increase in neurogenesis and a decrease in apoptosis. Consequently, the treatment resulted in an important animal recovery and improved long-term memory.

3.6. Intracardiac administration

Conventional management of myocardial infarction and stroke can only relieve symptoms and slow the deterioration progress, it does not restore the cardiac function. To overcome this problem, stem MSC-based therapies hold great promise because of their regenerative properties. Indeed, MSCs have been reported to secrete multiple active molecules that assist

the tissue regeneration, including growth factors such as hepatocyte growth factor (HGF) or VEGF. Nonetheless, implanted cell retention and the maintenance of their effects remains a challenge since the continuous contraction of the heart leads to the mechanical loss of the injected cells [87]. In fact, studies have shown that no more than 0.1-15% of cells is retained within the myocardium [88-90]. To avoid this problem, the encapsulation of cells has emerged as a proper alternative. The higher size of microcapsules favors the retention in the cardiac tissue, and therefore, the local and continuous delivery of healing factors from the entrapped cells is enabled [91]. In an interesting example, adipose tissue-derived MSCs (ASCs) were enclosed in genipin-crosslinked alginate chitosan microcapsules for *in vivo* xenotransplantation in infarcted rat hearts. The enhanced retention led to a reduction in fibrosis and cardiac dysfunction [92].

Furthermore, the possibility to track the survival and migration of encapsulated cells *in vivo* may represent an asset to improve the safety of the cardiac implant. In a recent study, a cell encapsulation system was designed in which porcine ASCs were enveloped in alginate microcapsules and labeled with superparamagnetic iron oxide (SPIO) nanoparticles [93]. Cells showed an adequate viability and were detected up to the end of the *in vivo* studies. In a different approach, encapsulated human MSCs were tracked by means of bioluminescence, which revealed that encapsulation enhanced cell retention and minimized scar formation in the cardiac tissue [94]. Despite the promising results, further investigation is needed in order to fully comprehend the underlying biological effects of such cells in cardiac repair.

4. Clinical trials

The proof of principle of cell encapsulation has been successfully demonstrated in experimental animal models and should pave the way towards clinical translation. Indeed, the great potential this technology holds has led researches to the conduction of several clinical trials. In this regard, Table 1 gathers some relevant studies.

Trials involving cell microencapsulation have been carried out for the treatment of multiple diseases. In particular, much effort has been devoted to materialize the leap to clinics of this technology for the treatment diabetes. The first human clinical trial in cell encapsulation

Table 1. Clinical trials using cell microcapsules classified attending to their application.
APA: alginate-PLL/PLO-alginate. MSCs: mesenchymal stromal cells, GLP1: glucagon-like peptide-1, PLO: poly-L-ornithine, PLL: poly-L-lysine, CYP2B1: cytochrome P450 2B1.

Investigator or company	Cell source	Biomaterial	Transplant site	Immunosupression	Ref.
Diabetes					
Soon-Shiong *et al.*	Allogeneic islets	APA microcapsule	Peritoneal cavity	Yes	[95]
Calafiore *et al.*	Allogeneic islets	APA microcapsule	Peritoneal cavity	No	[96,97]
Tuch *et al.*	Allogeneic islets	Ba^{+2} alginate microbeads	Peritoneal cavity	No	[98]
Jacobs-Tulleneers-Thevissen *et al.*	Allogeneic islets	Ca^{+2}/Ba^{+2} alginate microbeads	Peritoneal cavity	No	[48]
Living Cell Technologies (LCT)	Xenogeneic islets	APA microcapsule	Peritoneal cavity	No	[99]
Intracerebral hemorrhage					
Brinker *et al.*	Allogeneic MSCs transfected w/ GLP-1	Alginate microcapsule	Brain tissue cavity	No	[104]
Parkinson's disease					
Living Cell Technologies (LCT)	Xenogeneic choroid plexus cells (porcine)	Alginate-PLO microcapsules	Intracranial (Brain)	No	[105]
Pancreatic cancer					
Löhr *et al.*	Allogeneic 293 cells secreting CYP2B1	Cellulose-sulfate microcapsule	Tumor blood vessels	-	[109-112]

dates back to 1994 [95] and today, new strategies are still being tested in the ongoing studies [10]. After the pioneering work carried out by Soon-Shiong *et al.*, in which glycemic control was achieved for nine months in an immunosuppressed patient [95], several studies have demonstrated the potential of cell encapsulation for the treatment of diabetes. Concomitant immunosuppressive protocols have been left aside and the technique has been demonstrated valuable for immunoisolation showing neither side effects of the grafting procedure nor evidences of immune sensitization [96,97]. Moreover, results have shown the maintenance of insulin levels for long periods of time [98] and adequate cell viability and glucose-responsiveness after three months of transplantation [48]. Nowadays, the company Living Cell Technologies (LCT) is working in late-stage clinical trials of the DIABECELL® product. Studies started in 1996, and from then on, promising outcomes have been presented [99-103]. The retention of capsules was reported up to nine and a half years after transplantation. Recently, a website newsletter announced the launch of Phase IIb/III studies [10].

In the case of intracerebral hemorrhage, the aim of the therapy is to improve the outcome after surgical evacuation of the hematoma by enabling the local delivery of the neuroprotective and anti-inflammatory factor GLP-1. Phase I/II clinical trials have evaluated the safety of the treatment and neither side effects from the surgical intervention, nor implant-related side effects were shown in the interim evaluation of patients [104].

Concerning PD, the contribution of the LCT company is remarkable [105]. Their product, branded NTCELL®, obtained encouraging preclinical data in a model of PD [106]. In a Phase I/IIa clinical trial completed in June 2015, in which the safety and clinical effect of the capsules were investigated, implants were demonstrated to be safe and well tolerated. Moreover, the clinical symptoms of PD in all the patients improved, maintaining the effect up to 26 weeks after transplantation [107]. In March 2016, a Phase IIb study started in order to confirm the most effective dose [105]. The 24 month follow-up preliminary results were released on May 2019, suggesting a clinically relevant effect. However, the company claims that despite the successful outcomes, a confirmative larger Phase III study is still required. [108].

Pancreatic cancer treatment through cell microcapsules has also been studied in human patients. In particular, clinical trials have been conducted administering encapsulated cells

over-expressing cytochrome P450 2B1 (CYP2B1). CYP2B1 is an ifosfamide-converting enzyme that transforms the cytotoxic agent ifosfamide into its active metabolites. In normal conditions, this conversion occurs in the liver, and the cytotoxic metabolites reach the site of the tumor via the systemic circulation. However, the systemic side effects significantly limit the therapy. Consequently, the implementation of cell encapsulation may overcome this problem. Microcapsules may be directly implanted in the tumor site with the subsequent low-dose ifosfamide administration. Thus, the targeted transformation of the prodrug is enabled in the affected area. In a Phase I/II study, with a ifosfamide dose of 1g/m^2/day, the median patient survival was doubled and safety was assessed as only one patient experienced adverse effects related to the treatment [109-111]. A posterior Phase II trial, where the ifosfamide dose was increased to 2g/m^2/day, showed similar efficacy profiles but more severe side effects [112]. Altogether, these promising results suggest that later stage clinical trials may be carried out.

In brief, currently, multiple approaches intend to bring this technology closer to the clinical utility, and their success would represent a landmark in clinics and a starting point for an improved management of myriad health problems.

5. Future perspectives and challenges

In spite of the significant advancements in cell encapsulation since the pioneering studies almost four decades ago, various challenges should be resolved prior to the leap to clinical translation. Biocompatibility is one of the major issues that remains unsolved. The performance of the implant directly depends on its interaction with the host immune system. When capsules are administered, the immune recognition triggers a cascade of cellular processes that comprise inflammation, formation of foreign body giant cells, fibrosis and injury in the implantation site. This response is deleterious not only for the grafted cells but also for patients, as it may cause discomfort and pain. With the aim of analyzing in depth the factors related to biocompatibility, a recent study characterized the expression of immunological and fibrosis markers on microcapsules retrieved after transplantation into streptozotocin (STZ)-induced C57B6/J diabetic mice (Fig. 6A) [113]. To address this problem, several approaches have been studied, some of which presented successful results. For instance, the co-administration of

anti-inflammatory drugs seems a suitable alternative, since inflammation implies the vasodilatation and the subsequent augmented permeability that promotes the migration of immune cells and proteins to the damaged tissue. Anti-inflammatory molecules including curcumin [113], dexamethasone [114,115] or pentoxifylline [116] and antagonists of inflammation mediators such as high mobility group box protein 1 (HMGB1) [117] have demonstrated successful outcomes. In particular, microcapsules containing dexamethasone or curcumin retrieved two months post-transplantation showed that the latter significantly improved significantly the capsule biocompatibility (Fig. 6B-J) [113]. A different strategy is the addition of diverse motifs with anti-inflammatory properties to the capsule configuration. Among them, sulfated alginates [118] or interleukin-1 receptor (IL-1R) [29] have shown to reduce the expression of disparate inflammatory cytokines and to improve viability when exposed to them. Finally, it is relevant to note that the co-encapsulation of target effector cells with MSCs [32,119-122] or Sertoli cells [123,124] is particularly interesting. These cells exert valuable immunomodulatory effects that may improve the fate of implanted effector cells giving rise to an enhanced system functionality and long-term duration.

Also regarding biocompatibility, the size of capsules has been subject to an intense debate. Importantly, a recent study reported that spherical materials of 1.5 mm in diameter or greater turned out to be noticeably more biocompatible when compared to their smaller-sized or counterparts. In this work, the key role of shape was also highlighted, stating that simply increasing the size was not sufficient to avoid foreign body reactions. In particular, alginate spheres of 1.5 mm in diameter were tested and results revealed that these particles were able to ward off cellular deposition for at least 6 months [125]. However, traditionally, much effort has been made with the purpose to reduce the size of the encapsulating particle [8], since small capsules are required for administration in locations such as the brain or the eye. Moreover, it has been widely accepted that small capsules (< 350 μm) allow better diffusion and transportation of nutrients and oxygen to the encapsulated cells compared to medium (350 - 700 μm) and larger capsules (>700 μm) [126]. Consequently, the selection of a proper size remains a challenge since a balance between a correct transport and a minimal immune response ought to be reached.

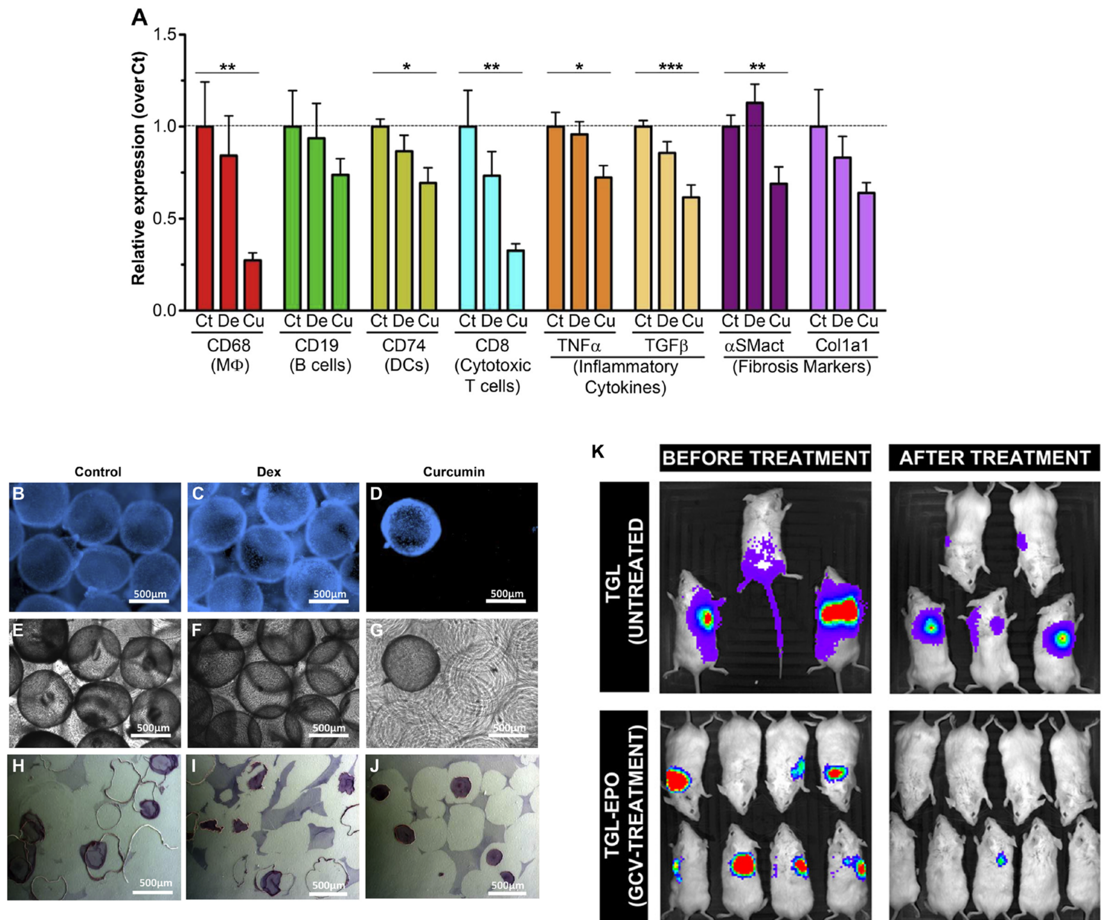


Fig. 6. Future challenges in cell encapsulation: biocompatibility and biosafety. (A) Characterization of fibrotic pericapsular overgrowth on microcapsules retrieved after transplantation into STZ-induced C57B6/J diabetic mice. qPCR analysis of host (mouse) expression of immunological and fibrosis markers on alginate capsules and surrounding fat pad tissue retrieved one month post-transplantation. The markers were macrophage (Mf) marker CD68, B cell marker CD19, dendritic cell marker CD74, cytotoxic T cell marker CD8, inflammatory cytokines TNFα and TGFβ, and fibrosis-associated activated-fibroblast marker α-smooth muscle actin (aSMact) and collagen 1A1 (Col1a1). Data: mean ± s.e.m, (n = 7). *, **, *** denote $p < 0.05, 0.01, 0.001$, respectively. Fluorescent images of DNA-stained control microcapsules **(B)** and microcapsules with dexamethasone **(C)** or curcumin **(D)** retrieved two months post-transplantation. Phase contrast images of the same control microcapsules **(E)** and microcapsules with dexamethasone **(F)** or curcumin **(G)**. Histology H&E sections of retrieved control microcapsules **(H)** and microcapsules with dexamethasone **(I)** or curcumin **(J)**. **(K)** Light emission was captured before and after ganciclovir (GCV) treatment for both TGL-EPO (GCV-treated) and TGL (untreated) mice. (A-J) reprinted from ref. [113], © 2013, and (K) reprinted from ref. [135], © 2012, with permission from Elsevier.

Controlling the intra-capsular microenvironment remains another important challenge. Culturing cells out of their natural niches demands a broad insight into cell biology, since cells are not individual entities but part of a sophisticated and dynamic ensemble of the cell itself, extracellular matrix (ECM) molecules, soluble morphogens / growth factors and the neighboring cells [127]. Considering that the connections among these elements govern the tissue physiology, the recreation of the natural scenario of cells will have nothing but significant benefits in cell function and viability. Therefore, in order to mimic the native microenvironment, the capsule can be modified with different motifs. Full-length ECM protein incorporation is possible [22] and also the use of short-peptide sequences containing functional domains from these molecules. Successful results have been obtained with arginine-glycine-aspartic acid (RGD), which is part of plenty of ECM proteins such as fibronectin or laminin, and undoubtedly, one of the most extensively utilized moieties [31,128-130]. Other examples are the YIGSR (Tyr-Ile-Gly-Ser-Arg) and IKVAV (Ile-Lys-Val-Ala-Val) motifs present in laminin [51,131]. Alternatively, other strategies have been explored, including the addition of ECM powder [132], the inclusion of growth factor binding domains [133] or the attachment of galactosylated chitosan in the polymeric core [134].

Undeniably, one of the most relevant aspects concerning the clinical feasibility of cell encapsulation technology is the safety of the implant. Indeed, the security of use is mandatory, and unfortunately, it still cannot be fully provided nowadays. Thus, several studies have devoted their efforts to design capsules which are inducible or which can be inactivated. For instance, the incorporation of suicide genes in the genome of the entrapped cells has resulted in the efficacious cease of biologically active factor release. In particular, a successful study analyzed the ganciclovir (GCV) mediated inactivation of entrapped cells transfected with the TGL triple-fusion reporter gene, which codifies for the suicide gene Herpes Simplex virus type 1 thymidine-kinase (HSV1-TK), green fluorescent protein (GFP) and Firefly Luciferase ($SFG_{NES}TGL$), to block their therapeutic effects (Fig. 6K) [135]. Similarly, TetOn/off systems provide a tool for controlled delivery of therapeutics [12,136].

Lastly, it is relevant to mention that the scalability of the biosystem is also a vital parameter to bear in mind when it comes to a conceivable adaptation to clinics. Currently, the available high throughput encapsulation systems enable the manufacture of large numbers of

capsules [137,138]. Nevertheless, the existence of multiple lab-to-lab variations represents an obstacle in the search of a proper scalability, since adequate reproducibility is not achieved. In an outstanding attempt to overcome this issue, studies were carried out around the inventarisation and comparison of diverse technologies to identify the technical aspects that need further characterization and to understand the existing lab-to-lab variations. To this end, five distinct factors that influence the final properties of the capsule were reported to be mandatory for a suitable description of the system: the applied polymer, permeability, surface properties, biocompatibility, and storage conditions [139]. Furthermore, the production of cell encapsulation technologies is notably pricey considering the complexity of the technology, the long periods required to expand cell cultures or the high-prized reagents. This fact represents another hurdle for scalability since reasonable prices are crucial for patient accessibility [22]. Moreover, prior to the scale up of the products, it is mandatory to take the regulatory approval. The European institutions agreed on a regulation for Advanced Therapies Medical Products (Regulation (EC) 1394/2007). It was designed to ensure the free movement of those products within Europe, to facilitate access to the European market and to foster the competitiveness of European companies in the field, while guaranteeing the highest level of health protection for patients. Interestingly, this regulation considers that multiple advanced therapy products combine biological materials (tissues, cells) with chemical structures such as polymer scaffolds, and that they need adapted requirements since they lie at the border of the traditional pharmaceutical field [140].

6. Conclusions

The continuous advancement in cell encapsulation is giving rise to multiple therapeutic treatments that hold great potential. The major advantage of this technology lies in the extraordinary adaptability of the system. Depending on the nature of the biomaterials that structure the capsule, the functionality will vary, resulting in different applications. On the one hand, non-biodegradable biomaterials that form a polymeric core surrounded by a semipermeable membrane lead to systems that act as biofactories by releasing the desired therapeutic factor *de novo* and in a sustained fashion. Different cell sources and the bioengineering techniques facilitate the delivery of disparate bioactive molecules, multiplying the applicability of the approach. On the other, biodegradable materials, both intrinsically degradable and

non-degradable appropriately modified to be so, enable the delivery of the encapsulated cells with regenerative purposes.

The development in cell encapsulation has been significant because of the great number of preclinical studies carried out. Consequently, invaluable knowledge has been gained around the system, moving this strategy towards clinical translation. Although still optimization needs to take place in order to achieve the clinical approval regarding biosafety or biocompatibility, the clinical trials conducted in the field are notably increasing the feasibility of this promising technology.

Acknowledgments

A. Gonzalez-Pujana thanks the Basque Government (Department of Education, Universities and Research) for the PhD fellowship. This project was partially supported by the Basque Government (Consolidated Groups, IT-907-16) and the University of the Basque Country UPV/EHU (UFI11/32).

References

[1] R.M. Hernandez, G. Orive, A. Murua, J.L. Pedraz, Microcapsules and microcarriers for in situ cell delivery, Adv. Drug Deliv. Rev. 62 (2010) 711-730. doi:10.1016/j.addr.2010.02.004.

[2] R. Mahou, S. Passemard, M. Carvello, A. Petrelli, F. Noverraz, S. Gerber-Lemaire, C. Wandrey, Contribution of polymeric materials to progress in xenotransplantation of microencapsulated cells: a review, Xenotransplantation 23 (2016) 179-201. doi:10.1111/xen.12240.

[3] G. Orive, A.R. Gascon, R.M. Hernandez, M. Igartua, J. Luis Pedraz, Cell microencapsulation technology for biomedical purposes: novel insights and challenges, Trends Pharmacol. Sci. 24 (2003) 207-210. doi:10.1016/S0165-6147(03)00073-7.

[4] V. Bisceglie, Über die antineoplastische Immunität, Zeitschrift Für Krebsforschung 40 (1934) 122-140.

[5] T.M. Chang, Semipermeable Microcapsules, Science 146 (1964) 524-525.

[6] F. Lim, A.M. Sun, Microencapsulated islets as bioartificial endocrine pancreas, Science 210 (1980) 908-910.

[7] D.F. Emerich, G. Orive, C. Thanos, J. Tornoe, L.U. Wahlberg, Encapsulated cell therapy for neurodegenerative diseases: from promise to product, Adv. Drug Deliv. Rev. 67-68 (2014) 131-141. doi:10.1016/j.addr.2013.07.008.

[8] G. Orive, E. Santos, D. Poncelet, R.M. Hernandez, J.L. Pedraz, L.U. Wahlberg, P. De Vos, D. Emerich, Cell encapsulation: technical and clinical advances, Trends Pharmacol. Sci. 36 (2015) 537-546. doi:10.1016/j.tips.2015.05.003.

[9] D.W. Scharp, P. Marchetti, Encapsulated islets for diabetes therapy: history, current progress, and critical issues requiring solution, Adv. Drug Deliv. Rev. 67-68 (2014) 35-73. doi:10.1016/j.addr.2013.07.018.

[10] M. Qi, Transplantation of Encapsulated Pancreatic Islets as a Treatment for Patients with Type 1 Diabetes Mellitus, Advances in Medicine 2014 (2013) 429710. doi:10.1155/2014/429710.

[11] G.A. Paredes Juarez, M. Spasojevic, M.M. Faas, P. de Vos, Immunological and technical considerations in application of alginate-based microencapsulation systems, Front. Bioeng. Biotechnol. 2 (2014) 26. doi:10.3389/fbioe.2014.00026.

[12] D. Qian, B. Bai, G. Yan, S. Zhang, Q. Liu, Y. Chen, X. Tan, Y. Zeng, Construction of doxycycline-mediated BMP-2 transgene combining with APA microcapsules for bone repair, Artif. Cells Nanomed Biotechnol. 44 (2016) 270-276. doi:10.3109/21691401.2014.942458.

[13] J. Hu, H. Li, G. Chi, Z. Yang, Y. Zhao, W. Liu, C. Zhang, IL-1RA gene-transfected bone marrow-derived mesenchymal stem cells in APA microcapsules could alleviate rheumatoid arthritis, Int. J. Clin. Exp. Med. 8 (2015) 706-713.

[14] V. Ibarra, A.A. Appel, M.A. Anastasio, E.C. Opara, E.M. Brey, This paper is a winner in the Undergraduate category for the SFB awards: Evaluation of the tissue response to alginate encapsulated islets in an omentum pouch model, J. Biomed. Mater. Res. 104 (2016) 1581-1590. doi:10.1002/jbm.a.35769.

[15] R. Pareta, J.P. McQuilling, S. Sittadjody, R. Jenkins, S. Bowden, G. Orlando, A.C. Farney, E.M. Brey, E.C. Opara, Long-term function of islets encapsulated in a re-designed alginate microcapsule construct in omentum pouches of immune-competent diabetic rats, Pancreas 43 (2014) 605-613. doi:10.1097/MPA.0000000000000107.

[16] J.M. Anderson, A. Rodriguez, D.T. Chang, Foreign body reaction to biomaterials, Semin. Immunol. 20 (2008) 86-100. doi:10.1016/j.smim.2007.11.004.

[17] A.M. Rokstad, O.L. Brekke, B. Steinkjer, L. Ryan, G. Kollarikova, B.L. Strand, G. Skjak-Braek, J.D. Lambris, I. Lacik, T.E. Mollnes, T. Espevik, The induction of cytokines by polycation containing microspheres by a complement dependent mechanism, Biomaterials 34 (2013) 621-630. doi:10.1016/j.biomaterials.2012.10.012.

[18] P. Orning, K.S. Hoem, A.E. Coron, G. Skjak-Braek, T.E. Mollnes, O.L. Brekke, T. Espevik, A.M. Rokstad, Alginate microsphere compositions dictate different mechanisms of complement activation with consequences for cytokine release and leukocyte activation, J. Control. Release 229 (2016) 58-69. doi:10.1016/j.jconrel.2016.03.021.

[19] A.M. Rokstad, O.L. Brekke, B. Steinkjer, L. Ryan, G. Kollarikova, B.L. Strand, G. Skjak-Braek, I. Lacik, T. Espevik, T.E. Mollnes, Alginate microbeads are complement compatible, in contrast to polycation containing microcapsules, as revealed in a human whole blood model, Acta Biomater. 7 (2011) 2566-2578. doi:10.1016/j.actbio.2011.03.011.

[20] S.K. Tam, S. Bilodeau, J. Dusseault, G. Langlois, J.P. Halle, L.H. Yahia, Biocompatibility and physicochemical characteristics of alginate-polycation microcapsules, Acta Biomater. 7 (2011) 1683-1692. doi:10.1016/j.actbio.2010.12.006.

[21] S. Juste, M. Lessard, N. Henley, M. Menard, J.P. Halle, Effect of poly-L-lysine coating on macrophage activation by alginate-based microcapsules: assessment using a new in vitro method, J. Biomed. Mater. Res. A. 72 (2005) 389-398. doi:10.1002/jbm.a.30254.

[22] E. Santos, J.L. Pedraz, R.M. Hernandez, G. Orive, Therapeutic cell encapsulation: ten steps towards clinical translation, J. Control. Release 170 (2013) 1-14. doi:10.1016/j.jconrel.2013.04.015.

[23] G. Zheng, X. Liu, X. Wang, L. Chen, H. Xie, F. Wang, H. Zheng, W. Yu, X. Ma, Improving stability and biocompatibility of alginate/chitosan microcapsule by fabricating bi-functional membrane, Macromol. Biosci. 14 (2014) 655-666. doi:10.1002/mabi.201300474.

[24] H.K. Yang, D.S. Ham, H.S. Park, M. Rhee, Y.H. You, M.J. Kim, J. Shin, O.Y. Kim, G. Khang, T.H. Hong, J.W. Kim, S.H. Lee, J.H. Cho, K.H. Yoon, Long-term Efficacy and Biocompatibility of Encapsulated Islet Transplantation With Chitosan-Coated Alginate Capsules in Mice and Canine Models of Diabetes, Transplantation 100 (2016) 334-343. doi:10.1097/TP.0000000000000927.

[25] G. Orive, R.M. Hernandez, A.R. Gascon, M. Igartua, J.L. Pedraz, Development and optimisation of alginate-PMCG-alginate microcapsules for cell immobilisation, Int. J. Pharm. 259 (2003) 57-68. doi:10.1016/S0378-5173(03)00201-1.

[26] M. Spasojevic, G.A. Paredes-Juarez, J. Vorenkamp, B.J. de Haan, A.J. Schouten, P. de Vos, Reduction of the inflammatory responses against alginate-poly-L-lysine microcapsules by anti-biofouling surfaces of PEG-b-PLL diblock copolymers, PLoS One 9 (2014) e109837. doi:10.1371/journal.pone.0109837.

[27] M. Spasojevic, S. Bhujbal, G. Paredes, B.J. de Haan, A.J. Schouten, P. de Vos, Considerations in binding diblock copolymers on hydrophilic alginate beads for providing an immunoprotective membrane, J. Biomed. Mater. Res. A. 102 (2014) 1887-1896. doi:10.1002/jbm.a.34863.

[28] T. Chen, J. Yuan, S. Duncanson, M.L. Hibert, B.C. Kodish, G. Mylavaganam, M. Maker, H. Li, M. Sremac, M. Santosuosso, B. Forbes, S. Kashiwagi, J. Cao, J. Lei, M. Thomas, C. Hartono, D. Sachs, J. Markmann, A. Sambanis, M.C. Poznansky, Alginate encapsulant incorporating CXCL12 supports long-term allo- and xenoislet transplantation without systemic immune suppression, Am. J. Transplant. 15 (2015) 618-627. doi:10.1111/ajt.13049.

[29] J. Su, B.H. Hu, W.L. Lowe Jr, D.B. Kaufman, P.B. Messersmith, Anti-inflammatory peptide-functionalized hydrogels for insulin-secreting cell encapsulation, Biomaterials 31 (2010) 308-314. doi:10.1016/j.biomaterials.2009.09.045.

[30] I. Horiguchi, M.M. Chowdhury, Y. Sakai, Y. Tabata, Proliferation, morphology, and pluripotency of mouse induced pluripotent stem cells in three different types of alginate beads for mass production, Biotechnol. Prog. 30 (2014) 896-904. doi:10.1002/btpr.1891.

[31] A. Garate, J. Ciriza, J.G. Casado, R. Blazquez, J.L. Pedraz, G. Orive, R.M. Hernandez, Assessment of the Behavior of Mesenchymal Stem Cells Immobilized in Biomimetic Alginate Microcapsules, Mol. Pharm. 12 (2015) 3953-3962. doi:10.1021/acs.molpharmaceut.5b00419.

[32] L. Zanotti, A. Sarukhan, E. Dander, M. Castor, J. Cibella, C. Soldani, A.E. Trovato, C. Ploia, G. Luca, M. Calvitti, F. Mancuso, I. Arato, M. Golemac, N. Jonjic, A. Biondi, R. Calafiore, M. Locati, G. D'Amico, A. Viola, Encapsulated mesenchymal stem cells for in vivo immunomodulation, Leukemia 27 (2013) 500-503. doi:10.1038/leu.2012.202.

[33] S. Ma, N. Xie, W. Li, B. Yuan, Y. Shi, Y. Wang, Immunobiology of mesenchymal stem cells, Cell Death Differ. 21 (2014) 216-225. doi:10.1038/cdd.2013.158.

[34] K.Y. Lee, D.J. Mooney, Alginate: properties and biomedical applications, Prog. Polym. Sci. 37 (2012) 106-126. doi:10.1016/j.progpolymsci.2011.06.003.

[35] O. Jeon, K.H. Bouhadir, J.M. Mansour, E. Alsberg, Photocrosslinked alginate hydrogels with tunable biodegradation rates and mechanical properties, Biomaterials 30 (2009) 2724-2734. doi:10.1016/j.biomaterials.2009.01.034.

[36] H.J. Kong, E. Alsberg, D. Kaigler, K.Y. Lee, D.J. Mooney, Controlling Degradation of Hydrogels via the Size of Cross-Linked Junctions, Adv Mater 16 (2004) 1917-1921. doi:10.1002/adma.200400014.

[37] T. Boontheekul, H.J. Kong, D.J. Mooney, Controlling alginate gel degradation utilizing partial oxidation and bimodal molecular weight distribution, Biomaterials 26 (2005) 2455-2465. doi:10.1016/j.biomaterials.2004.06.044.

[38] S.K. Leslie, D.J. Cohen, J. Sedlaczek, E.J. Pinsker, B.D. Boyan, Z. Schwartz, Controlled release of rat adipose-derived stem cells from alginate microbeads, Biomaterials 34 (2013) 8172-8184. doi:10.1016/j.biomaterials.2013.07.017.

[39] H. Zhou, H.H. Xu, The fast release of stem cells from alginate-fibrin microbeads in injectable scaffolds for bone tissue engineering, Biomaterials 32 (2011) 7503-7513. doi:10.1016/j.biomaterials.2011.06.045.

[40] M.P. Lutolf, J.L. Lauer-Fields, H.G. Schmoekel, A.T. Metters, F.E. Weber, G.B. Fields, J.A. Hubbell, Synthetic matrix metalloproteinase-sensitive hydrogels for the conduction of tissue regeneration: engineering cell-invasion characteristics, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 5413-5418. doi:10.1073/pnas.0737381100.

[41] M.W. Tibbitt, K.S. Anseth, Hydrogels as extracellular matrix mimics for 3D cell culture, Biotechnol. Bioeng. 103 (2009) 655-663. doi:10.1002/bit.22361.

[42] L. Draghi, D. Brunelli, S. Fare, M.C. Tanzi, Programmed cell delivery from biodegradable microcapsules for tissue repair, J. Biomater. Sci. Polym. Ed. 26 (2015) 1002-1012. doi:10.1080/09205063.2015.1070706.

[43] P. de Vos, C.G. van Hoogmoed, J. van Zanten, S. Netter, J.H. Strubbe, H.J. Busscher, Long-term biocompatibility, chemistry, and function of microencapsulated pancreatic islets, Biomaterials 24 (2003) 305-312. doi:10.1016/S0142-9612(02)00319-8.

[44] J.A. Steele, J.P. Halle, D. Poncelet, R.J. Neufeld, Therapeutic cell encapsulation techniques and applications in diabetes, Adv. Drug Deliv. Rev. 67-68 (2014) 74-83. doi:10.1016/j.addr.2013.09.015.

[45] V. Iacovacci, L. Ricotti, A. Menciassi, P. Dario, The bioartificial pancreas (BAP): Biological, chemical and engineering challenges, Biochem. Pharmacol. 100 (2016) 12-27. doi:10.1016/j.bcp.2015.08.107.

[46] M. Qi, Y. Morch, I. Lacik, K. Formo, E. Marchese, Y. Wang, K.K. Danielson, K. Kinzer, S. Wang, B. Barbaro, G. Kollarikova, D. Chorvat Jr, D. Hunkeler, G. Skjak-Braek, J. Oberholzer, B.L. Strand, Survival of human islets in microbeads containing high guluronic acid alginate crosslinked with Ca2+ and Ba2+, Xenotransplantation 19 (2012) 355-364. doi:10.1111/xen.12009.

[47] I.K. Hals, A.M. Rokstad, B.L. Strand, J. Oberholzer, V. Grill, Alginate microencapsulation of human islets does not increase susceptibility to acute hypoxia, J. Diabetes Res. 2013 (2013) 374925. doi:10.1155/2013/374925.

[48] D. Jacobs-Tulleneers-Thevissen, M. Chintinne, Z. Ling, P. Gillard, L. Schoonjans, G. Delvaux, B.L. Strand, F. Gorus, B. Keymeulen, D. Pipeleers, Beta Cell Therapy Consortium EU-FP7, Sustained function of alginate-encapsulated human islet cell implants in the peritoneal cavity of mice leading to a pilot study in a type 1 diabetic patient, Diabetologia 56 (2013) 1605-1614. doi:10.1007/s00125-013-2906-0.

[49] A.L. Hillberg, M. Oudshoorn, J.B. Lam, K. Kathirgamanathan, Encapsulation of porcine pancreatic islets within an immunoprotective capsule comprising methacrylated glycol chitosan and alginate, J. Biomed. Mater. Res. B. Appl. Biomater. 103 (2015) 503-518. doi:10.1002/jbm.b.33185.

[50] P. Montanucci, I. Pennoni, T. Pescara, G. Basta, R. Calafiore, Treatment of diabetes mellitus with microencapsulated fetal human liver (FH-B-TPN) engineered cells, Biomaterials 34 (2013) 4002-4012. doi:10.1016/j.biomaterials.2013.02.026.

[51] K. Formo, C.H. Cho, L. Vallier, B.L. Strand, Culture of hESC-derived pancreatic progenitors in alginate-based scaffolds, J. Biomed. Mater. Res. A. 103 (2015) 3717-3726. doi:10.1002/jbm.a.35507.

[52] T. Richardson, P.N. Kumta, I. Banerjee, Alginate encapsulation of human embryonic stem cells to enhance directed differentiation to pancreatic islet-like cells, Tissue Eng. Part A. 20 (2014) 3198-3211. doi:10.1089/ten.TEA.2013.0659.

[53] A.A. Tomei, C. Villa, C. Ricordi, Development of an encapsulated stem cell-based therapy for diabetes, Expert Opin. Biol. Ther. 15 (2015) 1321-1336. doi:10.1517/14712598.2015.1055242.

[54] P.K. Ngoc, P.V. Phuc, T.H. Nhung, D.T. Thuy, N.T. Nguyet, Improving the efficacy of type 1 diabetes therapy by transplantation of immunoisolated insulin-producing cells, Hum. Cell 24 (2011) 86-95. doi:10.1007/s13577-011-0018-z.

[55] A.J. Vegas, O. Veiseh, M. Gurtler, J.R. Millman, F.W. Pagliuca, A.R. Bader, J.C. Doloff, J. Li, M. Chen, K. Olejnik, H.H. Tam, S. Jhunjhunwala, E. Langan, S. Aresta-Dasilva, S. Gandham, J.J. McGarrigle, M.A. Bochenek, J. Hollister-Lock, J. Oberholzer, D.L. Greiner, G.C. Weir, D.A. Melton, R. Langer, D.G. Anderson, Long-term glycemic control using polymer-encapsulated human stem cell-derived beta cells in immune-competent mice, Nat. Med. 22 (2016) 306-311. doi:10.1038/nm.4030.

[56] A. Murua, G. Orive, R.M. Hernandez, J.L. Pedraz, Emerging technologies in the delivery of erythropoietin for therapeutics, Med. Res. Rev. 31 (2011) 284-309. doi:10.1002/med.20184.

[57] G. Orive, M. De Castro, H.J. Kong, R.M. Hernandez, S. Ponce, D.J. Mooney, J.L. Pedraz, Bioactive cell-hydrogel microcapsules for cell-based drug delivery, J. Control. Release 135 (2009) 203-210. doi:10.1016/j.jconrel.2009.01.005.

[58] G. Orive, E. Santos, J.L. Pedraz, R.M. Hernandez, Application of cell encapsulation for controlled delivery of biological therapeutics, Adv. Drug Deliv. Rev. 67-68 (2014) 3-14. doi:10.1016/j.addr.2013.07.009.

[59] A. Acarregui, J.L. Pedraz, F.J. Blanco, R.M. Hernandez, G. Orive, Hydrogel-based scaffolds for enclosing encapsulated therapeutic cells, Biomacromolecules 14 (2013) 322-330. doi:10.1021/bm301690a.

[60] S.M. Selimoglu, M. Elibol, Alginate as an immobilization material for MAb production via encapsulated hybridoma cells, Crit. Rev. Biotechnol. 30 (2010) 145-159. doi: 10.3109/07388550903451652.

[61] J. Dubrot, A. Portero, G. Orive, R.M. Hernandez, A. Palazon, A. Rouzaut, J.L. Perez-Gracia, S. Hervas-Stubbs, J.L. Pedraz, I. Melero, Delivery of immunostimulatory monoclonal antibodies by encapsulated hybridoma cells, Cancer Immunol. Immunother. 59 (2010) 1621-1631. doi:10.1007/s00262-010-0888-z.

[62] L. Saenz del Burgo, M. Compte, M. Aceves, R.M. Hernandez, L. Sanz, L. Alvarez-Vallina, J.L. Pedraz, Microencapsulation of therapeutic bispecific antibodies producing cells: immunotherapeutic organoids for cancer management, J. Drug Target. 23 (2015) 170-179. doi: 10.3109/1061186X.2014.971327.

[63] N. Kwak, N. Okamoto, J.M. Wood, P.A. Campochiaro, VEGF is major stimulator in model of choroidal neovascularization, Invest. Ophthalmol. Vis. Sci. 41 (2000) 3158-3164.

[64] E. Santos, G. Orive, A. Calvo, R. Catena, P. Fernandez-Robredo, A.G. Layana, R.M. Hernandez, J.L. Pedraz, Optimization of 100 μm alginate-poly-L-lysine-alginate capsules for intravitreous administration, J. Control. Release 158 (2012) 443-450. doi:10.1016/j.jconrel.2011.09.079.

[65] A. Kang, J. Park, J. Ju, G.S. Jeong, S.H. Lee, Cell encapsulation via microtechnologies, Biomaterials 35 (2014) 2651-2663. doi:10.1016/j.biomaterials.2013.12.073.

[66] A.R. Short, D. Koralla, A. Deshmukh, B. Wissel, B. Stocker, M. Calhoun, D. Dean, J.O. Winter, Hydrogels That Allow and Facilitate Bone Repair, Remodeling, and Regeneration, J. Mater. Chem. B. Mater. Biol. Med. 3 (2015) 7818-7830. doi:10.1039/C5TB01043H.

[67] A. Moshaverinia, S. Ansari, C. Chen, X. Xu, K. Akiyama, M.L. Snead, H.H. Zadeh, S. Shi, Co-encapsulation of anti-BMP2 monoclonal antibody and mesenchymal stem cells in alginate microspheres for bone tissue engineering, Biomaterials 34 (2013) 6572-6579. doi:10.1016/j.biomaterials.2013.05.048.

[68] A. Moshaverinia, C. Chen, K. Akiyama, X. Xu, W.W. Chee, S.R. Schricker, S. Shi, Encapsulated dental-derived mesenchymal stem cells in an injectable and biodegradable scaffold for applications in bone tissue engineering, J. Biomed. Mater. Res. A. 101 (2013) 3285-3294. doi:10.1002/jbm.a.34546.

[69] N. Sykaras, L.A. Opperman, Bone morphogenetic proteins (BMPs): how do they function and what can they offer the clinician?, J. Oral Sci. 45 (2003) 57-73. doi:10.2334/josnusd.45.57.

[70] P. Wang, Y. Song, M.D. Weir, J. Sun, L. Zhao, C.G. Simon, H.H. Xu, A self-setting iPSMSC-alginate-calcium phosphate paste for bone tissue engineering, Dent. Mater. 32 (2016) 252-263. doi:10.1016/j.dental.2015.11.019.

[71] Y. Shen, H. Qiao, Q. Fan, S. Zhang, T. Tang, Potentiated Osteoinductivity via Cotransfection with BMP-2 and VEGF Genes in Microencapsulated C2C12 Cells, Biomed. Res. Int. 2015 (2015) 435253. doi:10.1155/2015/435253.

[72] S. Agarwal, R. Sane, R. Oberoi, J.R. Ohlfest, W.F. Elmquist, Delivery of molecularly targeted therapy to malignant glioma, a disease of the whole brain, Expert Rev. Mol. Med. 13 (2011) e17. doi:10.1017/S1462399411001888.

[73] S.V. Bhujbal, P. de Vos, S.P. Niclou, Drug and cell encapsulation: alternative delivery options for the treatment of malignant brain tumors, Adv. Drug Deliv. Rev. 67-68 (2014) 142-153. doi:10.1016/j.addr.2014.01.010.

[74] C. Spuch, D. Antequera, A. Portero, G. Orive, R.M. Hernandez, J.A. Molina, F. Bermejo-Pareja, J.L. Pedraz, E. Carro, The effect of encapsulated VEGF-secreting cells on brain amyloid load and behavioral impairment in a mouse model of Alzheimer's disease, Biomaterials 31 (2010) 5608-5618. doi:10.1016/j.biomaterials.2010.03.042.

[75] D. Antequera, A. Portero, M. Bolos, G. Orive, R.M. Hernandez, J.L. Pedraz, E. Carro, Encapsulated VEGF-secreting cells enhance proliferation of neuronal progenitors in the hippocampus of AbetaPP/Ps1 mice, J. Alzheimers Dis. 29 (2012) 187-200. doi:10.3233/JAD-2011-111646.

[76] O. Lindvall, L.U. Wahlberg, Encapsulated cell biodelivery of GDNF: a novel clinical strategy for neuroprotection and neuroregeneration in Parkinson's disease?, Exp. Neurol. 209 (2008) 82-88. doi:10.1016/j.expneurol.2007.08.019.

[77] L. Grandoso, S. Ponce, I. Manuel, A. Arrue, J.A. Ruiz-Ortega, I. Ulibarri, G. Orive, R.M. Hernandez, A. Rodriguez, R. Rodriguez-Puertas, M. Zumarraga, G. Linazasoro, J.L. Pedraz, L. Ugedo, Long-term survival of encapsulated GDNF secreting cells implanted within the striatum of parkinsonized rats, Int. J. Pharm. 343 (2007) 69-78. doi:10.1016/j.ijpharm.2007.05.027.

[78] I. Date, T. Ohmoto, T. Imaoka, T. Ono, J.P. Hammang, J. Francis, C. Greco, D.F. Emerich, Cografting with polymer-encapsulated human nerve growth factor-secreting cells and chromaffin cell survival and behavioral recovery in hemiparkinsonian rats, J. Neurosurg. 84 (1996) 1006-1012. doi:10.3171/jns.1996.84.6.1006.

[79] I. Date, T. Ohmoto, T. Imaoka, T. Shingo, D.F. Emerich, Chromaffin cell survival from both young and old donors is enhanced by co-grafts of polymer-encapsulated human NGF-secreting cells, Neuroreport 7 (1996) 1813-1818.

[80] S. Knippenberg, N. Thau, R. Dengler, T. Brinker, S. Petri, Intracerebroventricular injection of encapsulated human mesenchymal cells producing glucagon-like peptide 1 prolongs survival in a mouse model of ALS, PLoS One 7 (2012) e36857. doi:10.1371/journal.pone.0036857.

[81] P.M. Klinge, K. Harmening, M.C. Miller, A. Heile, C. Wallrapp, P. Geigle, T. Brinker, Encapsulated native and glucagon-like peptide-1 transfected human mesenchymal stem cells in a transgenic mouse model of Alzheimer's disease, Neurosci. Lett. 497 (2011) 6-10. doi:10.1016/j.neulet.2011.03.092.

[82] E.J. Wright, K.A. Farrell, N. Malik, M. Kassem, A.L. Lewis, C. Wallrapp, C.M. Holt, Encapsulated glucagon-like peptide-1-producing mesenchymal stem cells have a beneficial effect on failing pig hearts, Stem Cells Transl. Med. 1 (2012) 759-769. doi:10.5966/sctm.2012-0064.

[83] A.M.B. Heile, C. Wallrapp, P.M. Klinge, A. Samii, M. Kassem, G. Silverberg, T. Brinker, Cerebral transplantation of encapsulated mesenchymal stem cells improves cellular pathology after experimental traumatic brain injury, Neurosci. Lett. 463 (2009) 176-181. doi:10.1016/j.neulet.2009.07.071.

[84] S.J.M. Skinner, M.S. Geaney, H. Lin, M. Muzina, A.K. Anal, R.B. Elliott, P.L.J. Tan, Encapsulated living choroid plexus cells: potential long-term treatments for central nervous system disease and trauma, - Journal of Neural Engineering (2009) 065001.

[85] S.L. Huang, J. Wang, X.J. He, Z.F. Li, J.N. Pu, W. Shi, Secretion of BDNF and GDNF from free and encapsulated choroid plexus epithelial cells, Neurosci. Lett. 566 (2014) 42-45. doi:10.1016/j.neulet.2014.02.017.

[86] A. Aliaghaei, H. Digaleh, F. Khodagholi, A. Ahmadiani, Encapsulated Choroid Plexus Epithelial Cells Actively Protect Against Intrahippocampal Abeta-induced Long-Term Memory Dysfunction; Upregulation of Effective Neurogenesis with the Abrogated Apoptosis and Neuroinflammation, J. Mol. Neurosci. 56 (2015) 708-721. doi:10.1007/s12031-015-0492-y.

[87] M. Mazo, J.J. Gavira, B. Pelacho, F. Prosper, Adipose-derived stem cells for myocardial infarction, J. Cardiovasc. Transl. Res. 4 (2011) 145-153. doi:10.1007/s12265-010-9246-y.

[88] C.J. Teng, J. Luo, R.C. Chiu, D. Shum-Tim, Massive mechanical loss of microspheres with direct intramyocardial injection in the beating heart: implications for cellular cardiomyoplasty, J. Thorac. Cardiovasc. Surg. 132 (2006) 628-632. doi:10.1016/j.jtcvs.2006.05.034.

[89] D. Hou, E.A. Youssef, T.J. Brinton, P. Zhang, P. Rogers, E.T. Price, A.C. Yeung, B.H. Johnstone, P.G. Yock, K.L. March, Radiolabeled cell distribution after intramyocardial, intracoronary, and interstitial retrograde coronary venous delivery: implications for current clinical trials, Circulation 112 (2005) I150-6. doi:10.1161/CIRCULATIONAHA.104.526749.

[90] A. Paul, Y. Ge, S. Prakash, D. Shum-Tim, Microencapsulated stem cells for tissue repairing: implications in cell-based myocardial therapy, Regen. Med. 4 (2009) 733-745. doi:10.2217/rme.09.43.

[91] A.H. Al Kindi, J.F. Asenjo, Y. Ge, G.Y. Chen, J. Bhathena, R.C. Chiu, S. Prakash, D. Shum-Tim, Microencapsulation to reduce mechanical loss of microspheres: implications in myocardial cell therapy, Eur. J. Cardiothorac. Surg. 39 (2011) 241-247. doi:10.1016/j.ejcts.2010.03.066.

[92] A. Paul, G. Chen, A. Khan, V.T. Rao, D. Shum-Tim, S. Prakash, Genipin-cross-linked microencapsulated human adipose stem cells augment transplant retention resulting in attenuation of chronically infarcted rat heart fibrosis and cardiac dysfunction, Cell Transplant. 21 (2012) 2735-2751. doi:10.3727/096368912X637497.

[93] R.G. Gomez-Mauricio, A. Acarregui, F.M. Sanchez-Margallo, V. Crisostomo, I. Gallo, R.M. Hernandez, J.L. Pedraz, G. Orive, M.F. Martin-Cancho, A preliminary approach to the repair of myocardial infarction using adipose tissue-derived stem cells encapsulated in magnetic resonance-labelled alginate microspheres in a porcine model, Eur. J. Pharm. Biopharm. 84 (2013) 29-39. doi:10.1016/j.ejpb.2012.11.028.

[94] R.D. Levit, N. Landazuri, E.A. Phelps, M.E. Brown, A.J. Garcia, M.E. Davis, G. Joseph, R. Long, S.A. Safley, J.D. Suever, A.N. Lyle, C.J. Weber, W.R. Taylor, Cellular encapsulation enhances cardiac repair, J. Am. Heart Assoc. 2 (2013) e000367. doi:10.1161/JAHA.113.000367.

[95] P. Soon-Shiong, R.E. Heintz, N. Merideth, Q.X. Yao, Z. Yao, T. Zheng, M. Murphy, M.K. Moloney, M. Schmehl, M. Harris, Insulin independence in a type 1 diabetic patient after encapsulated islet transplantation, Lancet 343 (1994) 950-951.

[96] R. Calafiore, G. Basta, G. Luca, A. Lemmi, M.P. Montanucci, G. Calabrese, L. Racanicchi, F. Mancuso, P. Brunetti, Microencapsulated pancreatic islet allografts into nonimmunosuppressed patients with type 1 diabetes: first two cases, Diabetes Care 29 (2006) 137-138. doi:10.2337/diacare.29.01.06.dc05-1270.

[97] G. Basta, P. Montanucci, G. Luca, C. Boselli, G. Noya, B. Barbaro, M. Qi, K.P. Kinzer, J. Oberholzer, R. Calafiore, Long-term metabolic and immunological follow-up of nonimmunosuppressed patients with type 1 diabetes treated with microencapsulated islet allografts: four cases, Diabetes Care 34 (2011) 2406-2409. doi:10.2337/dc11-0731.

[98] B.E. Tuch, G.W. Keogh, L.J. Williams, W. Wu, J.L. Foster, V. Vaithilingam, R. Philips, Safety and viability of microencapsulated human islets transplanted into diabetic humans, Diabetes Care 32 (2009) 1887-1889. doi:10.2337/dc09-0744.

[99] R.B. Elliott, L. Escobar, P.L. Tan, M. Muzina, S. Zwain, C. Buchanan, Live encapsulated porcine islets from a type 1 diabetic patient 9.5 yr after xenotransplantation, Xenotransplantation 14 (2007) 157-161. doi:10.1111/j.1399-3089.2007.00384.x.

[100] Living Cell Technologies: DIABECELL®. Available via http://www.lctglobal.com/products/diabecell/development-to-date. Accessed November 2016.

[101] Open-label Investigation if the Safety and Efficacy of DIABECELL® in Patients With Type 1 Diabetes Mellitus. Available via https://clinicaltrials.gov/ct2/show/NCT01736228. Accessed November 2016.

[102] Open-label Investigation of the Safety and Effectiveness of DIABECEL® in Patients With Type I Diabetes Mellitus. Available via https://clinicaltrials.gov/ct2/show/NCT00940173. Accessed November 2016.

[103] Open-label Investigation of the Safety and Effectiveness of DIABECELL® in Patients With Type 1 Diabetes Mellitus. Available via https://clinicaltrials.gov/ct2/show/NCT01739829. Accessed November 2016.

[104] A. Heile, T. Brinker, Clinical translation of stem cell therapy in traumatic brain injury: the potential of encapsulated mesenchymal cell biodelivery of glucagon-like peptide-1, Dialogues Clin. Neurosci. 13 (2011) 279-286.

[105] Investigation of the Safety and Efficacy of NTCELL® [Immunoprotected (Alginate-Encapsulated) Porcine Choroid Plexus Cells for Xenotransplantation] in Patients With Parkinson's Disease. Available via https://clinicaltrials.gov/ct2/show/NCT02683629. Accessed November 2016.

[106] X.M. Luo, H. Lin, W. Wang, M.S. Geaney, L. Law, S. Wynyard, S.B. Shaikh, H. Waldvogel, R.L. Faull, R.B. Elliott, S.J. Skinner, J.E. Lee, P.L. Tan, Recovery of neurological functions in non-human primate model of Parkinson's disease by transplantation of encapsulated neonatal porcine choroid plexus cells, J. Parkinsons Dis. 3 (2013) 275-291. doi:10.3233/JPD-130214.

[107] Open-label Investigation of the Safety and Clinical Effects of NTCELL® in Patients With Parkinson's Disease. Available via https://clinicaltrials.gov/ct2/show/NCT01734733. Accessed November 2016.

[108] Living Cell Technologies: NTCELL®. Available via http://www.lctglobal.com/ntcell/ntcell/development-to-date. Accessed October 2019.

[109] M. Lohr, Z.T. Bago, H. Bergmeister, M. Ceijna, M. Freund, W. Gelbmann, W.H. Gunzburg, R. Jesnowski, J. Hain, K. Hauenstein, W. Henninger, A. Hoffmeyer, P. Karle, J.C. Kroger, G. Kundt, S. Liebe, U. Losert, P. Muller, A. Probst, K. Puschel, M. Renner, R. Renz, R. Saller, B. Salmons, I. Walter, Cell therapy using microencapsulated 293 cells transfected with a gene construct expressing CYP2B1, an ifosfamide converting enzyme, instilled intra-arterially in patients with advanced-stage pancreatic carcinoma: a phase I/II study, J. Mol. Med. (Berl) 77 (1999) 393-398.

[110] M. Lohr, A. Hoffmeyer, J. Kroger, M. Freund, J. Hain, A. Holle, P. Karle, W.T. Knofel, S. Liebe, P. Muller, H. Nizze, M. Renner, R.M. Saller, T. Wagner, K. Hauenstein, W.H. Gunzburg, B. Salmons, Microencapsulated cell-mediated treatment of inoperable pancreatic carcinoma, Lancet 357 (2001) 1591-1592. doi:10.1016/S0140-6736(00)04749-8.

[111] B. Salmons, M. Lohr, W.H. Gunzburg, Treatment of inoperable pancreatic carcinoma using a cell-based local chemotherapy: results of a phase I/II clinical trial, J. Gastroenterol. 38 Suppl 15 (2003) 78-84.

[112] J.M. Lohr, S.L. Haas, J.C. Kroger, H.M. Friess, R. Hoft, P.E. Goretzki, C. Peschel, M. Schweigert, B. Salmons, W.H. Gunzburg, Encapsulated cells expressing a chemotherapeutic activating enzyme allow the targeting of subtoxic chemotherapy and are safe and efficacious: data from two clinical trials in pancreatic cancer, Pharmaceutics 6 (2014) 447-466. doi:10.3390/pharmaceutics6030447.

[113] T.T. Dang, A.V. Thai, J. Cohen, J.E. Slosberg, K. Siniakowicz, J.C. Doloff, M. Ma, J. Hollister-Lock, K.M. Tang, Z. Gu, H. Cheng, G.C. Weir, R. Langer, D.G. Anderson, Enhanced function of immuno-isolated islets in diabetes therapy by co-encapsulation with an anti-inflammatory drug, Biomaterials 34 (2013) 5792-5801. doi:10.1016/j.biomaterials.2013.04.016.

[114] A. Murua, E. Herran, G. Orive, M. Igartua, F.J. Blanco, J.L. Pedraz, R.M. Hernandez, Design of a composite drug delivery system to prolong functionality of cell-based scaffolds, Int. J. Pharm. 407 (2011) 142-150. doi:10.1016/j.ijpharm.2010.11.022.

[115] A. Acarregui, E. Herran, M. Igartua, F.J. Blanco, J.L. Pedraz, G. Orive, R.M. Hernandez, Multifunctional hydrogel-based scaffold for improving the functionality of encapsulated therapeutic cells and reducing inflammatory response, Acta Biomater. 10 (2014) 4206-4216. doi:10.1016/j.actbio.2014.06.038.

[116] S.A. Azadi, E. Vasheghani-Farahani, S. Hashemi-Najafbabadi, A. Godini, Co-encapsulation of pancreatic islets and pentoxifylline in alginate-based microcapsules with enhanced immunosuppressive effects, Prog. Biomater. 5 (2016) 101-109. doi:10.1007/s40204-016-0049-3.

[117] E.H. Jo, Y.H. Hwang, D.Y. Lee, Encapsulation of pancreatic islet with HMGB1 fragment for attenuating inflammation, Biomater. Res. 19 (2015) 21-015-0042-2. eCollection 2015. doi:10.1186/s40824-015-0042-2.

[118] O. Arlov, G. Skjak-Braek, A.M. Rokstad, Sulfated alginate microspheres associate with factor H and dampen the inflammatory cytokine response, Acta Biomater. 42 (2016) 180-188. doi:10.1016/j.actbio.2016.06.015.

[119] M. Figliuzzi, B. Bonandrini, S. Silvani, A. Remuzzi, Mesenchymal stem cells help pancreatic islet transplantation to control type 1 diabetes, World J. Stem Cells 6 (2014) 163-172. doi:10.4252/wjsc.v6.i2.163.

[120] V. Vaithilingam, M.D. Evans, A. Rowe, P.A. Bean, B.E. Tuch, Coencapsulation of Target Effector Cells With Mesenchymal Stem Cells Reduces Pericapsular Fibrosis and Improves Graft Survival in a Xenotransplanted Animal Model, Cell Transplant. 25 (2016) 1299-1317. doi:10.3727/096368915X688975.

[121] A. Alunno, P. Montanucci, O. Bistoni, G. Basta, S. Caterbi, T. Pescara, I. Pennoni, V. Bini, E. Bartoloni, R. Gerli, R. Calafiore, In vitro immunomodulatory effects of microencapsulated umbilical cord Wharton jelly-derived mesenchymal stem cells in primary Sjogren's syndrome, Rheumatology (Oxford) 54 (2015) 163-168. doi:10.1093/rheumatology/keu292.

[122] K. English, Mechanisms of mesenchymal stromal cell immunomodulation, Immunol. Cell Biol. 91 (2013) 19-26. doi:10.1038/icb.2012.56.

[123] G. Luca, I. Arato, F. Mancuso, M. Calvitti, G. Falabella, G. Murdolo, G. Basta, D.F. Cameron, B.C. Hansen, F. Fallarino, T. Baroni, M.C. Aglietti, C. Tortoioli, M. Bodo, R. Calafiore, Xenograft of microencapsulated Sertoli cells restores glucose homeostasis in db/db mice with spontaneous diabetes mellitus, Xenotransplantation (2016). doi:10.1111/xen.12274.

[124] G. Luca, I. Bellezza, I. Arato, A. Di Pardo, F. Mancuso, M. Calvitti, G. Falabella, S. Bartoli, V. Maglione, E. Amico, M. Favellato, G. Basta, M. Bodo, A. Minelli, R. Calafiore, L. Frati, F. Squitieri, Terapeutic Potential of Microencapsulated Sertoli Cells in Huntington Disease, CNS Neurosci. Ther. 22 (2016) 686-690. doi:10.1111/cns.12569.

[125] O. Veiseh, J.C. Doloff, M. Ma, A.J. Vegas, H.H. Tam, A.R. Bader, J. Li, E. Langan, J. Wyckoff, W.S. Loo, S. Jhunjhunwala, A. Chiu, S. Siebert, K. Tang, J. Hollister-Lock, S. Aresta-Dasilva, M. Bochenek, J. Mendoza-Elias, Y. Wang, M. Qi, D.M. Lavin, M. Chen, N. Dholakia, R. Thakrar, I. Lacik, G.C. Weir, J. Oberholzer, D.L. Greiner, R. Langer, D.G. Anderson, Size- and shape-dependent foreign body immune response to materials implanted in rodents and non-human primates, Nat. Mater. 14 (2015) 643-651. doi:10.1038/nmat4290.

[126] R. Robitaille, J.F. Pariseau, F.A. Leblond, M. Lamoureux, Y. Lepage, J.P. Halle, Studies on small (<350 microm) alginate-poly-L-lysine microcapsules. III. Biocompatibility of smaller versus standard microcapsules, J.Biomed.Mater.Res. 44 (1999) 116-120.

[127] E. Santos, G. Orive, R. Hernandez M., J.L. Pedraz, Cell-Biomaterial Interaction: Strategies To Mimic The Extracellular Matrix, in: L. Pramatarova (Ed.), On Biomimetics, InTech, Rijeka, 2011.

[128] A. Garate, E. Santos, J.L. Pedraz, R.M. Hernandez, G. Orive, Evaluation of different RGD ligand densities in the development of cell-based drug delivery systems, J. Drug Target. 23 (2015) 806-812. doi:10.3109/1061186X.2015.1020428.

[129] J.E. Marturano, N.R. Schiele, Z.A. Schiller, T.V. Galassi, M. Stoppato, C.K. Kuo, Embryonically inspired scaffolds regulate tenogenically differentiating cells, J. Biomech. 49 (2016) 3281-3288. doi:10.1016/j.jbiomech.2016.08.011.

[130] E. Santos, A. Garate, J.L. Pedraz, G. Orive, R.M. Hernandez, The synergistic effects of the RGD density and the microenvironment on the behavior of encapsulated cells: in vitro and in vivo direct comparative study, J. Biomed. Mater. Res. A. 102 (2014) 3965-3972. doi:10.1002/jbm.a.35073.

[131] A. Llacua, B.J. de Haan, S.A. Smink, P. de Vos, Extracellular matrix components supporting human islet function in alginate-based immunoprotective microcapsules for treatment of diabetes, J. Biomed. Mater. Res. A. 104 (2016) 1788-1796. doi:10.1002/jbm.a.35706.

[132] S. Mazzitelli, G. Luca, F. Mancuso, M. Calvitti, R. Calafiore, C. Nastruzzi, S. Johnson, S.F. Badylak, Production and characterization of engineered alginate-based microparticles containing ECM powder for cell/tissue engineering applications, Acta Biomater. 7 (2011) 1050-1062. doi:10.1016/j.actbio.2010.10.005.

[133] O. Jeon, C. Powell, L.D. Solorio, M.D. Krebs, E. Alsberg, Affinity-based growth factor delivery using biodegradable, photocrosslinked heparin-alginate hydrogels, J. Control. Release 154 (2011) 258-266. doi:10.1016/j.jconrel.2011.06.027.

[134] R. Lou, H. Xie, H. Zheng, Y. Ren, M. Gao, X. Guo, Y. Song, W. Yu, X. Liu, X. Ma, Alginate-based microcapsules with galactosylated chitosan internal for primary hepatocyte applications, Int. J. Biol. Macromol. 93 (2016) 1133-1140. doi:10.1016/j.ijbiomac.2016.09.078.

[135] E. Santos, L. Larzabal, A. Calvo, G. Orive, J.L. Pedraz, R.M. Hernandez, Inactivation of encapsulated cells and their therapeutic effects by means of TGL triple-fusion reporter/biosafety gene, Biomaterials 34 (2013) 1442-1451. doi:10.1016/j.biomaterials.2012.10.076.

[136] H.K. Chang, P.H. Kim, H.M. Cho, S.Y. Yum, Y.J. Choi, Y. Son, D. Lee, I. Kang, K.S. Kang, G. Jang, J.Y. Cho, Inducible HGF-secreting Human Umbilical Cord Blood-derived MSCs Produced via TALEN-mediated Genome Editing Promoted Angiogenesis, Mol. Ther. 24 (2016) 1644-1654. doi:10.1038/mt.2016.120.

[137] S. Swioklo, P. Ding, A.W. Pacek, C.J. Connon, Process parameters for the high-scale production of alginate-encapsulated stem cells for storage and distribution throughout the cell therapy supply chain, Process Biochemistry. doi:10.1016/j.procbio.2016.06.005.

[138] E. Erro, J. Bundy, I. Massie, S.A. Chalmers, A. Gautier, S. Gerontas, M. Hoare, P. Sharratt, S. Choudhury, M. Lubowiecki, I. Llewellyn, C. Legallais, B. Fuller, H. Hodgson, C. Selden, Bioengineering the liver: scale-up and cool chain delivery of the liver cell biomass for clinical targeting in a bioartificial liver support system, Biores Open Access 2 (2013) 1-11. doi:10.1089/biores.2012.0286.

[139] P. de Vos, M. Bucko, P. Gemeiner, M. Navratil, J. Svitel, M. Faas, B.L. Strand, G. Skjak-Braek, Y.A. Morch, A. Vikartovska, I. Lacik, G. Kollarikova, G. Orive, D. Poncelet, J.L. Pedraz, M.B. Ansorge-Schumacher, Multiscale requirements for bioencapsulation in medicine and biotechnology, Biomaterials 30 (2009) 2559-2570. doi:10.1016/j.biomaterials.2009.01.014.

[140] Medicinal products for human use - Advanced therapies. Available via https://ec.europa.eu/health/human-use/advanced-therapies_en. Accessed February 2017.

Hypothesis & Objectives

Mesenchymal stromal cells (MSCs) present the ability to secrete important immunomodulatory factors, and therefore, hold great potential for the treatment of multiple inflammatory and immune disorders. However, for the success of MSC-based therapies, the optimization of administration protocols is fundamental. Intravenously infused nude MSCs are undetectable shortly after administration and their clearance by host immune cells importantly limits cell retention. Additionally, disease indications requiring tissue targeting demand new strategies adapted to administration of MSCs in extravascular compartments.

In such regard, three-dimensional (3D) cell encapsulation systems emerge as a valuable strategy for MSCs administration, enhancing cell persistence and therefore, prolonging their effects. An interesting approach is the immobilization of cells within alginate-poly-L-lysine-alginate (APA) microcapsules. In this strategy, cells are encapsulated in an alginate core which is surrounded by a semipermeable membrane. The main advantage this platform offers is the immunoprotection of the graft, since the semipermeable barrier allows the diffusion of oxygen, nutrients and therapeutic factors produced by the cells, but avoids the entrance of immune cells and antibodies. Therefore, cells are protected from the host immune response, which enables a sustained release of bioactive factors. However, considering that the performance of the system is dependent on cell behavior, uncontrolled cell responses importantly compromise the safety and efficacy of cell microencapsulation technology.

Moreover, preserving the viability of encapsulated cells is crucial for a long-term functionality of the system. In this sense, special interest has been paid to biofunctionalize 3D matrices with cues present in the natural extracellular matrix (ECM). Cells interact with their microenvironment through integrins, cell adhesion receptors that recognize ECM ligands. Such integrin-ECM binding results in the transduction of mechanical and biochemical signals that regulate diverse cellular processes. To enhance cell survival, it is possible to biofunctionalize materials by incorporating full-length ECM proteins or short peptides recapitulating only minimal cell recognizable sequences. Since the affinity for these ECM cues varies among the different integrin classes, in the design of effective functionalization strategies, it is vital to gain knowledge on the particular integrins expressed by the specific cell being studied.

Another crucial variable that directly influences the outcome of MSC-based therapies is the immunomodulatory capacity of the cells. This potential is strongly influenced by the particular

local conditions where cells are implanted, which may not provide an adequate microenvironment to polarize MSCs towards an immunosuppressive phenotype. Consequently, the development of effective strategies that boost the immunomodulatory potential of MSCs is vital and still remains a challenge. Among the alternatives being currently explored, MSC 3D culture and licensing with inflammatory cytokines such as interferon γ (IFN-γ) are remarkable.

Bearing in mind all the above mentioned, we hypothesized that the development of a multifunctional system consisting of a hydrogel incorporating APA-microencapsulated MSCs, an adequate biofunctionalization strategy and the continuous presence of IFN-γ would represent an integrated solution to (1) protect MSCs from the host immune system, (2) to promote their viability and (3) to boost their immunomodulatory properties.

However, for the development of such a complex approach, a previous validation of the effectivity of each individual component is fundamental. Therefore, the present doctoral thesis aims to provide a deep insight and optimize each one of the three main aspects in the multifunctional system: the APA microcapsules, the matrix biofunctionalization and the strategy to boost the immunomodulatory properties of MSCs based on the inclusion of IFN-γ and the 3D cell culture itself (Fig. 1).

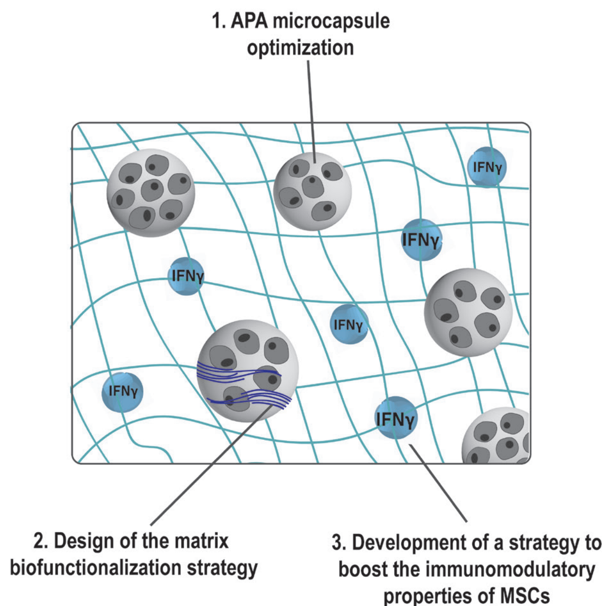


Fig. 1. Schematic representation of the objectives of the present doctoral thesis.
APA: alginate-poly-L-lysine-alginate. MSCs: mesenchymal stromal cells.

Thus, the specific objectives of the present work are the following:

1. To control the behavior of MSCs encapsulated in APA microcapsules in order to enhance the biosafety of the therapy and obtain an adequate release of therapeutic factors *in vivo*.

2. To develop an ECM protein dot microarray biosensor for an in depth characterization of the specific requirements of a particular cell type regarding cell adhesion, as a tool to design successful biofunctionalization strategies.

3. To develop a bioinspired multifunctional system combining 3D biomimetic cell culture and sustained inflammatory licensing as an integral solution to boost the immunomodulatory potential of MSCs.

Additionally, considering that the immunomodulatory capacity of MSCs is usually assessed by means of RT-qPCR, we aimed at analyzing the influence of these particular experimental conditions on the stability of widely employed housekeeping genes, to avoid inaccurate results and flawed conclusions.

Experimental section

Chapter 1

The role of osmolarity adjusting agents in the regulation of encapsulated cell behavior to provide a safer and more predictable delivery of therapeutics

Drug delivey, 24 (2017), 1654-1666.

DOI: https://doi.org/10.1080/10717544.2017.1391894

The role of osmolarity adjusting agents in the regulation of encapsulated cell behavior to provide a safer and more predictable delivery of therapeutics

Ainhoa Gonzalez-Pujana[a,b], Aitor Rementeria[c], Francisco Javier Blanco[d], Manoli Igartua[a,b], Jose Luis Pedraz[a,b], Edorta Santos-Vizcaino[a,b,*], Rosa Maria Hernandez[a,b,*]

[a] NanoBioCel Group, Laboratory of Pharmaceutics, School of Pharmacy, University of the Basque Country (UPV/EHU), Spain.
[b] Biomedical Research Networking Centre in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Spain.
[c] Fungal and Bacterial Biomics Group, Faculty of Science and Technology, University of the Basque Country (UPV/EHU), Spain.
[d] CIBER-BBN-Bioscaff Cartílago, INIBIC Institute for Biomedical research, Spain.
* Corresponding authors: Edorta Santos-Vizcaino, Rosa Maria Hernandez.

ABSTRACT

Transplantation of cells within alginate microspheres has been extensively studied for sustained drug delivery. However, the lack of control over cell behavior represents a major concern regarding the efficacy and the safety of the therapy. Here, we demonstrated that when formulating the biosystem, an adequate selection of osmolarity adjusting agents significantly contributed to the regulation of cell responses. Our data showed that these agents interacted in the capsule formation process, influencing the alginate crosslinking degree. Therefore, when selecting inert or electrolyte-based osmolarity adjusting agents to encapsulate erythropoietin (EPO) producing D1 mesenchymal stromal cells, alginate microcapsules with differing mechanical properties were obtained. Since mechanical forces acting on cells influence their behavior, contrasting cell responses were observed both, *in vitro* and *in vivo*. When employing mannitol as an inert osmolarity adjusting agent, microcapsules presented a more permissive matrix, allowing a tumoral-like behavior. This resulted in the formation of giant cell-aggregates that presented necrotic cores and protruding peripheral cells, rendering the therapy unpredictable, dysfunctional and unsafe. Conversely, the use of electrolyte osmolarity adjusting agents, including calcium or sodium, provided the capsule with a suitable crosslinking degree that established a tight control over cell proliferation and enabled an adequate EPO secretion *in vivo*. The crucial impact of these agents was confirmed when gene expression studies reported pivotal divergences not only in proliferative pathways, but also in genes involved in survival, migration and differentiation. Altogether, our results prove osmolarity adjusting agents as an effective tool to regulate cell behavior and obtain safer and more predictable therapies.

---

1. Introduction

The entrapment of therapeutically active cells within alginate-poly-L-lysine-alginate (APA) microcapsules has been widely employed for the sustained delivery of therapeutics (Strand, Coron and Skjak-Braek, 2017). The long-term function of the system allows the continuous production of therapeutics, avoiding the necessity of frequent administrations and hence, improving patient's quality of life. However, the performance of the system is directly dependent on cell responses and nowadays implant functionality and safety are still importantly limited by a lack of control over cell behavior (Santos *et al.*, 2013). Among the erratic cell responses, excessive proliferation rates represent one of the major hurdles, especially when it comes to using immortalized cell lines (Bhujbal *et al.*, 2014). Cellular overgrowth results in aggregates that present a characteristic dualism. On the one hand, the limited diffusion of nutrients and oxygen to the core of the aggregates results in inner cell death (de Vos, 2017). This does not only lead to a diminished therapeutic effect, but has additional consequences, since intracellular components derived from dying cells, namely danger-associated molecular patterns (DAMPs), function as alarm molecules that evoke important immune and inflammatory responses (Paredes Juarez *et al*., 2014). On the other hand, protruding peripheral cells may leak out of the matrix, constituting a major safety concern (Bhujbal *et al.*, 2014).

In attempting to develop systems with improved control over cell behavior, tuning the mechanical properties of the matrix has been suggested as a valuable strategy. This is possible because of the mechanotransduction process, by which mechanical forces acting on cells influence their biochemical behavior and viability (Humphrey, Dufresne and Schwartz, 2014). Taking advantage of it, we focused on osmolarity adjusting agents as a tool to modify the mechanics of the capsule and therefore, regulate cell behavior. The process of cell encapsulation requires the dissolution of the employed biomaterials. Therefore, to meet the standards of cell culture, the solutions of e.g. alginate or poly-L-lysine (PLL) should present physiological osmolarity values between 260-320 mOsm/L (Ozturk and Palsson, 1991). To this end, osmolarity adjusting agents are included in the solutions, which can be classified as electrolyte or inert agents. Considering that microcapsules are ionically crosslinked matrices, the presence or absence of electrolytes, especially divalent cations, may influence the capsule formation process. Therefore, the use of different types of osmolarity adjusting agents may alter the

mechanical properties of the matrix, having an impact on cell responses. To the best of our knowledge, the effect of these agents in cell behavior and consequently, in the outcome of the therapy, has not been previously studied in depth.

Here, we designed two sets of solutions (each one including all the solutions required for the elaboration of microcapsules: 1.5% alginate, 0.05% PLL, 0.1% alginate and washing solutions), which differed in the selected type of osmolarity adjusting agent. The Biological set contained electrolytes including calcium, sodium or phosphates and the Technological was based on mannitol as an inert agent. These solutions were used to encapsulate D1 multipotent mesenchymal stromal cells (MSCs). MSCs were genetically modified to express erythropoietin (EPO), a model therapeutic molecule that can be easily traced *in vivo* through hematocrit measurements to assess the functionality of the implant. The resulting Biological and Technological microcapsules were characterized and our data showed that the distinct mechanical properties of the matrix influenced cells at a genic level. This resulted in a contrasting cell behavior, which led to divergent therapeutic profiles *in vitro* and *in vivo*, highlighting the pivotal importance of an accurate formulation to obtain systems with suitable properties that control cell behavior.

2. Methods

2.1 Characterization of the solutions

pH was determined by means of the pH-Meter GLP 21, Crison®. Osmotic pressure was assessed using a cryoscopic osmometer (Osmomat 030, Gonotec®) that measures the freezing point depression, which is directly proportional to the concentration of osmotically active compounds in aqueous solutions. Each sample was assayed in triplicate.

2.2. Cell Culture

MSCs (ATCC® CRL 12424TM) were genetically modified with the lentiviral vector pSIN-EF2-Epo-Pur to express erythropoietin (D1-MSCs-EPO) (Gurruchaga *et al.*, 2015). Cells were seeded in T-flasks and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin. They were

maintained at 37ºC in a 5% CO_2 / 95% air atmosphere and passaged every 2-3 days using trypsin-EDTA. All reagents were purchased from Fisher Scientific, Spain.

2.3. Cell microencapsulation

D1-MSCs-EPO were encapsulated using an electrostatic droplet generator (Nisco®), following the procedure designed by Lim and Sun (Lim and Sun, 1980). Briefly, cells were suspended in 1.5% (w/v) sodium alginate obtaining a cell density of 5 x 10^6 cells/mL. This suspension was extruded through a 0.35 mm needle at a 5.9 mL/h flow rate by means of a peristaltic pump. Beads were collected in a 100 mM $CaCl_2$ bath and maintained in agitation for 10 min to ensure a complete ionic gelation. After washing the particles, they were suspended in 0.05% (w/v) PLL for 5 min. Once washed, a second coating was performed by suspending the particles in 0.1% (w/v) alginate for 5 min, giving rise to APA microcapsules. Since different solutions of 1.5% alginate, 0.05% PLL, 0.1% alginate and washings were designed, three types of APA microcapsules were obtained: Biological microcapsules (made of Biological solutions), Technological microcapsules (made of Technological solutions) and Hybrid microcapsules (made of Biological 1.5% alginate and Technological 0.05% PLL, 0.1% alginate and washing solutions) (Supplementary Fig. 1). This last group was used in the structural studies to separately determine the influence of core and coating solutions in the final properties of the capsule. The same 100 mM $CaCl_2$ washing solution was employed for every group, since the presence of the gelling ion Ca^{+2} was mandatory. Microcapsules were cultured in complete medium at 37ºC in a 5% CO_2 / 95% air atmosphere. All the process was carried out under aseptic conditions at room temperature. Ultra-pure low-viscosity high guluronic acid alginate (UPLVG, code #4200006) was purchased from FMC Biopolymer, Norway and PLL (hydrobromide Mw 15,000–30,000 Da, code P7890-500MG) from Sigma-Aldrich, Spain.

2.4. Cell aggregate area quantification

Micrographs were captured with an inverted microscope (Nikon TMS) and processed by means of ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2016) to quantify the cell-aggregate area. For each point, aggregates of at least 20 random capsules were analyzed.

2.5. Cell proliferation: Bromodeoxyuridine (BrdU) uptake

BrdU uptake was determined by means of Cell Proliferation Biotrak ELISA System (code GERPN250, Sigma Aldrich, Spain) in 5 independent samples per group. In a 96 well-plate, the equivalent of 2 x 10^4 cells was placed in each well (≈ 80 microcapsules). All groups were incubated for 24 h in complete medium supplemented with 10% FBS, except for the starving control group, which was incubated in medium supplemented with 0.1% FBS. An additional 24 h incubation was carried out in presence of 10 μM BrdU, except for the non-specific binding control group, in which the reagent was not added. Subsequently, cells were de-encapsulated by using a 500 μg/mL alginate lyase solution (code A1603, Sigma Aldrich, Spain) and the assay was performed following manufacturer's instructions. Absorbance measurements were normalized with the non-specific binding control.

2.6. Viability assays

Three different assays were performed to evaluate the viability of the immobilized cells: live/dead staining, flow cytometry and cell metabolism studies. For live/dead staining 10 μL of microcapsules containing D1-MSCs-EPO were dyed with the LIVE/DEAD® kit (code L3224, Fisher Scientific, Spain) following manufacture's indications. After 30 min, fluorescence micrographs were taken using an epi-fluorescence microscope (Nikon TSM). At least 6 independent experiments were analyzed for each group. For flow cytometry (BD FACSCalibur), cells were de-encapsulated using a 500 μg/mL solution of alginate lyase. Cells were then treated with trypsin-EDTA to eliminate possible cell-aggregates and stained with the LIVE/DEAD® kit. After a 20 min incubation, protected from light and at room temperature, cells were acquired. 3 independent samples of cells from each group were assayed. Metabolic activity was determined using the Cell Counting Kit-8 (CCK-8, code 96992-3000TESTS-F, Sigma Aldrich, Spain). Approximately 50 microcapsules (≈ 12,300 cells) were suspended in 100 μL of DMEM and placed in a 96-well plate. Subsequently, 10 μL of CCK-8 were added to each well. Plates were incubated for 4 h at 37ºC and subsequently read on an Infinite M200 TECAN plate reader at 450 nm, with reference wavelength at 650 nm. At least 7 independent experiments were analyzed for each group.

2.7. EPO secretion

EPO secretion was determined by means of the Quantikine IVD Human EPO ELISA Kit (code DEP00, R&D Systems, Spain). 100 μL of microcapsules were incubated for 24 h at 37°C and supernatants were assayed. Samples and standards were run in duplicate following manufacturer's instructions. 3 independent samples were assayed per study group.

2.8. Diameter determination and Osmotic resistance test

Micrographs were obtained with an inverted microscope and analyzed using the ImageJ software. To evaluate osmotic resistance 100 μL of microcapsules were suspended in 1 mL of deionized water (ddH_2O) and placed in 12 well plates. After 5 min, supernatants were replaced with fresh ddH_2O to perform a second washing. The process was repeated to a total of 5 washings. Micrographs were taken previous to the assay and after each washing to determine diameter and integrity of the capsules. At least 30 microcapsules were analyzed in both assays.

2.9. Fluorescein isothiocyanate (FITC)-Dextran diffusion

In order to determine the microcapsule membrane molecular weight cut-off (MWCO), FITC-dextrans were employed (Mw 10, 20, 40, 70 and 150 kDa). A volume of 10 μL of microcapsule suspension (≈ 170 capsules) was incubated with 0.5 mg/mL FITC-dextran solution for 24 h at room temperature and observed by confocal microscopy (Leica TCS SP2 AOBS Spectral Confocal Scanner mounted on a Leica DM IRE2 inverted fluorescent microscope). Micrographs were analyzed using ImageJ. Equal area squares were defined and the relative intensity of 20 microcapsules and 20 background areas was determined to obtain the dextran diffusion percentage. 4 independent samples were assayed per study group.

2.10. Crosslinking ion determination

Microcapsules with no cell load were treated with a 500 μg/mL solution of alginate lyase in order to cause their rupture and release all the calcium forming the matrix. A colorimetric Calcium Detection Kit (code ab102505, Abcam, Spain) was employed to perform the calcium determination. Each group was assayed in triplicate.

2.11. Cell cycle

In this method, BrdU (an analogous of the DNA precursor thymidine) was incorporated into newly synthesized DNA. Additionally, cells were stained with 7-aminoactinomycin D (7-AAD), which binds to the total DNA and resolves cell cycle phases in our populations: G0/1 (resting phase) or S/G2/M (DNA synthesis and division). With that purpose, cells were previously de-encapsulated using a 500 µg/mL solution of alginate lyase and treated using the BrdU Flow Kit Staining Protocol (code 559619, BD Biosciences, Spain). Finally, 3 independent samples from each group were analyzed by flow cytometry.

2.12. Animal experimentation

In vivo studies were performed according to the ethical guidelines established by the institutional animal care and use committee of the University of Basque Country UPV/EHU (Permit number: CEEA_411_2015_HERNÁNDEZ MARTÍN). 15 female 6-week-old C57BL/6 mice were chosen as allogenic immunocompetent murine models (n = 5 per group: Biological, Technological and control). Animals were anesthesised by isofluorane inhalation and subcutaneously implanted with a total volume of 60 µL of microcapsules (suspended in additional 300 µL of Dulbecco's Phosphate-Buffered Saline (DPBS) code BE17-513F, Lonza, Spain) by means of a 20-gauge catheter. For the control group, sole DPBS was administered. At days 15, 30 and 45 blood samples were collected in heparinized capillary tubes by facial vein puncture. Whole blood was centrifuged at 760 x *g* for 15 min and hematocrit levels were determined using a standard microhematocrit method. At day 45 mice were sacrificed. Samples were retrieved and fixed in 4% paraformaldehyde for histological analysis. Hematoxylin and eosin staining was performed and results were blindly evaluated by a pathologist.

2.13 RNA isolation, Microarray hybridization and Transcriptomic analysis

These procedures were performed at the Gene Expression Unit of the Genomics Facility in the UPV/EHU (SGIker platforms; Leioa, Spain). Total RNA was extracted from four independent samples of cells within Biological or Technological microcapsules, using Tri Reagent solution (Fisher Scientific, Spain), and the modified precipitation protocol recommended for sources rich in polysaccharides and proteoglycans. After the addition of 10 µg of glycogen,

RNA was further purified with PureLink RNA Mini kit (Fisher Scientific, Spain). RNA concentration and purity were assessed by NanoDrop 1000 spectrophotometer, and RNA quality and integrity were assayed by Lab-chip technology on an Agilent 2100 Bioanalyzer with Agilent RNA 6000 Nano Chips (Agilent technologies, Santa Clara, CA USA). All obtained RNA samples presented RNA integrity number (RIN) values 1-2. Whole mouse gene expression microarray analysis was performed using the available SurePrint G3 Mouse GE v2 8x60K Microarray (Agilent Microarray Design ID: ID 074809, Agilent Technologies). Nucleic acid from each replicate, 100 ng, were labeled following the Agilent protocol "Gene Expression FFPE Workflow", which is specific for low quality RNA samples. Feature Extraction Software vs 10.7.3.1 (Agilent Technologies) was used to convert the image into expression data. Raw data were preprocessed, normalized and filtered using GeneSpring GX V 13.0 software (Agilent Technologies). Data were normalized with Quantile method and filtered based on Coefficient of Variation (CV < 100%), obtaining the log2 of the average value of signal intensity for each probe. LIMMA statistical package (Smyth, 2004) from the Multi Experiment Viewer (MEV) software version 4.9 (Saeed *et al.*, 2006) was used for differential gene expression analysis. Conventional statistical criteria (adjusted p-value < 0.05) were used for the selection of differentially expressed genes. Microarray data have been deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-5786. (For further details, see supplementary methods).

2.14. Data analysis and statistics

To detect significant differences between two groups Student's t-test was used. One-way ANOVA was used for multiple comparisons. Depending on the results of the Levene test of homogeneity of variances, Bonferroni or Tamhane post-hoc test was applied. For non-normally distributed data, Mann-Whitney nonparametric analysis was applied. p values less than 0.05 were assumed to be significant in all analyses. All statistical computations were performed using SPSS 23 (IBM SPSS, Chicago, IL).

3. Results

3.1 Characterization of the solutions

To determine the influence of selecting inert or electrolyte osmolarity adjusting agents, two sets of solutions were designed: a Biological set (containing electrolytes as osmolarity adjusting agents) and a Technological set (with mannitol as an inert agent). During the design, preliminary experiments were performed to determine the best vehicle containing electrolytes for the Biological 1.5% alginate solution. Among DMEM w/o Ca^{+2} and Mg^{+2}, Hank's balanced salt solution (HBSS) and phosphate buffered saline (PBS), the former was selected. Despite presenting osmolarity values slightly above the limits, it was chosen for providing the best results in terms of cell viability and metabolic activity (Supplementary Fig. 2). The resulting sets were characterized (Table 1) showing physiological pH and osmolarity values.

Table 1. Characterization of the Biological and Technological sets of solutions. Each value represents mean ± SD (n = 3). DMEM: Dulbecco's modified Eagle's medium. PLL: Poly-L-Lysine. DPBS: Dulbecco's Phosphate-Buffered Saline. HEPES: N'-2-Hydroxyethylpiperazine-N'-2 ethanesulphonic acid. ddH_2O: deionized water.

Solution	Composition	pH	Osmolarity (mOsm/L)
Biological set			
1.5% Alginate	1.5% alginate, DMEM w/o Ca^{2+},Mg^{2+}	8.01 ± 0.30	345 ± 17
0.05% PLL	0.05% PLL, DPBS w/ Ca^{2+}, Mg^{2+}	7.23 ± 0.05	277 ± 14
0.1% Alginate	0.1% alginate, DPBS w/ Ca^{2+}, Mg^{2+}	7.12 ± 0.04	282 ± 16
100 mM $CaCl_2$	100 mM $CaCl_2$, 25 mM HEPES, 0.7% mannitol, ddH_2O	7.04 ± 0.05	284 ± 17
Washings	$CaCl_2$ to 2.5 mM calcium, DPBS w/ Ca^{2+}, Mg^{2+}	7.13 ± 0.04	286 ± 06
Technological set			
1.5% Alginate	1.5% alginate, 25 mM HEPES, 4.3% mannitol, ddH_2O	7.22 ± 0.16	305 ± 07
0.05% PLL	0.05% PLL,25 mM HEPES, 4.7% mannitol, ddH_2O	7.20 ± 0.10	289 ± 11
0.1% Alginate	0.1% alginate, 25 mM HEPES, 4.7% mannitol, ddH_2O	7.09 ± 0.04	306 ± 04
100 mM $CaCl_2$	100 mM $CaCl_2$, 25 mM HEPES, 0.7% mannitol, ddH_2O	7.04 ± 0.05	284 ± 17
1° Washing	$CaCl_2$ to 2.5 mM calcium, 25 mM HEPES, 4.5% mannitol, ddH_2O	7.06 ± 0.10	292 ± 03
Rest of Washings	25 mM HEPES, 4.8% mannitol, ddH_2O	7.10 ± 0.02	292 ± 06

3.2. APA microcapsules formulated with different types of osmolarity adjusting agents led to contrasting cell behavior in vitro

The Biological and Technological sets of solutions were employed to encapsulate D1-MSCs-EPO, obtaining Biological and Technological microcapsules, respectively. To study cell behavior in each group, microcapsules were cultured *in vitro* for 45 days. During the course of the study, important cell-aggregates started to emerge in Technological microcapsules, fact

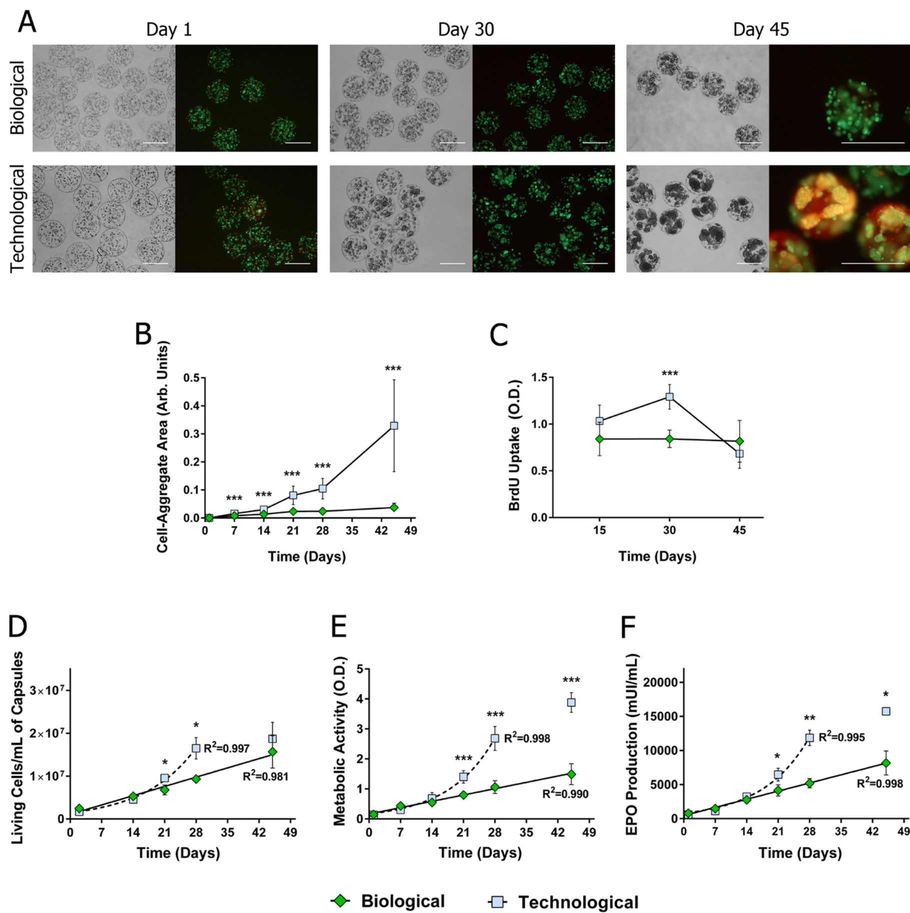


Fig. 1. APA microcapsules formulated with different types of osmolarity adjusting agents led to contrasting cell behavior *in vitro*. (A) Brightfield and LIVE/DEAD fluorescence micrographs revealed diverging cell behavior. Scale bars = 400 μm. **(B)** Subsequent quantification of cell-aggregate areas demonstrated statistically significant differences. **(C)** Bromodeoxyuridine (BrdU) uptake studies confirmed these data by showing an important increase in proliferation in the Technological group by day 30. Consequently, the Biological biosystem presented a linear tendency for cell viability **(D)**, metabolic activity **(E)** and erythropoietin (EPO) production **(F)**, whereas Technological capsules followed an exponential trend up to day 30. From that point on, this group withdrew from its tendency due to an increase in cell death. Graphs show mean ± SD (n = 5 for BrdU uptake, n = 3 for cell viability and EPO production studies, n = 7 for metabolic assays). Statistical significance: *p < 0.05, **p < 0.01 and ***p < 0.001.

that was not so evident in the Biological group (Fig. 1A). Their area was quantified confirming significant differences from day 21 (Fig. 1B). BrdU uptake assays supported these data by demonstrating that the proliferation rate remained constant in the Biological group whereas it importanty increased in Technological microcapsules until day 30 and drastically decreased by day 45 (Fig. 1C). This drop at the end of the study may be originated by reduced cell viability, as shown in calcein/ethidium fluorescent micrographs (Fig. 1A). This phenomenon was also observed in flow cytometry analyses (Fig. 1D). Although the number of viable cells increased in both groups over the study, Biological microcapsules adjusted to a linear tendency ($R^2 = 0.981$), whereas the Technological group followed an exponential one ($R^2 = 0.997$) (Fig. 1D). Nevertheless, by day 45, the latter withdrew from its trend, pointing out, again, to increased cell death. The exact same profiles were shown in cell metabolism (Fig. 1E) and EPO production (Fig. 1F).

3.3. Mechanical studies revealed differences in the structural properties of microcapsules

Next, we studied if the choice of different types of osmolarity adjusting agents influenced the mechanical configuration of the capsule. When comparing Biological *versus* Technological beads (microspheres with no coatings), larger diameters were observed in the latter (Fig. 2A). Subsequently, the diameters of coated microcapsules (APA microcapsules) were analyzed, obtaining, again, higher values for the Technological group (Fig. 2B). In order to elucidate if the increase in size was only due to the core solution (1.5% alginate) or on the contrary, to a synergistic effect between core and coating solutions (0.05% PLL and 0.1% alginate), Biological microcapsules were compared to Hybrid capsules (Biological core and Technological coatings). Hybrid particles presented larger size (Fig. 2C) confirming the direct effect of both core and coating solutions in the size of the capsule.

We next evaluated the osmotic resistance by performing ddH_2O washings. Higher swelling values were observed in the Biological group when compared to Technological (Fig. 2D) or Hybrid microcapsules (Fig. 2E). The number of broken capsules after each washing was also quantified (Fig. 2F). For the Biological group, the rupture of the 40% of microcapsules was observed after the second washing, while the rest broke in the third. Contrarily, both Technological and Hybrid microcapsules presented a slightly higher resistance: around

a 40% broke in the third washing, and the remaining did it in the fourth. Concerning permeability, confocal fluorescent micrographs (Supplementary Fig. 3) of FITC-dextran diffusion showed no statistical differences in the MWCO (Fig. 2G). To finish with the mechanical characterization, the calcium-mediated crosslinking of the alginate matrix was analyzed. A significantly higher release of calcium from Biological matrices suggested they contained a greater concentration of it, and consequently, a higher crosslinking degree (Fig. 2H).

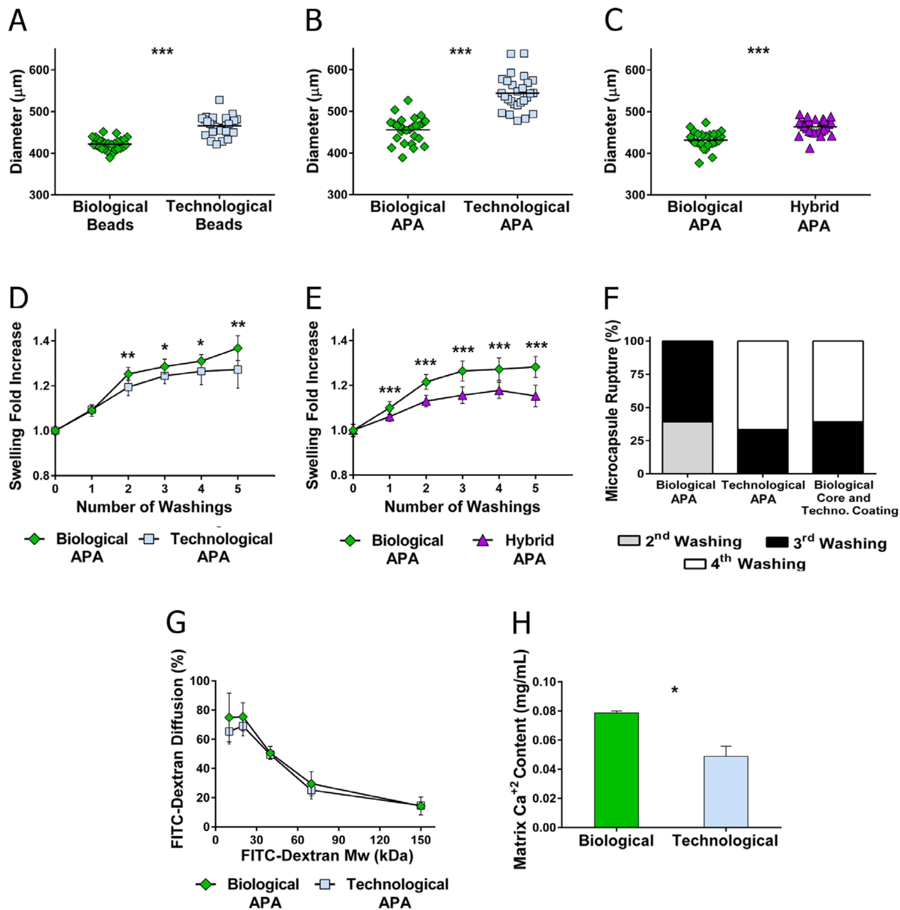


Fig. 2. Mechanical studies revealed important differences in the structural properties of microcapsules. Diameter quantification of **(A)** uncoated beads, **(B)** alginate-poly-L-lysine-alginate (APA) microcapsules and **(C)** a comparison between Biological and Hybrid microcapsules (Biological core and Technological coatings). In all cases, greater sizes were observed when employing Technological solutions. Osmotic resistance was assessed for **(D)** APA microcapsules and **(E)** a comparison between Biological and Hybrid microcapsules and both showed an increased swelling for the Biological group. **(F)** Despite the three types of microcapsules presented a good resistance to rupture, it was enhanced in the Hybrid and Technological groups. **(G)** FITC labeled dextran diffusion showed no differences in permeability. **(H)** Calcium release assays proved a higher crosslinking degree in Biological matrices. Graphs show mean ± SD (n = 30 for diameter quantification and osmotic resistance, n = 3 for calcium determination assays, n = 4 for FITC dextran diffusion). Statistical significance: *p < 0.05, **p < 0.01 and ***p < 0.001.

3.4. Cell cycle analysis demonstrated that mechanical differences led to differing cell behavior in vitro

Since the mechanical characterization of microcapsules determined a higher crosslinking degree in the Biological matrix, we hypothesized this may be the factor that restricted the formation of aggregates in this group. To shed light on this issue, we simultaneously studied cell cycle and proliferation. At day 1 after encapsulation, the majority of cells in both groups presented an S/G2/M state with no BrdU uptake (63.3% for Biological and 66.1% for Technological) (Fig. 3A). According to these results, before the addition of BrdU cells had already duplicated their genetic material and invested the following 48 h, in presence of BrdU, in the steps previous to cell division.

At day 30, the majority of cells in both groups presented a G0/1 state with no BdrU uptake (55.3% for Biological and 57.9% for Technological) (Fig. 3B). However, interesting differences were observed in the remaining states. The Biological group showed a similar behavior than the observed at day 1, having the highest percentage of those remaining cells in S/G2/M state with no BrdU uptake. Nevertheless, the trend changed for the Technological group. The majority of the remaining cells presented a G0/1 state with BrdU uptake. This means that after the addition of BrdU, cells duplicated their genetic material (incorporating BrdU), and divided completing cell cycle. Thus, for this group, cell division occurred in a remarkably shorter period of time. A schematic representation is depicted in Fig. 3C.

3.5. In vivo *studies exhibited divergent therapeutic profiles*

To assess the therapeutic effect *in vivo*, D1-MSCs-EPO were encapsulated in Biological or Technological microcapsules and subcutaneously implanted in C57BL/6 mice for 45 days. Progressive increases in hematocrit levels were observed in mice in which Biological microcapsules were administered, with a narrowed distribution of the replicates. Contrarily, Technological implants showed erratic profiles with high dispersion values (Fig. 4A-B). After implant retrieval, we showed that Biological microcapsules maintained their spherical shape and integrity, but the vast majority of Technological capsules were broken and had released their content in form of enormous cell-aggregates (Fig. 4C). Despite cell-aggregates were also observed in the Biological group, their size was significantly smaller (Fig. 4D).

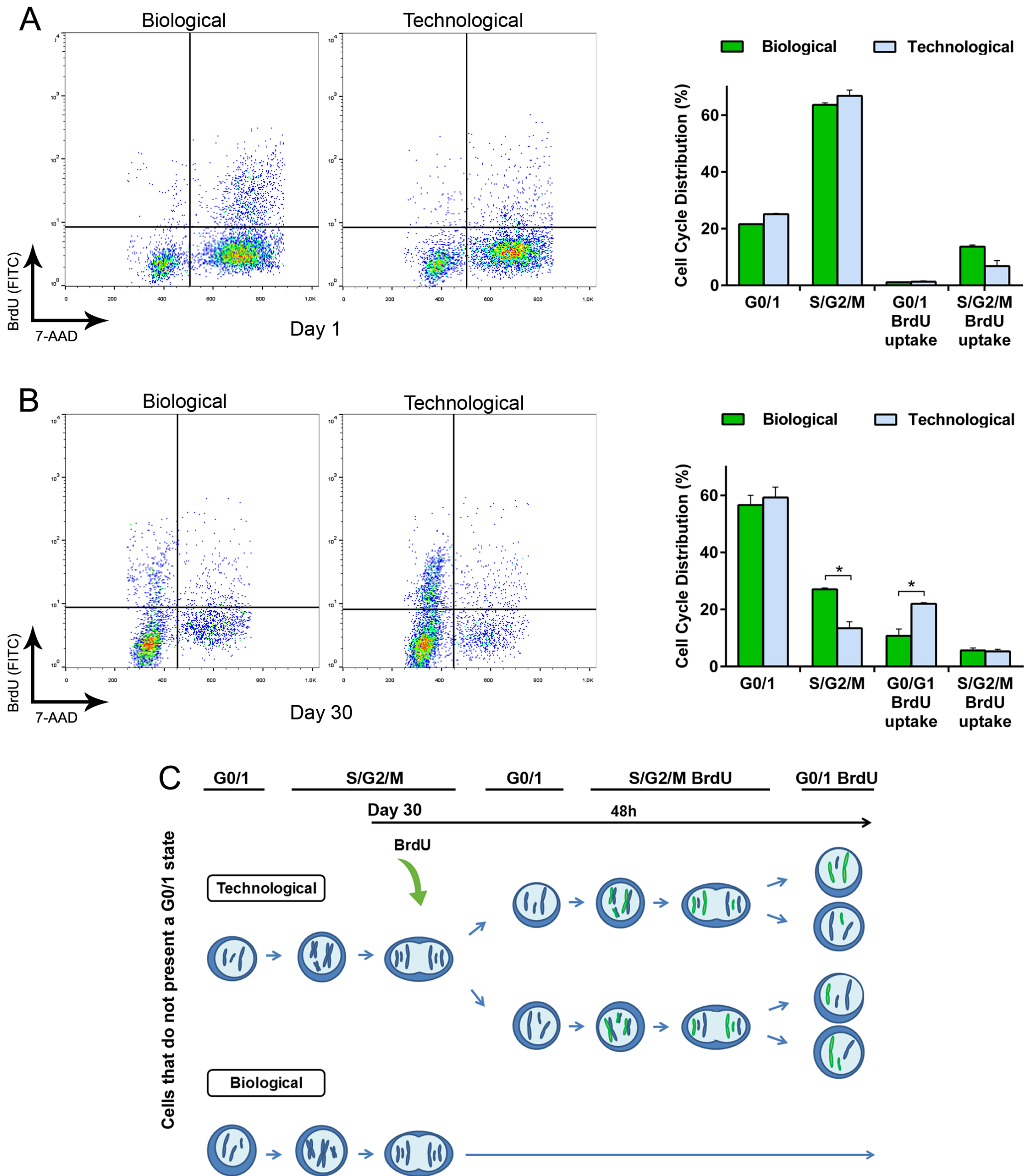


Fig. 3. Cell cycle analysis demonstrated that mechanical differences led to differing cell behavior *in vitro*. (A) Flow cytometry combining bromodeoxyuridine (BrdU) uptake and 7-aminoactinomycin D (7-AAD) staining showed no statistically significant differences at the very beginning of the study. **(B)** This tendency dramatically changed by day 30 in the Technological group where the proliferation rate significantly increased. **(C)** Schematic representation of the results obtained in the combined flow cytometry assay at day 30. In particular, the scheme shows the proliferative capacity of the remaining cells that did not present a G0/1 state (44.7% Biological, 42.1% Technological). Graphs symbolize mean ± SD (n = 3). Statistical significance: $^{*}p < 0.05$.

Hematoxylin and eosin staining revealed that all implants in the Biological group presented a similar behavior, contrasting with Technological implants, where the 2 grafts that failed to raise the hematocrit levels showed empty microcapsules (Fig. 4E.I). The inflammatory response was remarkably more intense in the Technological group (Fig. 4E.II), except for the 2 implants showing capsules with no cell load (Fig. 4E.I). Moreover, Technological microcapsules showed enormous cell-aggregates, which caused mesenchymal microtumors (Fig. 4E.III). Further, broken capsules were observed in the Technological group (Fig. 4E.IV), which led to the release of the cellular content and subsequent inflammatory response and extracapsular tumor-like mass formation (Fig. 4E.V). Such behavior was not observed in the Biological group, where microcapsules maintained their integrity (Fig. 4E.VI-X).

3.6. Behavioral differences were caused at a genic level

For gene expression studies, four independent samples of cells within each type of matrix were analyzed. One Technological replicate was removed from this expression analysis due to technical problems during the hybridization with the microarray. Our study recovered a set of 1,570 genes with significant changes ($p < 0.05$) in expression. In particular, we focused on differentially expressed genes showing a FC > 3 or < -3. Under such restriction, cells within Technological microcapsules showed 176 up-regulated and 88 down-regulated genes in comparison to the Biological group.

It is noteworthy that 86 out of the 264 sequences presented unknown function. Among the rest, many of these genes pointed out to a higher activation of proliferative pathways in the Technological group (Fig. 5). Such is the case of the PI3K/Akt/mTOR pathway, which was promoted by the up-regulation of genes such as *Gnb3* (FC 3.51), *Pik3cg* (FC 3.65) and *Igbp1b* (FC 6.01) together with the down-regulation of *Inpp5d* (FC -3.48). Similarly, the Ras/Raf/MAPK pathway was reinforced by the up-regulation of genes such as *Ros1* (FC 6.05) or *Cyp2c44* (FC 5.34). Additionally, the higher expression of *Usp2* (FC 3.03) and *Usp17le* (FC 6.06), together with the down-regulation of transcriptional co-repressors such as *Basp1* (FC -3.7) led to cell cycle progression.

The reorganization of the cytoskeleton was also enhanced in the Technological group due to the up-regulation of genes such as *Rhoj* (FC 4.46) or *Eps8l1* (FC 3.72). Further, the

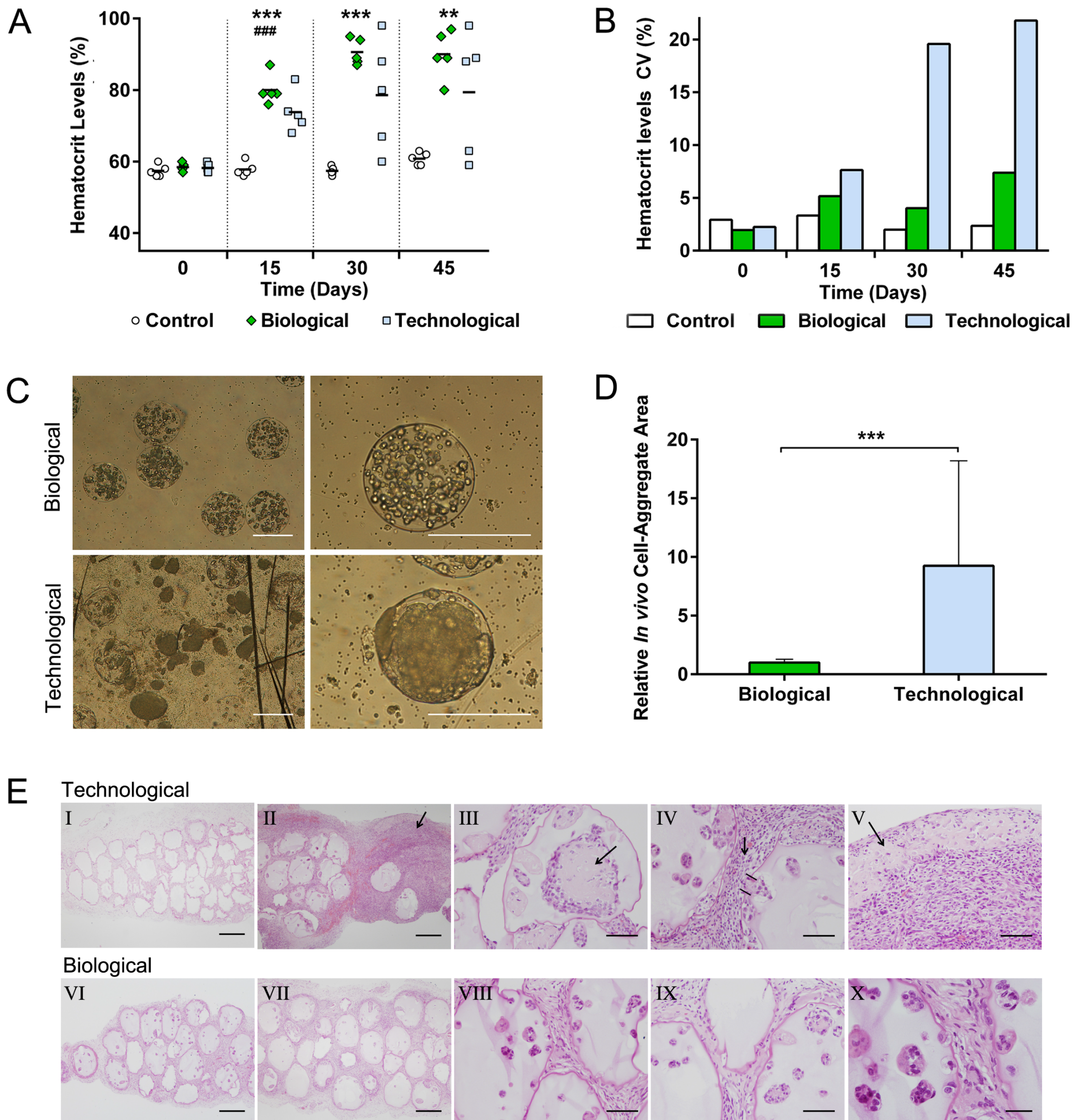
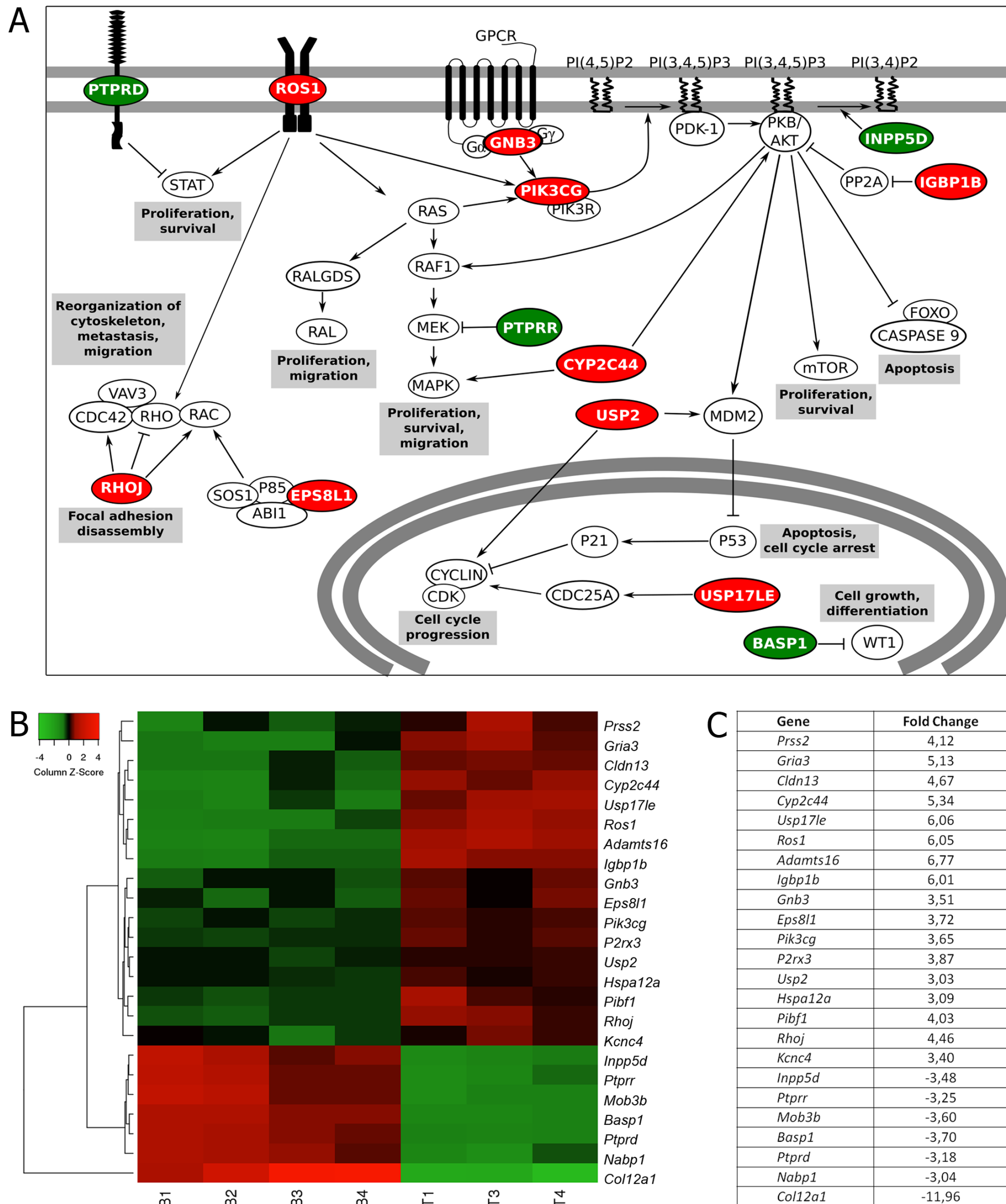


Fig. 4. *In vivo* studies exhibited divergent therapeutic profiles. (A-B) Hematocrit levels progressively increased in every mouse with Biological implants, whereas erratic profiles and big data dispersion were observed in mice with Technological implants. For the control group sole DPBS was administered. Statistical significance: between Control and Biological groups: $^{**}p < 0.01$ and $^{***}p < 0.001$. Between Control and Technological groups: ### $p < 0.001$ **(C)** Morphological characterization after graft explantation revealed enormous cell-aggregates and broken capsules in the Technological group. Scale bars = 400 µm. **(D)** Cell-aggregate area quantification proved statistically significant differences between the groups $^{***}p < 0.001$. **(E)** Histological studies showed empty microcapsules for the Technological implants that failed (I). The analyses also confirmed that Technological implants presented an intense inflammatory response (II), cell-aggregates causing microtumors (III), capsule rupture (IV) and the consequent extra-capsular tumor formation (V). Such behavior was not observed in Biological implants, where a lower inflammation (VI-VII) and capsule integrity with no cell extravasation (VIII-X) were observed. Scale bars I,II, VI, VII = 400 µm. III, IV, V, VIII, IX = 100 µm. X = 50 µm. Graphs show mean ± SD. (n = 5 for animal studies, n = 30 for diameter quantification).



Gene	Fold Change
Prss2	4,12
Gria3	5,13
Cldn13	4,67
Cyp2c44	5,34
Usp17le	6,06
Ros1	6,05
Adamts16	6,77
Igbp1b	6,01
Gnb3	3,51
Eps8l1	3,72
Pik3cg	3,65
P2rx3	3,87
Usp2	3,03
Hspa12a	3,09
Pibf1	4,03
Rhoj	4,46
Kcnc4	3,40
Inpp5d	-3,48
Ptprr	-3,25
Mob3b	-3,60
Basp1	-3,70
Ptprd	-3,18
Nabp1	-3,04
Col12a1	-11,96

Fig. 5. Gene expression studies proved that behavioral differences were caused at a genic level. (A) Schematic representation of signaling pathways in which gene expression presented a fold change > 3 or < -3 in cells within Technological microcapsules, in comparison to the Biological group. **(B)** Heatmap representation of differentially expressed genes. Results are expressed as a fold change ratio. Each column represents a replicate and each row represents a gene (n = 4 for gene studies, with the loss of a Technological replicate due to technical problems during hybridization process). B: Biological. T: Technological. **(C)** Fold change values in cells within Technological v Biological capsules. Red color indicates up-regulated genes and green color down-regulated genes in cells within Technological microcapsules in comparison to the Biological group. All these studies were performed at the Gene Expression Unit of the Genomics Facility in the UPV/ EHU (SGIker platforms; Leioa, Spain).

Ptprd (FC -3.18) and *Ptprr* (FC -3.25) tumor suppressor genes showed a lower expression in Technological cells, whereas the *Hspa12a* heat shock protein was significantly up-regulated (FC 3.09). Finally, the Technological group showed up-regulation of the *Adamts16* gene (FC 6.77), suggesting chondrogenic differentiation. On the contrary, the Biological group presented a high expression of *Col12a1* (FC -11.96), gene present in the osteogenic differentiation.

4. Discussion

Currently, there is an urgent need to regulate cell behavior in encapsulation systems, since the lack of control leads to unsuccessful therapies and represents an important hurdle towards clinical translation (Bhujbal *et al.*, 2014; Gonzalez-Pujana *et al.*, 2017). Interestingly, the stiffness of the matrix has been described to play an important role in the regulation of cell responses (Shin and Mooney, 2016). Therefore, to date, many have been the attempts to modulate this factor. Nonetheless, little attention has been paid to the osmolarity adjusting agents as a tool to regulate the final outcome of the therapy. Considering that microcapsule formation occurs by electrostatic interactions, this process is directly influenced by the presence of electrolytes, especially divalent cations, in the surrounding media (Chen *et al.*, 2014; Thu *et al.*, 1996). Hence, we hypothesized that in the attempt to design solutions that meet the standards for cell culture, the choice of different types of osmolarity adjusting agents may play a pivotal role in the mechanics of the capsule. To shed light on this premise, two different sets of solutions were designed. Both of them were adjusted to adequate osmolarity values, however, different types of osmolarity adjusting agents were selected to do so. For the Biological set of solutions, electrolytes such as sodium, potassium, calcium or phosphates were chosen. Contrarily, mannitol was included as an inert agent in the Technological set of solutions. Subsequently, D1-MSCs-EPO were encapsulated using these sets, obtaining Biological or Technological microcapsules. This cell type was selected because of its important proliferative capacity (Garate *et al.*, 2015; Gurruchaga *et al.*, 2015), which renders it a suitable model for studying the control that the mechanical configuration of the matrix may exert on cell behavior. Indeed, during the course of our study, these cells were able to proliferate even in starving conditions (Supplementary Fig. 4).

First, pivotal differences were observed when evaluating cell behavior *in vitro*. The morphological analysis, together with the BrdU uptake assay showed a significantly higher proliferation rate in the Technological group, with the subsequent cell-aggregate formation. These results could explain the so different tendencies that cells repeatedly followed in viability, metabolism and EPO secretion studies: linear for Biological and exponential for Technological microcapsules. The dramatic decrease in proliferation in the Technological group and the subsequent failure in the exponential trend by day 45 were likely caused by a decrease in viability. We point out to the enormous aggregates as the most probable factor that originated cell death, possibly due to pore collapse (Leal-Egana *et al.*, 2012), and subsequent limited diffusion of oxygen and nutrients to the inner core.

To determine if the contrasting cell behavior was originated by differences in the mechanical properties of the system, structural studies were carried out, which revealed significant differences in matrix configuration. Considering that the binding between the alginate and the crosslinking ion is responsible for conferring stiffness to the matrix, higher calcium levels, and thus a higher crosslinking degree, suggested a less permissive matrix in Biological capsules. According to these results, during the coating process, the calcium loss was lower when beads were put in contact with solutions that presented it, probably due to the gradient stabilization that could have prevented the leakage. Consequently, the Technological group presented a higher leakage of the ion, resulting in a more permissive matrix (Kleinberger *et al.*, 2013; Ma, Vacek and Sun, 1994). This property allowed an aggressive proliferation, which led to the formation of giant cell-aggregates. On the contrary, the more restrictive matrix of Biological microcapsules established a control in cell division (Richardson *et al.*, 2016; Liu *et al.*, 2015). Therefore, it was not the direct effect of osmolarity adjusting agents on cells which evoked so contrasting behavior, but the effect this agents have in the capsule formation process and thus, in the final crosslinking degree of the alginate matrix.

This may also explain the results obtained for particle size. When alginate beads were put in contact with Technological solutions, the calcium loss not only led to a decreased stiffness, but also to swelling, thus increasing microcapsule diameter (Kleinberger *et al.*, 2013). Additionally, a higher osmotic resistance in Technological microcapsules indicated an enhanced interaction between the alginate matrix and the PLL coating. This may have been caused

by the contribution of two phenomena. First, Technological microcapsules presented less calcium in their matrix, and consequently, a higher number of alginate chains were free to interact with the PLL. Secondly, since the membrane formation is governed by electrostatic interactions between the alginate and the polycation, the presence of ions in the surrounding media may weaken the bind (Thu *et al.*, 1996). Therefore, although both groups presented a good osmotic resistance, it was enhanced in the Technological group. Regarding permeability, no statistical differences were observed, with both groups maintaining an adequate MWCO to fulfill the objectives of the technology.

To thoroughly evaluate the effect of the matrix crosslinking degree in cell division, next step was to study in depth cell cycle. The proliferation rate was remarkably enhanced for Technological microcapsules by day 30. Although a 55.3% of Biological and a 57.9% of Technological cells presented a G0/1 state, important differences were detected in the remaining cells. A significant number of cells in the Biological group remained the 48 h after BrdU addition in the same S/G2/M state they were before inclusion of the thymidine analog. Contrarily, the Technological group was able to duplicate the genetic material and complete the cycle, returning to G0/1 phase with the BrdU label incorporated in the DNA. This indicates that softer matrices present lower resistance to deformation, allowing a significantly faster cell division (Leal-Egana *et al.*, 2012).

Because the *in vivo* studies represent the most similar approach to clinics, they were pivotal to determine if the obtained data remained significant. Hematocrit levels progressively increased in mice in which Biological microcapsules were implanted, obtaining similar values for every mouse in the group. Conversely, by day 30 two of the Technological implants had already failed and the remaining showed important data dispersion. When morphologically analyzing the explants after 45 days of study, great differences were observed. Giant cell aggregates were detected in Technological microcapsules and their size quantification confirmed, once again, the divergent proliferation rates in each group. Moreover, while Biological microcapsules remained spherical and maintained their integrity, for the Technological group, the majority of capsules were broken, allowing the cell content to be released to the surrounding tissue. That was probably originated by the giant aggregates whose aggressive growth triggered an increment in pressure that was not tolerated by the membranes of the system.

Histological analyses supported these results by showing a significantly higher capsule rupture in the Technological group, which resulted in tissue invasion and tumor-like mass formation. This fact may have also contributed to the severe inflammatory response observed in such group. In particular, dying cells released from Technological microcapsules might have secreted DAMPs, which are extensively recognized to play a role in the responses against grafts (Paredes Juarez *et al.*, 2014).

The final goal of cell microencapsulation technology is to maintain the bioactive factor levels within the therapeutic range in a sustained manner. Since Biological implants provided progressive increases with minimal dispersion values, they were able to fulfill this objective and give rise to a controlled regimen. On the contrary, the erratic behavior shown by cells encapsulated within Technological microcapsules led to an unpredictable secretion of EPO. Indeed, some implants failed and others maintained their functionality, making it difficult to foretell the result of the therapy. Moreover, it may be hypothesized that in the functional Technological implants, due to the extreme proliferation rate allowed by such type of matrix, hematocrit levels may continue to increase, reaching toxic levels.

Microarray analyses explained that the contrasting cell behavior observed was due to differences at a genic level. Important proliferative pathways were significantly activated in cells immobilized within Technological microcapsules. The up-regulation of genes such as *Gnb3*, *Pik3cg*, or *Igbp1b* together with the down-regulation of *Inpp5d* enhanced the PI3K/Akt/mTor route. Similarly, the Ras/Raf/MAPK pathway was promoted due to over-expression of genes like *Ros1* (Chin *et al.*, 2012) and *Cyp2c44*, which can also activate Akt (Yang *et al.*, 2009). Interestingly, it has been reported that when these two pathways are mutated or amplified, proliferation and survival signals are constitutively activated and, ultimately, lead to tumorigenesis (Wu *et al.*, 2017). Additionally, the over-expression of *Ros1* is related to reorganization of the cytoskeleton, process often related to metastasis and migration (Chin *et al.*, 2012). This effect was accentuated by the up-regulation of other implied genes such as *Rhoj*, which evokes focal adhesion disassembly (Wilson *et al.*, 2014), and *Eps8l1*, which activates *Rac*, leading to the reorganization of the actin cytoskeleton (Offenhauser *et al.*, 2004). Moreover, the repression of cell cycle arrest was enhanced due to the up-regulation of *Usp2* and *Usp17le*, which promote the stabilization of cyclin D1 (Shan, Zhao and Gu, 2009)

and Cdc25A (Hjortland and Mesecar, 2016), respectively. Supporting these results, *Ptprd* and *Ptprr* tumor suppressor genes were down-regulated in the Technological group. The former inhibits the Stat pathway, which leads to proliferation and survival (Ortiz *et al.*, 2014). The latter inhibits MEK, repressing the Ras/Raf/MAPK route (Su *et al.*, 2013). Furthermore, for the same group, we found up-regulation of *Hspa12a*, whose high expression has been proven in tumor tissues (Yang *et al.*, 2015). Consequently, cells enclosed in Technological microcapsules may have found less restriction to develop a tumor-like behavior.

Genes related to differentiation of D1-MSCs-EPO into other lineages presented a distinctive expression. In particular, we observed an up-regulation of *Col12a1* in cells within Biological microcapsules. This gene encodes type XII collagen, which is expressed by osteoblasts and localizes to areas of bone formation (Izu *et al.*, 2011). On the other hand, the *Adamts16* gene, up-regulated in Technological cells, has been reported to be expressed by MSCs during chondrogenesis (Boeuf *et al.*, 2012). These differences may be explained by the already described mechanosensitive differentiation of MSCs by which, according to the physical properties of each matrix, cells differentiate into varying lineages (Rape *et al.*, 2015).

These results demonstrate that gene expression was considerably influenced by the mechanical properties of the matrix in which cells were encapsulated. The pivotal differences regarding expression resulted in a contrasting cell behavior both, *in vitro* and *in vivo*. Since in our system cells are responsible for producing the therapeutic factor, there is a direct connection between cell behavior and drug delivery. Therefore, the different cell responses had a drastic impact on the release of the therapeutic factor, influencing key points such as the efficacy and safety of the therapy. Therefore, our research supports the data described in the literature pointing out to a mechanosensing process in the absence of integrin binding domains (Bhujbal *et al.*, 2014; Huang *et al.*, 2013). Further research should focus on the mechanism by which it occurs in order to gain knowledge over the factors involved in cell-microenvironment interactions.

5. Conclusion

The present work provides new insights regarding the regulation of uncontrolled cell responses in alginate microspheres. In particular, we proved the employment of osmolarity adjusting agents as a useful tool to modify the mechanical configuration of the matrix, with no need of altering the biomaterial or crosslinker type/proportion. Technological microcapsules, resulting from the use of inert osmolarity adjusting agents, presented a permissive matrix that allowed uncontrolled cell division, with the subsequent erratic and dysfunctional therapeutic regimen. Contrarily, when employing electrolytes, including calcium or sodium, as osmolarity adjusting agents, Biological capsules were formed. This type of matrix allowed establishing a tight control over cell proliferation, avoiding the enormous cell-aggregate formation, the risk of cell protrusion, the intense inflammatory response and the potential toxicity given by drug overdose. Overall, these data demonstrated that employing the Biological formulation represents a valuable strategy to control cell behavior and thus achieve a predictable, safe and sustained *de novo* release of therapeutics.

6. Acknowledgements

Authors thank the support provided by the Gene Expression Unit of the Genomics Facility in the UPV/ EHU (SGIker platforms; Leioa, Spain) where all gene expression studies were performed. Similarly, we thank Dr. Felipe Prosper at the University Clinic of Navarra (CUN) for his assistance on the development of the lentiviral vector pSIN-EF2-Epo-Pur. This work was supported by European funding (ERDF and ESF), ICTS “NANBIOSIS” (Drug Formulation Unit, U10) the Basque Government (Consolidated Groups, IT-907-16) and the University of the Basque Country UPV/EHU (UFI11/32). A. Gonzalez-Pujana thanks the Basque Government for the PhD grant

7. References

Bhujbal, S.V., de Haan, B., Niclou, S.P., et al, 2014. A novel multilayer immunoisolating encapsulation system overcoming protrusion of cells. Scientific reports, 4, 6856.

Boeuf, S., Graf, F., Fischer, J., et al, 2012. Regulation of aggrecanases from the ADAMTS family and aggrecan neoepitope formation during in vitro chondrogenesis of human mesenchymal stem cells. European cells & materials, 23, 320-332.

Chen, L., Zhang, Y., Li, S., et al, 2014. Effect of plasma components on the stability and permeability of microcapsule. Journal of biomedical materials research.Part A, 102 (7), 2408-2416.

Chin, L.P., Soo, R.A., Soong, R., et al, 2012. Targeting ROS1 with anaplastic lymphoma kinase inhibitors: a promising therapeutic strategy for a newly defined molecular subset of non-small-cell lung cancer. Journal of thoracic oncology: official publication of the International Association for the Study of Lung Cancer, 7 (11), 1625-1630.

de Vos, P. , 2017. Historical Perspectives and Current Challenges in Cell Microencapsulation. Methods in molecular biology (Clifton, N.J.), 1479, 3-21.

Garate, A., Ciriza, J., Casado, J.G., et al, 2015. Assessment of the Behavior of Mesenchymal Stem Cells Immobilized in Biomimetic Alginate Microcapsules. Molecular pharmaceutics, 12 (11), 3953-3962.

Gonzalez-Pujana, A., Santos, E., Orive, G., et al, 2017. Cell microencapsulation technology: Current vision of its therapeutic potential through the administration routes. Journal of Drug Delivery Science and Technology, .

Gurruchaga, H., Ciriza, J., Saenz Del Burgo, L., et al, 2015. Cryopreservation of microencapsulated murine mesenchymal stem cells genetically engineered to secrete erythropoietin. International journal of pharmaceutics, 485 (1-2), 15-24.

Hjortland, N.M. and Mesecar, A.D., 2016. Steady-state kinetic studies reveal that the anti-cancer target Ubiquitin-Specific Protease 17 (USP17) is a highly efficient deubiquitinating enzyme. Archives of Biochemistry and Biophysics, 612, 35-45.

Huang, X., Hang, R., Wang, X., et al, 2013. Matrix stiffness in three-dimensional systems effects on the behavior of C3A cells. Artificial Organs, 37 (2), 166-174.

Humphrey, J.D., Dufresne, E.R. and Schwartz, M.A. , 2014. Mechanotransduction and extracellular matrix homeostasis. Nature reviews.Molecular cell biology, 15 (12), 802-812.

Izu, Y., Sun, M., Zwolanek, D., et al, 2011. Type XII collagen regulates osteoblast polarity and communication during bone formation. The Journal of cell biology, 193 (6), 1115-1130.

Kleinberger, R.M., Burke, N.A., Dalnoki-Veress, K., et al, 2013. Systematic study of alginate-based microcapsules by micropipette aspiration and confocal fluorescence microscopy. Materials science & engineering.C, Materials for biological applications, 33 (7), 4295-4304.

Leal-Egana, A., Fritsch, A., Heidebrecht, F., et al, 2012. Tuning liver stiffness against tumours: an in vitro study using entrapped cells in tumour-like microcapsules. Journal of the mechanical behavior of biomedical materials, 9, 113-121.

Lim, F. and Sun, A.M. , 1980. Microencapsulated islets as bioartificial endocrine pancreas. Science (New York, N.Y.), 210 (4472), 908-910.

Liu, C., Liu, Y., Xu, X.X., et al, 2015. Potential effect of matrix stiffness on the enrichment of tumor initiating cells under three-dimensional culture conditions. Experimental cell research, 330 (1), 123-134.

Ma, X., Vacek, I. and Sun, A., 1994. Generation of alginate-poly-l-lysine-alginate (APA) biomicrocapsules: the relationship between the membrane strength and the reaction conditions. Artificial Cells, Blood Substitutes, and Immobilization Biotechnology, 22 (1), 43-69.

Offenhauser, N., Borgonovo, A., Disanza, A., et al, 2004. The eps8 family of proteins links growth factor stimulation to actin reorganization generating functional redundancy in the Ras/Rac pathway. Molecular biology of the cell, 15 (1), 91-98.

Ortiz, B., Fabius, A.W., Wu, W.H., et al, 2014. Loss of the tyrosine phosphatase PTPRD leads to aberrant STAT3 activation and promotes gliomagenesis. Proceedings of the National Academy of Sciences of the United States of America, 111 (22), 8149-8154.

Ozturk, S.S. and Palsson, B.O., 1991. Effect of medium osmolarity on hybridoma growth, metabolism, and antibody production. Biotechnology and bioengineering, 37 (10), 989-993.

Paredes Juarez, G.A., Spasojevic, M., Faas, M.M., et al, 2014. Immunological and technical considerations in application of alginate-based microencapsulation systems. Frontiers in bioengineering and biotechnology, 2, 26.

Rape, A.D., Zibinsky, M., Murthy, N., et al, 2015. A synthetic hydrogel for the high-throughput study of cell-ECM interactions. Nature communications, 6, 8129.

Richardson, T., Barner, S., Candiello, J., et al, 2016. Capsule stiffness regulates the efficiency of pancreatic differentiation of human embryonic stem cells. Acta biomaterialia, 35, 153-165.

Saeed, A.I., Bhagabati, N.K., Braisted, J.C., et al, 2006. TM4 microarray software suite. Methods in enzymology, 411, 134-193.

Santos, E., Pedraz, J.L., Hernandez, R.M., et al, 2013. Therapeutic cell encapsulation: ten steps towards clinical translation. Journal of controlled release: official journal of the Controlled Release Society, 170 (1), 1-14.

Shan, J., Zhao, W. and Gu, W., 2009. Suppression of cancer cell growth by promoting cyclin D1 degradation. Molecular cell, 36 (3), 469-476.

Shin, J.W. and Mooney, D.J., 2016. Extracellular matrix stiffness causes systematic variations in proliferation and chemosensitivity in myeloid leukemias. Proceedings of the National Academy of Sciences of the United States of America, 113 (43), 12126-12131.

Smyth, G.K., 2004. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Statistical applications in genetics and molecular biology, 3, Article3.

Strand, B.L., Coron, A.E. and Skjak-Braek, G., 2017. Current and Future Perspectives on Alginate Encapsulated Pancreatic Islet. Stem cells translational medicine, 6 (4), 1053-1058.

Su, P.H., Lin, Y.W., Huang, R.L., et al, 2013. Epigenetic silencing of PTPRR activates MAPK signaling, promotes metastasis and serves as a biomarker of invasive cervical cancer. Oncogene, 32 (1), 15-26.

Thu, B., Bruheim, P., Espevik, T., et al, 1996. Alginate polycation microcapsules. I. Interaction between alginate and polycation. Biomaterials, 17 (10), 1031-1040.

Wilson, E., Leszczynska, K., Poulter, N.S., et al, 2014. RhoJ interacts with the GIT-PIX complex and regulates focal adhesion disassembly. Journal of cell science, 127 (Pt 14), 3039-3051.

Wu, Y.L., Maachani, U.B., Schweitzer, M., et al, 2017. Dual Inhibition of PI3K/AKT and MEK/ERK Pathways Induces Synergistic Antitumor Effects in Diffuse Intrinsic Pontine Glioma Cells. Translational oncology, 10 (2), 221-228.

Yang, S., Wei, S., Pozzi, A., et al, 2009. The arachidonic acid epoxygenase is a component of the signaling mechanisms responsible for VEGF-stimulated angiogenesis. Archives of Biochemistry and Biophysics, 489 (1-2), 82-91.

Yang, Z., Zhuang, L., Szatmary, P., et al, 2015. Upregulation of heat shock proteins (HSPA12A, HSP90B1, HSPA4, HSPA5 and HSPA6) in tumour tissues is associated with poor outcomes from HBV-related early-stage hepatocellular carcinoma. International journal of medical sciences, 12 (3), 256-263.

Supporting information

Supplementary Methods

All microarray procedures were performed at the Gene Expression Unit of the Genomics Facility in the UPV/ EHU (SGIker platforms; Leioa, Spain). Microarray hybridization and Transcriptomic analysis Whole mouse gene expression microarray analysis was performed using the available SurePrint G3 Mouse GE v2 8x60K Microarray (Agilent Microarray Design ID: ID 074809, Agilent Technologies). Nucleic acid from each replicate, 100 ng, were labeled following the Agilent protocol “Gene Expression FFPE Workflow”. Briefly, cDNA library was generated and amplified using TransPlex Whole Transcriptome Amplification System (Sigma-Aldrich, Spain). The Titanium Taq DNA polymerase (Takara, Saint-Germain-en-Laye, France) was used during the cDNA library amplification step. Amplified cDNA was purified using the QIAquick PCR Purification kit (Qiagen, Hilden, Germany) and quantified using the NanoDrop 1000 spectrophotometer. The SureTag Labeling kit (Agilent Technologies) was used to enzymatically label 1.8 µg of amplified cDNA with cyanine 3-dUTP. Cy3-labeled cDNA samples were cleaned up using an Amicon Ultra-0.5 with Ultracel-30 Membrane, 30 kDa filter, provided with the SureTag Labeling kit. Yield and specific activity were determined using the NanoDrop 1000 spectrophotometer. The purified Cy3-labeled cDNA samples were hybridized to the Agilent SurePrint G3 Mouse GE v2 8x60K Microarray following the manufacturer instructions. Hybridized microarrays were scanned on a G2565CA DNA microarray scanner (Agilent Technologies).

Conventional statistical criteria (adjusted p-value < 0.05) were used for the selection of differentially expressed genes. For each differentially expressed gene the fold change (FC) was calculated as follows: log2 (FC) = log2 (Technological data) – log2 (Biological data), and the absolute FC was calculated according to the following formula [1]: if log2 (FC) > 0, FC = 2(log2(FC)); if log2 (FC) < 0, FC = (-1) x 2-(log2(FC)). Positive and negative values of FC showed upregulated or down-regulated genes on Technological microcapsules, respectively.

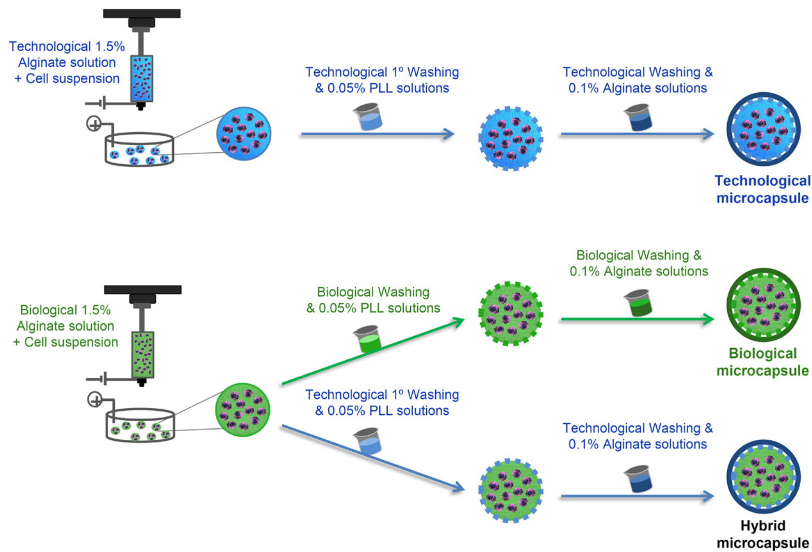
PANTHER Gene List Analysis Tools (http://pantherdb.org/) and National Center for Biotechnology Information databases (https://www.ncbi.nlm.nih.gov/) were used to detect the biological activity of genes. The heat maps and clustering of differentially expressed genes were constructed using Heatmapper (www.heatmapper.ca) [2]. For gene and protein nomenclature, the guidelines of Mouse Nomenclature Home Page of Mouse Genome Informatics (MGI) were followed (http://informatics.jax.org/mgihome/nomen/).

References

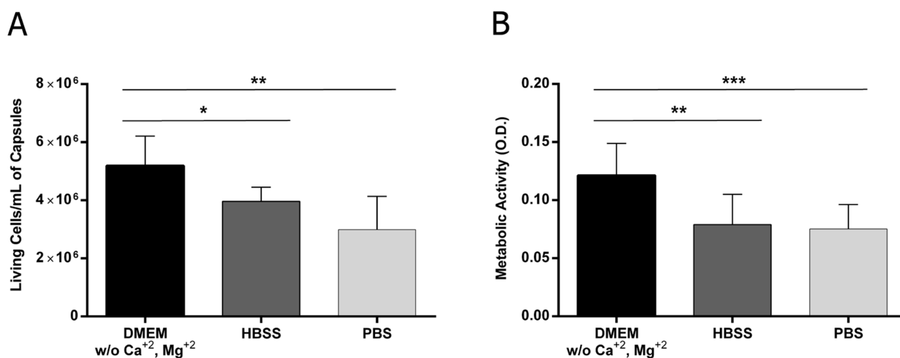
[1] N. Leonhardt, J.M. Kwak, N. Robert, D. Waner, G. Leonhardt, J.I. Schroeder, Microarray expression analyses of Arabidopsis guard cells and isolation of a recessive abscisic acid hypersensitive protein phosphatase 2C mutant, Plant Cell. 16 (2004) 596-615.

[2] S. Babicki, D. Arndt, A. Marcu, Y. Liang, J.R. Grant, A. Maciejewski, D.S. Wishart, Heatmapper: web-enabled heat mapping for all, Nucleic Acids Res. 44 (2016) W147-53.

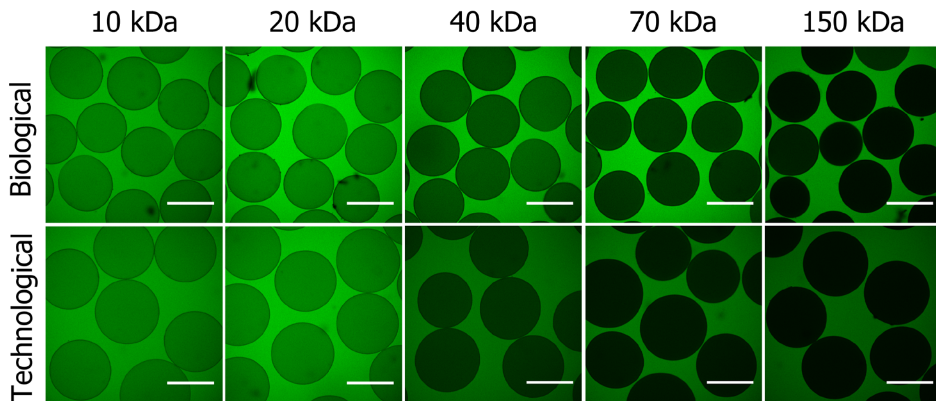
Supplementary Figures



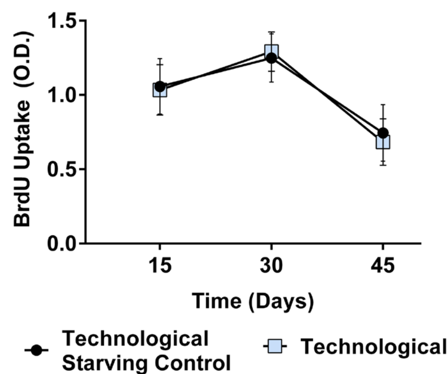
Supplementary Fig. 1. Schematic representation of the synthesis of Biological, Technological and Hybrid microcapsules. PLL: Poly-L-Lysine



Supplementary Fig. 2. Preliminary studies to design the Biological 1.5% alginate solution. (A) Live/dead flow cytometry and **(B)** CCK-8 metabolic assays were performed to select the most suitable vehicle containing electrolytes for the Biological 1.5% alginate solution. Among them, DMEM w/o Ca^{+2}, Mg^{+2} demonstrated to provide the best results for cell viability and metabolic activity. Graphs symbolize mean ± SD (n = 3 for cell viability studies, n = 7 for metabolic assays). Statistical significance: *p < 0.05, **p < 0.01 and ***p < 0.001. DMEM: Dulbecco's modified Eagle's medium. HBSS: Hank's balanced salt solution. PBS: Phosphate buffered saline.



Supplementary Fig. 3. Microcapsule permeability. FITC-labeled dextran diffusion showed no statistically significant differences in the molecular weight cut off between Biological and Technological microcapsules. (n = 4). Scale bars = 400 µm.



Supplementary Fig. 4. D1-MSCs-EPO presented an important proliferative capacity. During bromodeoxyuridine (BrdU) uptake assays, when comparing results of Technological samples (incubated in DMEM with 10% FBS supplementation) to the starving control (same Technological microcapsules incubated in DMEM with 0.1% FBS supplementation) no statistical differences were found, confirming the high proliferative capacity of these cells. Graphs symbolize mean ± SD (n = 5). DMEM: Dulbecco's modified Eagle's medium. FBS: fetal bovine serum.

Chapter 2

Extracellular matrix protein microarray-based biosensor with single cell resolution: integrin profiling and characterization of cell-biomaterial interactions

Sensors and Actuators B: Chemical, 299 (2019), 126954.

DOI: https://doi.org/10.1016/j.snb.2019.126954

Extracellular matrix protein microarray-based biosensor with single cell resolution: integrin profiling and characterization of cell-biomaterial interactions

Ainhoa Gonzalez-Pujana[a,b*], Edorta Santos-Vizcaino[a,b*], Maite García-Hernando[c,d], Beatriz Hernaez-Estrada[a,b], Marian M. de Pancorbo[d], Fernando Benito-Lopez[c], Manoli Igartua[a,b], Lourdes Basabe-Desmonts[e,f,#], Rosa Maria Hernandez[a,b,#]

[a] NanoBioCel Group, Laboratory of Pharmaceutics, School of Pharmacy, University of the Basque Country (UPV/EHU), Spain.
[b] Biomedical Research Networking Centre in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Spain.
[c] Analytical Microsystems & Materials for Lab-on-a-Chip (AMMa-LOAC) Group, University of the Basque Country (UPV/EHU), Spain.
[d] BIOMICs Research Group, Lascaray Ikergunea Research Center, University of the Basque Country (UPV/EHU), Spain.
[e] Ikerbasque, Basque Foundation for Science, Spain
[f] BIOMICs-microfluidics Research Group, Microfluidics Cluster University of the Basque Country (UPV/EHU), Spain.
* Authors contributed equally to this work. # Corresponding authors: Lourdes Basabe-Desmonts, Rosa Maria Hernandez.

ABSTRACT

In the search of biomaterials that promote cell adhesion, it is crucial to explore the integrin-substrate dynamic interactions given in a certain cell type to design successful biofunctionalization strategies. Here, we use a microarray platform for a thorough characterization of cell adhesion to a particular substrate. A biosensor based on an array of 20 µm fibronectin circular isles was adapted to tissue culture treated plates to facilitate the performance of cell adhesion assays and the posterior affinity analyses. This sensitive analytical tool enables not only the evaluation of the cell adhesion kinetics, but also the integrin profiling and their contribution to cell attachment and adhesion strengthening via clustering. In particular, the biosensor was able to detect a significantly slower adhesion kinetics in fibroblasts, namely baby hamster kidney fibroblasts (BHK) and human dermal fibroblasts (hDF), in comparison to other cell types such as C_2C_{12} mouse myoblasts (C_2C_{12}) or adipose-derived human mesenchymal stromal cells (hMSCa). When directly comparing hDF and hMSCa, the analysis determined that the differing kinetics were caused by a distinct integrin expression profile. Whereas β_1-presenting integrins were the major responsible for hDF attachment, hMSCa adherence was importantly dependent on β_1 but also on other integrin classes. Additionally, results revealed that concerning cell adhesion consolidation, in hMSCa, both $\alpha_v\beta_3$ and β_1-subunit-presenting integrins contributed similarly; whereas in hDF, the latter played a more important role. Hence, our biosensor provided crucial information for the development of new cell-adhesive biomaterials, which are key in multiple biomedical fields including cell therapy or tissue engineering.

1. Introduction

In their natural niche, cells are surrounded by the extracellular matrix (ECM), a complex three-dimensional (3D) network consisting of proteoglycans, glycosaminoglycans, glycoproteins and fibrous-forming proteins such as fibronectin (FN), collagen or elastin [1]. Cells interact with their microenvironment through integrins, cell adhesion receptors that recognize ECM ligands. Integrins are heterodimers composed of an α-subunit and a β-subunit. At least eighteen α and eight β subunits have been identified in humans, and the different combinations give rise to 24 different integrin classes [2]. Their role is vital, since cell adhesion is a paramount feature for a correct cell function. Indeed, integrin-ECM binding results in the transduction of mechanical and biochemical signals that regulate diverse cellular processes such as cell adhesion, migration, proliferation and differentiation [3].

For that reason, over the last decades, special interest has been paid to engineering 3D matrices that mimic the physical, mechanical and biological properties of the ECM. Numerous biomaterials have demonstrated suitability for 3D culture of cells with multiple applications, including regenerative medicine [4], controlled drug delivery [5] or stem cell differentiation [6]. For instance, hydrogels made of natural polymers such as alginate [7] have been widely explored because of their versatility, enabling the modification of mechanical and degradation properties [8,9]. Moreover, these matrices offer the possibility to attach peptides naturally present in the ECM, thereby allowing immobilized cells to interact with otherwise inert biomaterials. This biofunctionalization, key for cell adhesion, has also been employed in other materials, such as titanium medical implants, promoting their interaction with host cells and accelerating the bone healing process [10].

Therefore, in the search of adequate materials for different biomedical applications, the focus is on surface modification [11]. It is possible to incorporate full-length ECM proteins, such as collagen, FN, or gelatin. However, their use may be limited due to their heterogeneity, low protein stability and immunogenicity [12]. Moreover, the conformation and orientation of the protein hinder its correct presentation to the cells. An alternative that overcomes these limitations is the use of minimal cell recognizable sequences (short peptides responsible for cell adhesion) instead of the whole protein. Additional advantages comprise the more cost-effective synthesis and the easier characterization of peptides. Furthermore, because of

their smaller size, it is possible to decorate the material surface with a higher density of cell binding domains [13]. The most widely employed short-peptide motif is Arginine-Glycine-Aspartate (RGD), a tri-peptide present in multiple ECM proteins such as FN, vitronectin or laminin [14,15]. Its broad use is, in part, due to the fact that approximately half of the 24 integrins bind to ECM molecules in a RGD dependent manner. In fact, RGD peptides have been proven to stimulate cell adhesion on various materials, comprising polymers (synthetic or natural) and inorganic materials [16]. Some other relevant ECM moieties have also demonstrated cell attachment properties, either in combination with RGD or independently. Some examples are the sequences PHSRN, PRARI, YIGSR or IKVAV [16,17] (abbreviations are described in Supplementary Table 1).

Despite numerous studies highlight the potential of matrices with mechano-adhesive properties, the affinity of each integrin class for these ECM cues is different. Therefore, for the design of effective functionalization strategies, it is necessary to determine the specific integrins that contribute to cell adhesion in the particular cell type employed. To date, it is possible to study the integrin expression via complex techniques such as flow cytometry; however, simple methods that evaluate not only the expression, but also the contribution of integrins to the whole adhesion process, from the initiation of cell attachment to the adhesion consolidation via integrin clustering, are still required. In this regard, electrical impedance spectroscopy has been used to develop instruments to measure cell-substrate interactions and several equipment are commercially available [18]. However, these systems require complex fabrication techniques and they give an indirect measurement of cell adhesion and detachment based on electrical impedance, which can cause many artifacts [19]. Other optical [20,21] and piezoelectric [22] sensors have also successfully measured cell attachment and adhesion kinetics, but mainly for drug screening applications and not focused on integrin profiling.

On the other hand, sensor arrays represent an attractive analytical tool to explore cell-ECM interactions [23] by optical means. Protein dot microarrays are fabricated by immobilizing the protein of interest onto a surface, commonly glass or silicon [24]. The rest of the surface is blocked with molecules that do not promote cell adhesion, such as bovine serum albumin (BSA) or nonfat milk blocking buffers, making every printed spot an independent experimental replicate. Numerous studies have taken advantage of these arrays to investigate how

cell adhesion to printed ECM proteins regulates different cellular processes. These include the influence of cell spreading and shape in cell migration [25], cell-cell adhesion [26] or cell differentiation [27,28]. Additionally, arrays designed to hold a single cell per dot have been used to monitor adhesion kinetics of blood platelets to biofunctionalized substrates [29] as well as to measure platelet affinity to different ECM proteins [30] or to monitor the effect of antiplatelet drugs [31] by optical means. This platform enables a simple and accurate quantification of cell adhesion using a binary counting system of occupied and empty adhesion dots. The measurement is expressed by means of the dot array occupancy (DAO, percentage of occupied dots).

Following this path, and focused on gathering fundamental information directly applicable to the development of new materials suited to the specific requirements of multiple biomedical applications, we developed a sensitive FN dot array biosensor, with single cell resolution, to explore the integrin profile and characterize the cell-biomaterial dynamic interactions.

2. Materials and methods

2.1. Polydimethylsiloxane (PDMS) stamp fabrication

A patterned stamp master wafer was fabricated by means of photolithography. The pattern design consisted of areas presenting circular spots of a determined size (20, 50 or 100 μm) separated from each other by 50 μm. To obtain PDMS stamps, a 10:1 (v/v) mixture of Sylgard 184 Silicon Elastomer and curing agent (Sigma Aldrich, Cat. No. 761036-5EA) was poured over the stamp master and degassed under vacuum for 30 min. The mixture was cured 1 h at 70ºC, carefully demolded and left in the oven for an additional hour for complete curing.

2.2. Preparation of FN-patterned surfaces

The process was conducted at room temperature and 50-60% of humidity. Tissue culture plates (TCP) (Sigma-Aldrich, Cat. No. CLS3513-50EA) were employed as substrates. To improve the protein transference to the surface, the plates were treated with oxygen plasma by being introduced in a plasma reactor (Harrick Plasma cleaner, PDC-002) for 40 s at an oxygen pressure of 0.7 bar and a high power. PDMS stamps were washed with 70% ethanol and dried

prior to use. Subsequently, they were inked with 50 µL of a phosphate buffered saline (PBS) solution containing a mixture of 50 µg mL^{-1} of bovine plasma FN (Thermo Fisher Scientific, Cat. No. 33010-018,) and 6.25 µg mL^{-1} of carboxytetramethylrhodamine (TAMRA) (Thermo Fisher, Cat. No. A23016) labeled BSA. After 30 min, the excess ink solution was removed from the PDMS surface with a pipette. Subsequently, stamps were washed with ddH_2O and dried under a stream of nitrogen. Stamps were then placed in a plate well and incubated in contact with the substrate for additional 30 min. After removing the PDMS stamps from the plate, 1 mL of a solution of 1% BSA in PBS was added to each micro-patterned well. Plates were incubated for 1 h at 4ºC with this solution to block any uncovered region of the surface. The plate wells were then rinsed with PBS and stored at 4ºC in the dark. Typically, patterned surfaces were used within the next 24 h after fabrication. Verification of homogeneous printing was performed by fluorescence microscopy (Nikon TMS, Hampton, NH).

2.3. Cell culture

Four cell types were selected for testing the applicability of the platform. Cells were seeded in T-flasks and cultured in the corresponding medium. Baby hamster kidney fibroblasts (BHK) (ATCC®, Cat. No. CCL-10™) and C_2C_{12} Mouse Myoblasts (C_2C_{12}) (ATCC®, Cat. No. CRL-1772™) were grown in Dulbecco's Modified Eagle's Medium (DMEM) (ATCC®, Cat. No. 30-2002™) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution. Adipose-derived human mesenchymal stromal cells (hMSCa) (ATCC®, Cat. No. PCS-500-011) were cultured in mesenchymal stromal cell basal medium (ATCC®, Cat. No. PCS-500-030™) supplemented with the corresponding growth kit (ATCC®, Cat. No. PCS-500-040). Primary human dermal fibroblasts (hDF) (ATCC®, Cat. No. PCS-201-012) were grown in fibroblast basal medium (ATCC®, Cat. No. PCS-201-030™) supplemented with the corresponding growth kit (ATCC®, Cat. No. PCS-201-041). Cells were maintained at 37ºC in a 5% CO_2 / 95% air atmosphere and passaged according to the necessities of each cell type using trypsin-ethylenediaminetetraacetic acid (EDTA) (ATCC®, Cat. No. PCS-999-003).

2.4. Cell seeding and DAO determination

For cell seeding in the substrate, $2x10^5$ cells were suspended in 1 mL of the adequate FBS free medium and added to each well. Plates were protected from light and placed in a rocker

(Thermo Fisher, Cat. No. M48720-33Q). Agitation was maintained in an incubator, at 37ºC in a 5% CO_2 air atmosphere, for the time interval required for each experiment. Subsequently, unattached cells were washed off and samples were processed following the requirements of each experiment. For adhesion quantification, microscopy images were obtained by means of an inverted microscope (Nikon TMS, Hampton, NH). The 4X objective field of view was used to capture approximately 1000 dots of 20 μm in each micrograph. For the same substrate zone, two images were acquired: one displaying the TAMRA-labeled pattern, and a brightfield image capturing the adhered cells. At least three different substrate areas were processed for each experimental point, gathering at least 3000 dots, by means of ImageJ software (Rasband, W.S., ImageJ, US National Institutes of Health, Bethesda, Rockville, MD, https://imagej. nih.gov/ij/, 1997–2016). In particular, micrographs were analyzed using the cell counter tool and the DAO was expressed as the mean value of the percentage of FN dots occupied with adhered cells.

2.5. Integrin profiling assay

To determine the influence of blocking specific integrins on cell adhesion, cells were pretreated with the adequate anti-integrin antibodies (Abcam, anti-Integrin β_1 antibody, Cat. No. ab24693, anti-Integrin $\alpha_v\beta_3$ antibody, Cat. No. ab78289) for 1 h at 37ºC in 5% CO_2 and agitation. Afterwards, cells were seeded in the patterned plates and incubated an additional hour as previously described. Once the unattached cells were washed off, cells were fixed and the DAO was quantified as detailed above.

2.6. Immunostaining

Cells were pretreated with anti-integrin antibodies and incubated in the substrates for 1 h at 37ºC in 5% CO_2 and agitation. After washing off the non-attached excess, cells were fixed, permeabilized and incubated with anti-phosphorylated focal adhesion kinase (pFAK) primary antibody (Abcam, Anti-FAK phospho Y397, Cat. No. ab81298). Afterwards, samples were stained with the Alexa Fluor 488-labelled secondary antibody (Abcam, Cat. No. ab150077), Alexa Fluor 594- labelled phalloidin for F-actin (Thermo Fisher Scientific, Cat. No. A12381) and DAPI for the nuclei (Thermo Fisher Scientific, Cat. No. D1306). Fluorescent micrographs were acquired by means of an inverted fluorescence microscope (Nikon TMS, Hampton, NH).

2.7. Cell-substrate attachment strength determination

After being pretreated with anti-integrin antibodies, cells were incubated in the substrates in agitation for 1 h at 37°C in 5% CO_2. Once the non-adhered cells were washed, the DAO was quantified as previously described to obtain the initial occupancy values. Afterwards, a detachment assay was performed. Specifically, each micro-patterned well was subjected to 5 cycles of liquid rinse/aspiration, and the DAO was quantified again. Pre-detachment and post-detachment DAO values were compared to determine the strength of cell-FN binding.

2.8. Flow cytometry

A suspension of 10^6 cells was dyed for 10 min at 4°C in the dark with human CD29-PEVIO770 antibodies for β_1 detection (Miltenyi Biotec, Cat. No. 130-101-281) and human CD51/CD61-APC antibodies for $\alpha_v\beta_3$ determination (Miltenyi Biotec, Cat. No. 130-103-745) following the manufacturer's instructions. IgG1-APC (Miltenyi Biotec, Cat. No. 130-113-758) and IgG1-PE-vio770 (Miltenyi Biotec, No. 130-113-764) antibodies were used as isotype controls. Cells were then washed and re-suspended in a buffer containing 0.5 % BSA and 2 mM EDTA in PBS to be processed by flow cytometry (MACSQuant Analyzer, Miltenyi Biotec).

2.9. Data analysis and statistics

All samples were assayed at least in triplicate and results are shown as mean ± SD. The normal distribution of the data was checked by the Shapiro-Wilk test. One sample t-test was employed to determine if data differed significantly from a determined mean value. To detect statistical significances between two groups, a two-tailed t-test was performed, while one-way ANOVA was used for multiple comparisons. For the latter, the Levene test was used to determine the homogeneity of variances. If homogeneous, the Bonferroni post-hoc was applied and if non-homogeneous, the Tamhane post-hoc was employed. In the case of non-normally distributed data, a Mann-Whitney non-parametric test was applied. p values less than 0.05 were assumed to be significant in all analyses. All statistical computations were performed by means of SPSS 23 (IBM SPSS, Chicago, IL).

3. Results and discussion

3.1. Platform optimization for single cell studies

In the development of a platform to explore cell adhesive properties, micro-contact printing was adapted to print FN dot arrays on TCP wells (Fig. 1A). A patterned stamp master was obtained by photolithography and used to fabricate PDMS stamps presenting circular pillars

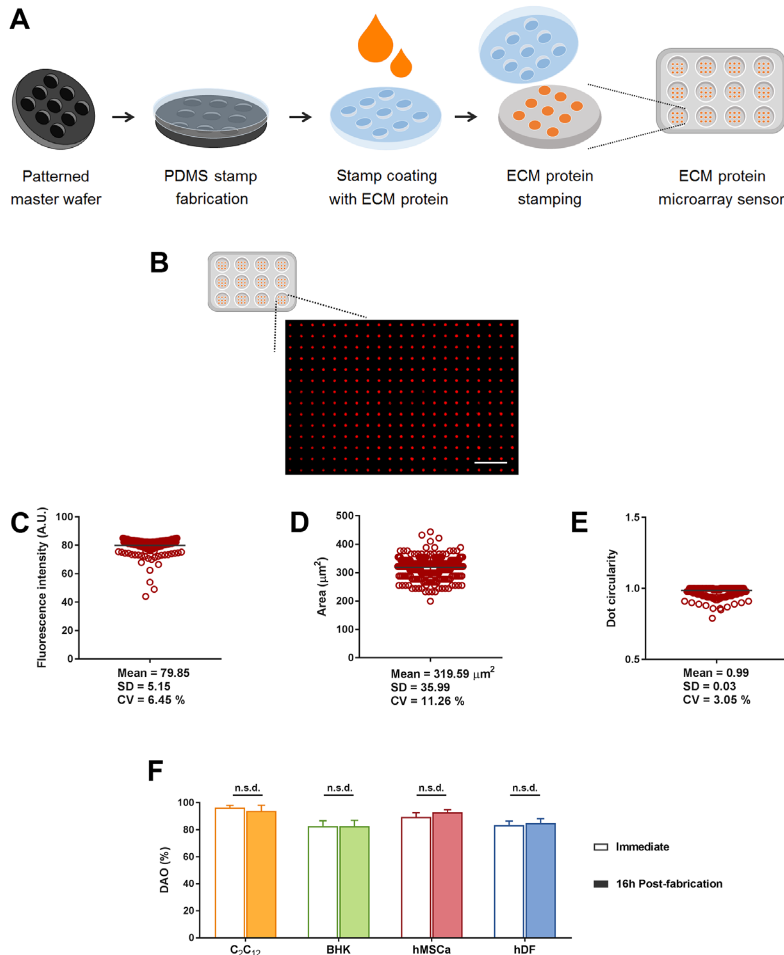
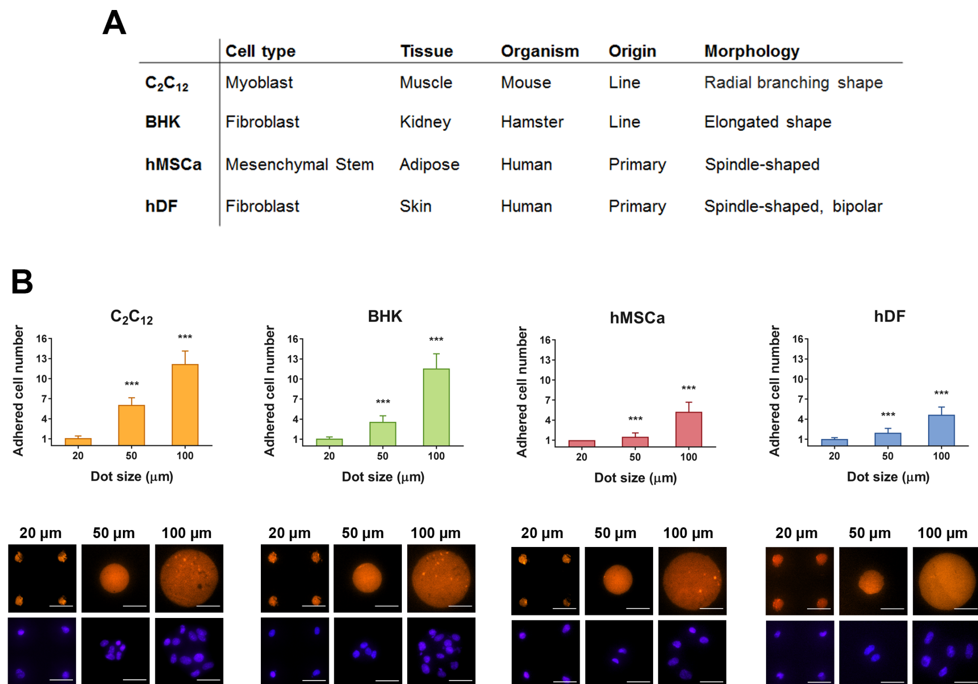


Fig. 1. Protein microarray biosensor development. (A) Scheme of the fabrication of the ECM protein microarray platform. **(B)** Homogeneous distribution of the fluorescently labeled fibronectin-BSA-TAMRA pattern. Scale bar = 200 μm. Pattern uniformity was confirmed by the analysis of dot fluorescence intensity **(C)**, size **(D)** and circularity **(E)** (n = 300 dots per image field). **(F)** Validation of the platform stability 16h post-fabrication. Error bars mean ± SD (n = 3 samples per experimental condition). Statistical significance: paired, two-tailed t-test: n.s.d.: non-significant differences ($p > 0.05$). PDMS: polydimethylsiloxane. ECM: extracellular matrix. SD: standard deviation. CV: coefficient of variation. DAO: dot array occupancy. C_2C_{12}: C_2C_{12} mouse myoblasts. BHK: baby hamster kidney fibroblasts. hMSCa: adipose-derived human mesenchymal stromal cells. hDF: human dermal fibroblasts.

of various sizes. Indeed, the versatility of the technique enables the printing of protein isles of different sizes and shapes (Supplementary Fig. 1). The stamps were incubated with a PBS solution containing FN and TAMRA-labeled BSA, to obtain a positive control for protein printing, and stamped in the TCP wells resulting in FN dot arrays. Fluorescence microscopy indicated a homogeneous printing of dots (Fig. 1B). To verify the uniformity of the pattern, we evaluated the CV of the dots' fluorescence intensity (Fig. 1C), size (Fig. 1D) and circularity (Fig. 1E), and obtained CV values of 6.45%, 11.26% and 3.05%, respectively (n = 300 dots per image field). The sample was therefore considered homogeneous, which permitted cells to adhere to the dots with equal probability, as observed throughout all the study. To confirm this observation, within the same sample, we calculated the CV of the dot array occupancy (DAO) of the replicates (n = 4). In particular, we evaluated samples from the different cell types employed in the study, and obtained low CV % values as observed in Supplementary Table 2. Subsequently, we assayed the stability of the pattern by incubating a suspension of cells



A

	Cell type	Tissue	Organism	Origin	Morphology
C_2C_{12}	Myoblast	Muscle	Mouse	Line	Radial branching shape
BHK	Fibroblast	Kidney	Hamster	Line	Elongated shape
hMSCa	Mesenchymal Stem	Adipose	Human	Primary	Spindle-shaped
hDF	Fibroblast	Skin	Human	Primary	Spindle-shaped, bipolar

Fig. 2. Micro-patterning optimization for single cell resolution analyses in C_2C_{12}, BHK, hMSCa and hDF cells. (A) Description of the cells employed in the study. **(B)** Dot size optimization for analyses with single cell resolution. Error bars mean ± SD (n = 4 samples per experimental condition). Statistical significance: one-sample t-test: ***p < 0.001 compared to 1. Scale bars = 50 µm. C_2C_{12}, C_2C_{12} mouse myoblasts. BHK, baby hamster kidney fibroblasts. hMSCa, adipose-derived human mesenchymal stromal cells. hDF, human dermal fibroblasts.

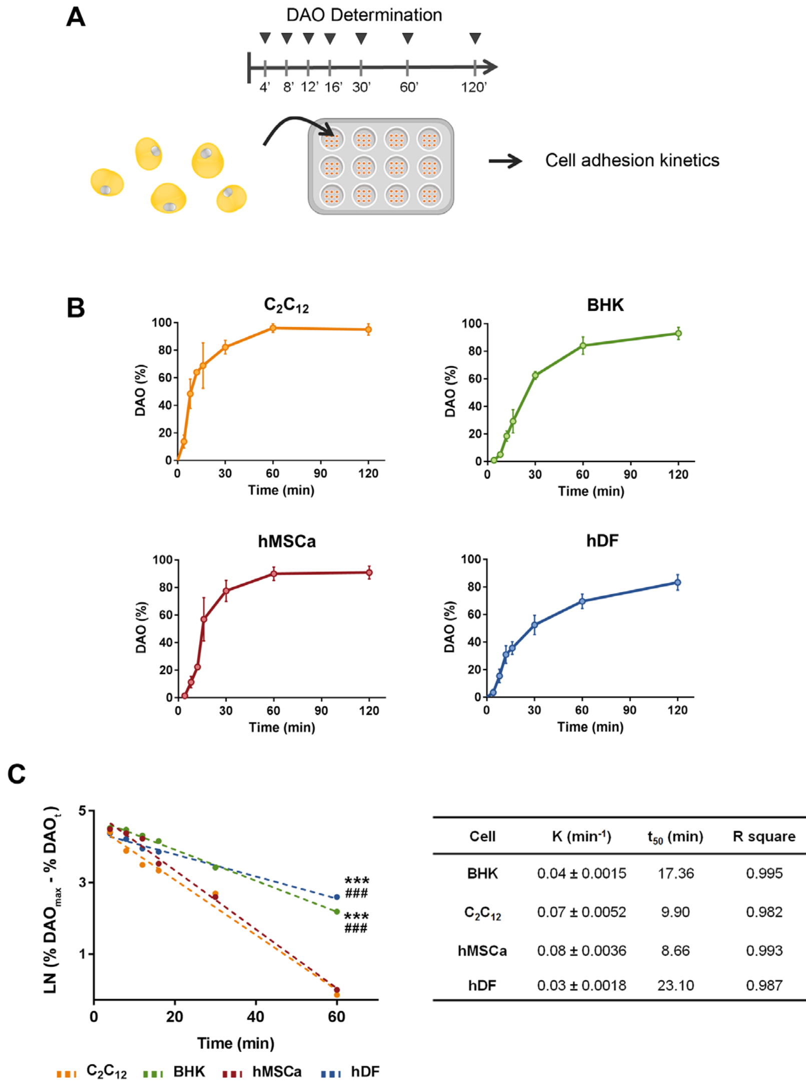
in both, a freshly fabricated substrate and an overnight 4ºC stored substrate. We showed no significant differences in the FN dot array occupancy (DAO), confirming the overnight stability of the platform (Fig. 1F). This represents a technical advantage, since it is possible to fabricate and store substrates on one day and perform the cell studies on the following.

To demonstrate the real applicability of the biosensor, cells with differing characteristics were selected. In particular, we chose established cell lines and primary cells with varying origin and morphology as shown in Fig. 2A. Moreover, because of their different sizes, it was necessary to determine the adequate dot diameter that resulted in single cell adhesion for each cell type. Therefore, cells were incubated in substrates presenting dots of 20, 50 and 100 µm (Fig. 2B). For the bigger isles, 50 and 100 µm dots, we did not obtain a single cell adhesion in any case. Multiple cells were attached to each of these dots, and interestingly, the number varied when using cell lines or primary cells. Specifically, the cell lines in this study, namely BHK and C_2C_{12}, presented a smaller size than primary hMSCa and hDF, and hence, a higher number of cells fitted each 50 or 100 µm dot. In spite of these differences, FN isles of 20 µm provided a single cell adhesion pattern for all the four cells. Therefore, 20 µm circular dots were printed for the rest of the study to carry out single cell assays.

3.2. Cell type determined cell adhesion kinetics

First, we studied cell adhesion kinetics following the procedure shown in Fig. 3A. BHK, C_2C_{12}, hMSCa and hDF cells were incubated in a FN dot array substrate for two hours and the DAO was determined at different time points. For every cell, we observed how the DAO increased over time until reaching a plateau (Fig. 3B). The adhesion curves were fitted to a first order kinetics, providing values for the rate constant (k) and the t_{50}, defined as the time point at which the 50% of the dot arrays are occupied. With these parameters, 2 different kinetics were identified. Presenting significantly higher k and lower t_{50} values, C_2C_{12} and hMSCa adhered significantly faster to the substrate than BHK and hDF cells (Fig. 3C). Although it was possible to hypothesize that the kinetics might be importantly influenced for being primary cells or a cell line, our results indicated that the cell type was the major determinant. We observed that the two fibroblasts included in the study, BHK and hDF, presented similar kinetics in spite of the fact that the former is a mouse cell line and the latter has a human primary

origin. Since cell-matrix interactions are mainly mediated by integrins [2], we hypothesized that the differences observed in the adhesion kinetics may be given by a distinct integrin expression profile in each cell type.



Cell	K (min^{-1})	t_{50} (min)	R square
BHK	0.04 ± 0.0015	17.36	0.995
C_2C_{12}	0.07 ± 0.0052	9.90	0.982
hMSCa	0.08 ± 0.0036	8.66	0.993
hDF	0.03 ± 0.0018	23.10	0.987

Fig. 3. Cell adhesion kinetics is dependent on cell type. (A) Representation of the experimental procedure followed for adhesion kinetics determination. **(B)** Dot array occupancy (DAO) for C_2C_{12}, BHK, hMSCa and hDF over time. Error bars mean ± SD (n = 4 samples per experimental condition). **(C)** First order kinetics fit for cell adhesion. Error bars mean ± SD (n = 4 samples per experimental condition). Statistical significance: one-way ANOVA with Bonferroni multiple comparisons test: ***$p < 0.001$ compared to hMSCa. ###$p < 0.001$ compared to C_2C_{12}. BHK: baby hamster kidney fibroblasts. C_2C_{12}: C_2C_{12} mouse myoblasts. hMSCa: adipose-derived human mesenchymal stromal cells. hDF: human dermal fibroblasts.

3.3. hMSCa and hDF presented a different integrin expression profile

Our next goal was to study the integrin contribution to cell attachment. For the following sets of experiments, we continued with two of the four cells. In particular, we selected hMSCa and hDF for presenting contrasting kinetics and for being the most attractive from a therapeutic standpoint because of their human and primary origin [32-34]. With that aim, we followed the procedure described in Fig. 4A. We first incubated the cells with anti-integrin antibodies, specifically blocking either the $\alpha_v\beta_3$ integrin, or all the integrins presenting the β_1 subunit. These integrins were selected for being primary FN binding receptors in both cell types [28,35]. Subsequently, cells were seeded in the FN dot array platform and the DAO was quantified. Regarding hMSCa (Fig. 4B), DAO was not significantly affected when blocking the $\alpha_v\beta_3$ integrin, but it was when blocking all the integrins presenting the β_1 subunit. Similar results were obtained for hDF (Fig. 4C). However, in this case, a significantly greater impact of β_1 blocking was observed, as demonstrated when normalizing the data to directly compare the two cell types (Fig. 4D).

When blocking β_1 subunit presenting integrins, approximately a 40% of the hMSCa cells were able to attach to the substrate, but only around a 2% of hDF. This indicates a different integrin profile; while in hDF β_1 integrin expression is major, in hMSCa, their influence is balanced up to a certain point by other integrins, including α_v-class integrins, which also play a role in cell attachment. Importantly, this assay proved the sensitivity of the platform, since the sensor was capable of discerning differences in DAO when blocking a single integrin, $\alpha_v\beta_3$, *versus* blocking the β_1 domain, which is present in multiple integrins [36].

3.4. $\alpha_v\beta_3$-mediated crosstalk was crucial for integrin clustering and cell-substrate interaction strengthening

In order to study the contribution of each integrin class to cell adhesion consolidation via focal adhesion formation, we performed an immunocytochemistry assay for focal adhesion kinase (FAK) staining. FAK is a non-receptor protein-tyrosine kinase that transforms the cell external biomechanical stimuli into biochemical signals, activating important intracellular signaling pathways [37]. This occurs preferentially by the specific phosphorylation of FAK internal tyrosine residues, being two of the most relevant the tyrosine residues Y397 and Y861

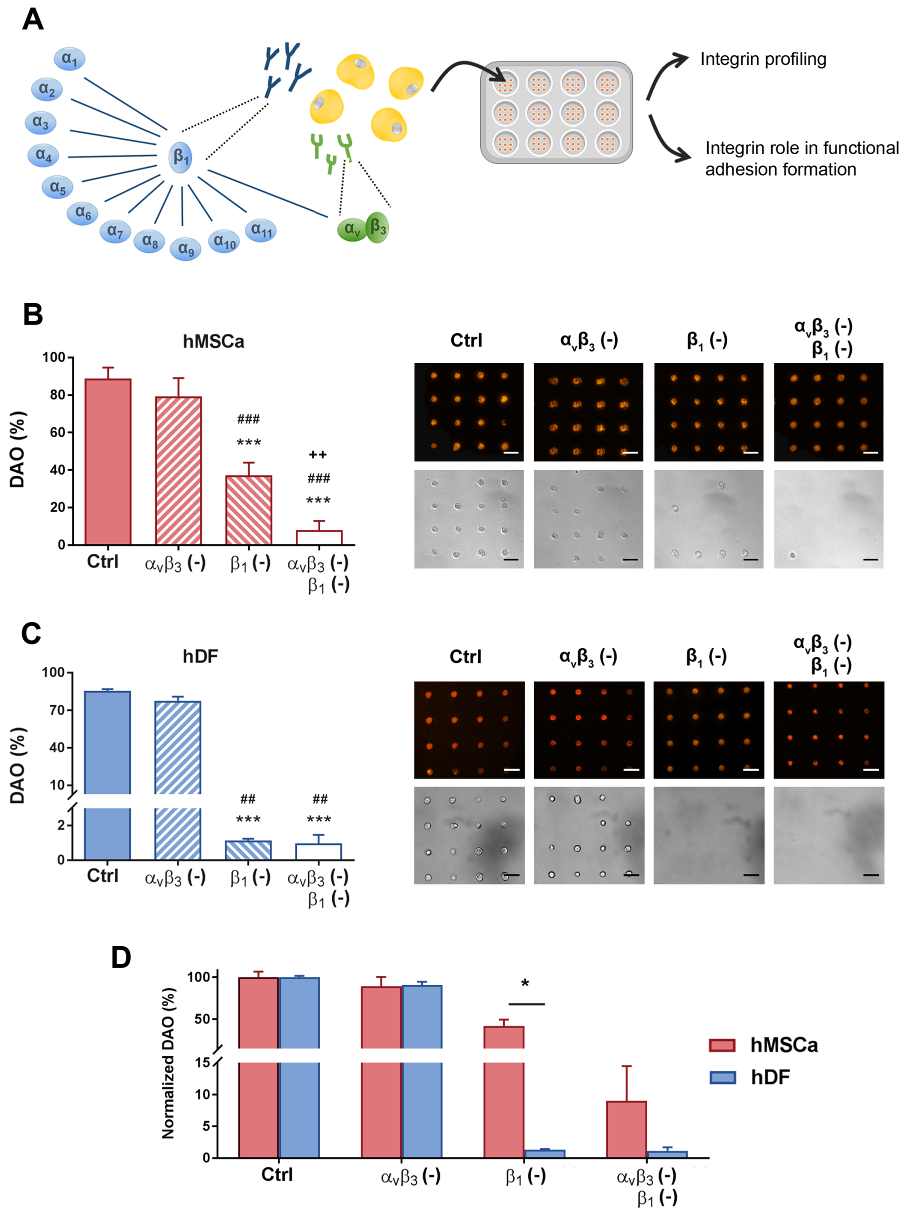


Fig. 4. hMSCa and hDF presented different integrin expression balance. (A) Experimental procedure followed for analyzing integrin profile and integrin contribution to adhesion strengthening. Influence of blocking $\alpha_v\beta_3$ and/or β_1-subunit presenting integrins on cell adhesion for **(B)** hMSCa and **(C)** hDF. Error bars mean ± SD (n = 4 samples per experimental condition). Statistical significance: one way ANOVA with Bonferroni multiple comparisons test for hMSCa, one-way ANOVA with Tamhane multiple comparisons test for hDF: ***p < 0.001 compared to Ctrl. ##p < 0.01 and ###p < 0.001 compared to $\alpha_v\beta_3$ (-). ++p < 0.01 compared to β_1 (-). Scale bars = 50μm. **(D)** Cell adhesion normalization and comparison between hMSCa and hDF. Error bars mean ± SD (n=4 samples per experimental condition). Statistical significance: paired, two-tailed t-test: *p < 0.05. DAO: dot array occupancy. hMSCa: adipose-derived human mesenchymal stromal cells. hDF: human dermal fibroblasts. Ctrl: control.

[38]. Therefore, we once again blocked the $\alpha_v\beta_3$ integrin or all the integrins presenting the β_1 subunit in hMSCa or hDF cells, and subsequently seeded them in the FN dot array substrate for 1h. Cells were fixed and stained for pFAK-Y397 or pFAK-Y861. Micrographs showed FAK-Y397 phosphorylation in all of the groups for both cell types (Fig. 5A). Similar results were obtained when staining for the tyrosine residue Y861 (Supplementary Fig. 2). Hence, this commonly used technique failed to determine the actual contribution of each integrin to the achievement of a functional adhesion and was not able to discriminate between blocking the $\alpha_v\beta_3$ integrin or all the β_1 integrins.

On the contrary, a detachment assay performed in our biosensor was able to provide a deeper insight into cell-substrate interaction. $\alpha_v\beta_3$ or β_1-presenting integrins were blocked and cells incubated in the FN dot array. The DAO was quantified and subsequently, a detachment force was applied by liquid aspiration. After five cycles, the DAO was quantified again and compared to the initial determination. Micrographs showing adhered cells prior and after the application of the detachment force are shown in Supplementary Figures 3 and 4. Although we previously detected that $\alpha_v\beta_3$ blocking did not alter the DAO, the adhered cells in this group presented less resistance to detachment forces than the control group in both, hMSCa and hDF (Fig. 5B). This phenomenon may be explained by the crosstalk that exists between α_v and β_1 integrins. It has been reported that once engaged, α_v integrins induce the clustering of integrins such as $\alpha_5\beta_1$, strengthening cell adhesion [39]. Therefore, the absence of α_v integrins would impede the crosstalk and as a result, weaken the cell-substrate interaction. Consequently, β_1 blocking also debilitated the cell binding, because of the absence of β_1-class integrin clusters which are responsible for providing a stronger attachment.

Although both cell types showed this behavior, β_1 subunit blocking prevented cell adhesion strengthening more importantly than $\alpha_v\beta_3$ blocking in hDF, but not in hMSCa (Fig. 5C). This indicates a greater impact of β_1 integrins in hDF in comparison to hMSCa, confirming the results previously obtained (Fig. 4D). This finding corroborates previous data highlighting the vital role of $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins in hDF cells [35].

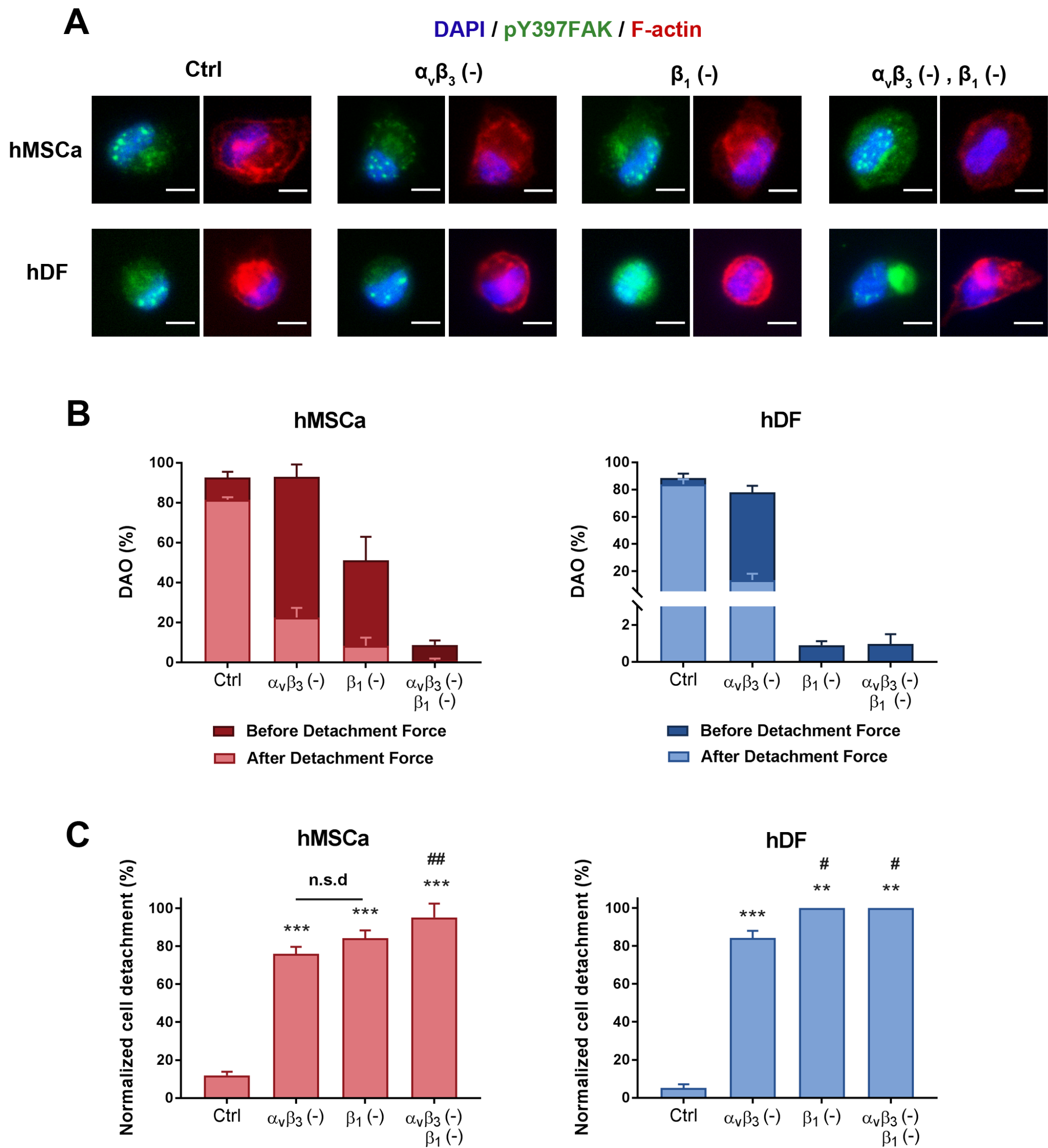


Fig. 5. Integrin role in the formation of a functional adhesion. (A) Focal adhesion determination by pFAK Y-397 immunofluorescence imaging. DAPI in blue, pY397-FAK in green and F-actin in red. Scale bars = 10 μm (n = 4 samples per experimental condition). **(B)** Effect of integrin blocking on cell-substrate interaction strength: dot array occupancy (DAO) determination before and after applying a detachment force. Error bars mean ± SD (n = 4 samples per experimental condition). **(C)** Data normalization in hMSCa and hDF cells. Error bars mean ± SD (n = 4 samples per experimental condition). Statistical significance: one-way ANOVA with Bonferroni multiple comparisons test for hMSCa, one-way ANOVA with Tamhane multiple comparisons test for hDF: **p < 0.01, ***p < 0.001 compared to Ctrl. #p < 0.05, ##p < 0.01 compared to $\alpha_v\beta_3$ (-). Ctrl: control. hMSCa: adipose-derived human mesenchymal stromal cells. hDF: human dermal fibroblasts. n.s.d.: non-significant differences (p > 0.05).

3.5. Validation of the method by means of flow cytometry

To validate the potential of the platform as a simple and rapid tool to determine integrin contribution to the cell adhesion process, $\alpha_v\beta_3$ and β_1 expression was determined by flow cytometry. Regarding $\alpha_v\beta_3$ integrin (Fig. 6A), whereas a 99.97% of hMSCa cells presented it, only a 51.62% of hDF expressed it above the detectable threshold (Fig. 6B). Consequently, when quantifying the mean fluorescence intensity (MFI), the expression of the integrin was significantly higher in hMSCa (Fig. 6B). Regarding β_1, (Fig. 6C) despite for both, hMSCa and hDF, all the cells presented it, the determination of the MFI showed an importantly higher expression in hDF (Fig. 6D). Altogether, these findings highlight the differences between the two cell types, showing that hDF cells present a lower $\alpha_v\beta_3$ and a higher β_1 expression than hMSCa.

These data support the three main conclusions provided by our method. First, considering that α_v-class integrins initiate cell attachment by binding FN quicker than β_1-presenting integrins such as $\alpha_5\beta_1$ [35], the faster kinetics indicated a higher density of α_v-class integrins in C_2C_{12} and hMSCa than in BHK and hDF. Second, the integrin expression profile was different in hMSCa and hDF, being major the role of β_1-presenting integrins in hDF, and important but not unique in hMSCa. Third, $\alpha_v\beta_3$ and β_1 contributed similarly to hMSCa adhesion strengthening, but for hDF, the role of β_1 integrins was still greater.

Although integrins can be explored through flow cytometry, this technique is more complex and only provides information regarding the integrin expression, but does not specify their actual contribution to the whole adhesion process. Moreover, it is important to note that for flow cytometry, cells are detached and studied in suspension. Hence, the biological processes dependent on cell adhesion, involving important phosphorylation pathways, are limited. Contrarily, our platform offered the possibility to study attached cells in an environment that mimics their natural niche and further, it predicted both, integrin expression profile and their role in functional adhesion formation via integrin crosstalk and clustering. This resulted in a valuable biosensor for the rapid determination of the adhesive properties of a specific cell type, generating crucial information directly applicable to the field of biomimetic material science.

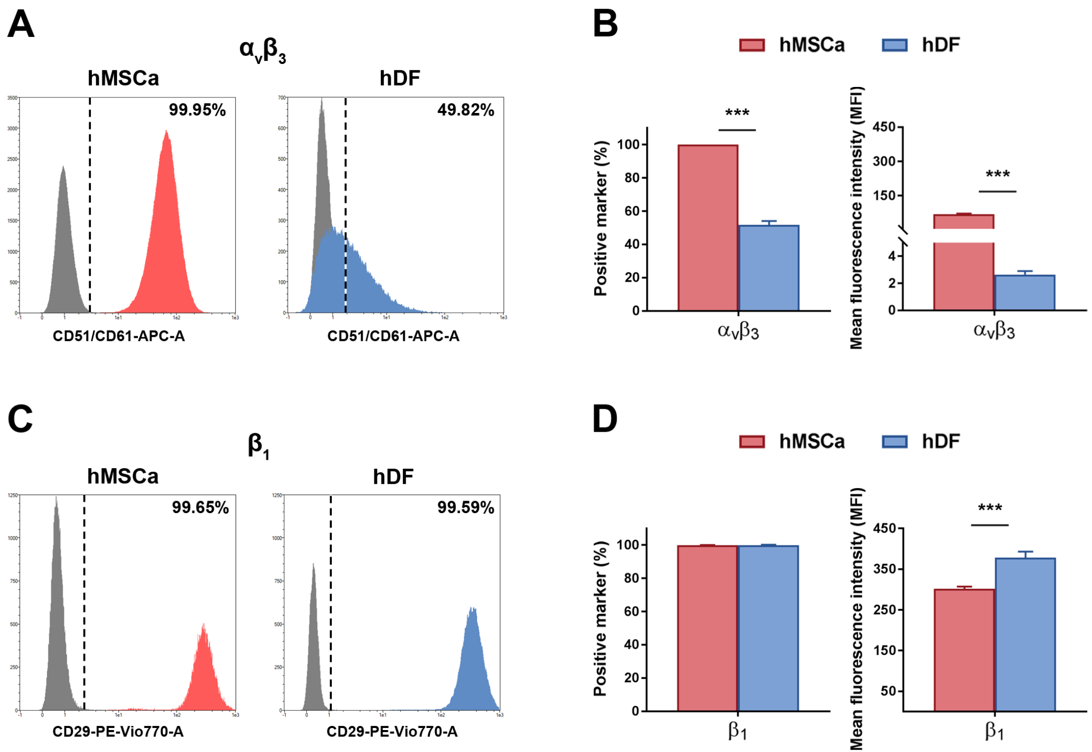


Fig. 6. Sensor validation by flow cytometry. (A) $\alpha_v\beta_3$ (CD51/CD61) integrin determination by flow cytometry. **(B)** Quantification of the percentage of cells expressing the $\alpha_v\beta_3$ integrin and its mean fluorescence intensity (MFI). Error bars mean ± SD (n = 4 samples per experimental condition). Statistical significance: paired, two-tailed t-test: ***p < 0.001. **(C)** β_1 (CD29) determination and **(D)** quantification. Error bars mean ± SD (n = 4 samples per experimental condition). Statistical significance: paired, two-tailed t-test: ***p < 0.001. In grey, the isotype control. In pink, hMSCa. In blue, hDF. hMSCa: adipose-derived human mesenchymal stromal cells. hDF: human dermal fibroblasts.

Multiple integrins bind the tri-peptide RGD, which has been recognized as the essential cell adhesion site in FN and other natural components of the ECM [14,40]. However, the affinity for this motif varies among the different integrins. For instance, while both $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins bind RGD, the latter also requires the PHSRN synergy site, present in natural FN in close proximity to RGD, to establish cell adhesion [35,41,42]. Likewise, $\alpha_4\beta_1$ integrins also bind a synergistic site, in this case the PRARI sequence [42]. Therefore, it is crucial to explore the contribution of integrins in a particular cell type in order to correctly design efficient biofunctionalization strategies [43]. By means of our biosensor, we were able to determine the contribution of $\alpha_v\beta_3$ and β_1 integrins in hDF and hMSCa, gathering fundamental information to

optimize material biomimetization. In particular, our data indicate that although the inclusion of synergy motifs such as PHSRN or PRARI may be beneficial in hMSCa, the presence of the sole RGD may promote an important cell response. Contrarily, the accompaniment of RGD with the synergistic peptides in hDF would be essential considering the smaller contribution of $\alpha_v\beta_3$ and the potent effects of β_1.

Generally, the design of biofunctionalized matrices goes through an optimization stage in which the material is modified with different ECM-derived peptides at different densities. Once all the materials are functionalized, complex 3D matrices are fabricated with each of the biomimetization alternatives and cells are cultured within them to determine their behavior. A preliminary screening in our biosensor would rapidly predict the best strategies to follow, importantly limiting the number of groups to test. Here, FN dot arrays were employed; however, it is possible to pattern other peptides or proteins of interest or even mixtures of different moieties that *a priori* may seem interesting [44]. This would not only save time but would be significantly more cost-effective, since the amount of peptide to be used for the screening is significantly lower than the required to modify materials and manufacture matrices with them. Moreover, the platform allows the study of any receptor class by only selecting the most suitable anti-integrin antibodies and can be easily performed in multiple cell types in parallel, resulting in a powerful and high throughput analytical tool.

4. Conclusion

In this study, we developed an ECM protein dot microarray biosensor for the characterization of cell adhesion to a particular substrate with single cell resolution. By exploring cell adhesion kinetics, integrin profile and integrin contribution to adhesion formation and consolidation, we were able to detect differences in the adhesive properties of various cell types, including primary and established cell lines from different sources. The sensor enables to study in depth the affinity of cells for different substrates, it is not limited to FN. This results in a useful tool for the design of biomimetic materials, which are key in multiple fields including cell therapy, tissue engineering or targeted drug delivery. The potential of this biosensor lies on its high sensitivity, accuracy, simplicity and low cost and will be of interest for the whole community of researchers interested in cell-substrate interactions.

5. Acknowledgements

Authors thank for the projects SAF2017-82292-R and BIO2016-80417-P (MINECO/AEI/FEDER, UE), ICTS "NANBIOSIS" (Drug Formulation Unit, U10) and the support from the Basque Country Government (Grupos Consolidados, Ref. No. IT907-16). A. Gonzalez-Pujana thanks the Basque Government (Department of Education, Universities and Research) for the PhD grant (PRE_2018_2_0133).

6. References

[1] A.D. Theocharis, S.S. Skandalis, C. Gialeli, N.K. Karamanos, Extracellular matrix structure, Adv. Drug Deliv. Rev. 97 (2016) 4-27. https://doi.org/10.1016/j.addr.2015.11.001.

[2] Y. Takada, X. Ye, S. Simon, The integrins, Genome Biol. 8 (2007) 215-2007-8-5-215. https://doi.org/10.1186/gb-2007-8-5-215.

[3] H.T. Aiyelabegan, E. Sadroddiny, Fundamentals of protein and cell interactions in biomaterials, Biomed. Pharmacother. 88 (2017) 956-970. https://doi.org/10.1016/j.biopha.2017.01.136.

[4] L. Wang, J. Huang, C. Huang, Q. Li, L. Liu, S. Luo, J. Xiao, Adult Stem Cells and Hydrogels for Cartilage Regeneration, Curr. Stem Cell. Res. Ther. 13 (2018) 533-546. https://doi.org/10.2174/1574888X12666170511142917.

[5] A.A. Tomei, C. Villa, C. Ricordi, Development of an encapsulated stem cell-based therapy for diabetes, Expert Opin. Biol. Ther. 15 (2015) 1321-1336. https://doi.org/10.1517/14712598.2015.1055242.

[6] Q. Huang, Y. Zou, M.C. Arno, S. Chen, T. Wang, J. Gao, A.P. Dove, J. Du, Hydrogel scaffolds for differentiation of adipose-derived stem cells, Chem. Soc. Rev. 46 (2017) 6255-6275. https://doi.org/10.1039/c6cs00052e.

[7] T. Andersen, P. Auk-Emblem, M. Dornish, 3D Cell Culture in Alginate Hydrogels, Microarrays (Basel). 4 (2015) 133-161. https://doi.org/10.3390/microarrays4020133.

[8] K.H. Vining, A. Stafford, D.J. Mooney, Sequential modes of crosslinking tune viscoelasticity of cell-instructive hydrogels, Biomaterials. 188 (2019) 187-197. https://doi.org/10.1016/j.biomaterials.2018.10.013.

[9] A. Gonzalez-Pujana, A. Rementeria, F.J. Blanco, M. Igartua, J.L. Pedraz, E. Santos-Vizcaino, R.M. Hernandez, The role of osmolarity adjusting agents in the regulation of encapsulated cell behavior to provide a safer and more predictable delivery of therapeutics, Drug Deliv. 24 (2017) 1654-1666. https://doi.org/10.1080/10717544.2017.1391894.

[10] M. Heller, V.V. Kumar, A. Pabst, J. Brieger, B. Al-Nawas, P.W. Kammerer, Osseous response on linear and cyclic RGD-peptides immobilized on titanium surfaces in vitro and in vivo, J. Biomed. Mater. Res. A. 106 (2018) 419-427. https://doi.org/10.1002/jbm.a.36255.

[11] A.L. Hook, D.G. Anderson, R. Langer, P. Williams, M.C. Davies, M.R. Alexander, High throughput methods applied in biomaterial development and discovery, Biomaterials. 31 (2010) 187-198. https://doi.org/10.1016/j.biomaterials.2009.09.037.

[12] K. Sivaraman, C. Shanthi, Matrikines for therapeutic and biomedical applications, Life Sci. 214 (2018) 22-33. https://doi.org/10.1016/j.lfs.2018.10.056.

[13] E. Santos, A. Garate, J.L. Pedraz, G. Orive, R.M. Hernandez, The synergistic effects of the RGD density and the microenvironment on the behavior of encapsulated cells: in vitro and in vivo direct comparative study, J. Biomed. Mater. Res. A. 102 (2014) 3965-3972. https://doi.org/10.1002/jbm.a.35073.

[14] J. Takagi, Structural basis for ligand recognition by RGD (Arg-Gly-Asp)-dependent integrins, Biochem. Soc. Trans. 32 (2004) 403-406. https://doi.org/10.1042/BST0320403.

[15] A. Garate, E. Santos, J.L. Pedraz, R.M. Hernandez, G. Orive, Evaluation of different RGD ligand densities in the development of cell-based drug delivery systems, J. Drug Target. 23 (2015) 806-812. https://doi.org/10.3109/1061186X.2015.1020428.

[16] U. Hersel, C. Dahmen, H. Kessler, RGD modified polymers: biomaterials for stimulated cell adhesion and beyond, Biomaterials. 24 (2003) 4385-4415. https://doi.org/10.1016/S0142-9612(03)00343-0.

[17] A. Farrukh, F. Ortega, W. Fan, N. Marichal, J.I. Paez, B. Berninger, A.D. Campo, M.J. Salierno, Bifunctional Hydrogels Containing the Laminin Motif IKVAV Promote Neurogenesis, Stem Cell. Reports. 9 (2017) 1432-1440. https://doi.org/10.1016/j.stemcr.2017.09.002.

[18] ACEA Biosciences - xCELL Ligence System, Cell Index, E-Plate. https://www.aceabio.com/about/, Last accessed March 2019 (2014).

[19] A. Susloparova, D. Koppenhofer, J.K. Law, X.T. Vu, S. Ingebrandt, Electrical cell-substrate impedance sensing with field-effect transistors is able to unravel cellular adhesion and detachment processes on a single cell level, Lab. Chip. 15 (2015) 668-679. https://doi.org/10.1039/c4lc00593g.

[20] B. Peter, R. Ungai-Salanki, B. Szabo, A.G. Nagy, I. Szekacs, S. Bosze, R. Horvath, High-Resolution Adhesion Kinetics of EGCG-Exposed Tumor Cells on Biomimetic Interfaces: Comparative Monitoring of Cell Viability Using Label-Free Biosensor and Classic End-Point Assays, ACS Omega. 3 (2018) 3882-3891. https://doi.org/10.1021/acsomega.7b01902.

[21] I. Szekacs, N. Orgovan, B. Peter, B. Kovacs, R. Horvath, Receptor specific adhesion assay for the quantification of integrin–ligand interactions in intact cells using a microplate based, label-free optical biosensor, Sensors and Actuators B: Chemical. 256 (2018) 729-734. https://doi.org/10.1016/j.snb.2017.09.208.

[22] Z. Fohlerová, J. Turánek, P. Skládal, The cell adhesion and cytotoxicity effects of the derivate of vitamin E compared for two cell lines using a piezoelectric biosensor, Sensors and Actuators B: Chemical. 174 (2012) 153-157. https://doi.org/10.1016/j.snb.2012.08.047.

[23] B. Delalat, D.M. Rojas-Canales, S. Rasi Ghaemi, M. Waibel, F.J. Harding, D. Penko, C.J. Drogemuller, T. Loudovaris, P.T. Coates, N.H. Voelcker, A Combinatorial Protein Microarray for Probing Materials Interaction with Pancreatic Islet Cell Populations, Microarrays (Basel). 5 (2016). https://doi.org/10.3390/microarrays5030021.

[24] Y. Huang, H. Zhu, Protein Array-based Approaches for Biomarker Discovery in Cancer, Genomics Proteomics Bioinformatics. 15 (2017) 73-81. https://doi.org/10.1016/j.gpb.2017.03.001.

[25] P. Monzo, Y.K. Chong, C. Guetta-Terrier, A. Krishnasamy, S.R. Sathe, E.K. Yim, W.H. Ng, B.T. Ang, C. Tang, B. Ladoux, N.C. Gauthier, M.P. Sheetz, Mechanical confinement triggers glioma linear migration dependent on formin FHOD3, Mol. Biol. Cell. 27 (2016) 1246-1261. https://doi.org/10.1091/mbc.E15-08-0565.

[26] J.Y. Sim, J. Moeller, K.C. Hart, D. Ramallo, V. Vogel, A.R. Dunn, W.J. Nelson, B.L. Pruitt, Spatial distribution of cell-cell and cell-ECM adhesions regulates force balance while main-taining E-cadherin molecular tension in cell pairs, Mol. Biol. Cell. 26 (2015) 2456-2465. https://doi.org/10.1091/mbc.E14-12-1618.

[27] R.M. Duffy, Y. Sun, A.W. Feinberg, Understanding the Role of ECM Protein Composition and Geometric Micropatterning for Engineering Human Skeletal Muscle, Ann. Biomed. Eng. 44 (2016) 2076-2089. https://doi.org/10.1007/s10439-016-1592-8.

[28] J. Lee, A.A. Abdeen, X. Tang, T.A. Saif, K.A. Kilian, Geometric guidance of integrin mediated traction stress during stem cell differentiation, Biomaterials. 69 (2015) 174-183. https://doi.org/10.1016/j.biomaterials.2015.08.005.

[29] A. Lopez-Alonso, B. Jose, M. Somers, K. Egan, D.P. Foley, A.J. Ricco, S. Ramstrom, L. Basabe-Desmonts, D. Kenny, Individual platelet adhesion assay: measuring platelet function and antiplatelet therapies in whole blood via digital quantification of cell adhesion, Anal. Chem. 85 (2013) 6497-6504. https://doi.org/10.1021/ac401076s.

[30] L. Basabe-Desmonts, S. Ramstrom, G. Meade, S. O'Neill, A. Riaz, L.P. Lee, A.J. Ricco, D. Kenny, Single-step separation of platelets from whole blood coupled with digital quantification by interfacial platelet cytometry (iPC), Langmuir. 26 (2010) 14700-14706. https://doi.org/10.1021/la9039682.

[31] B. Jose, P. McCluskey, N. Gilmartin, M. Somers, D. Kenny, A.J. Ricco, N.J. Kent, L. Basabe-Desmonts, Self-Powered Microfluidic Device for Rapid Assay of Antiplatelet Drugs, Langmuir. 32 (2016) 2820-2828. https://doi.org/10.1021/acs.langmuir.5b03540.

[32] V. Thulabandu, D. Chen, R.P. Atit, Dermal fibroblast in cutaneous development and healing, Wiley Interdiscip. Rev. Dev. Biol. 7 (2018) 10.1002/wdev.307. Epub 2017 Dec 15.https://doi.org/10.1002/wdev.307.

[33] Y. Wang, X. Chen, W. Cao, Y. Shi, Plasticity of mesenchymal stem cells in immunomodulation: pathological and therapeutic implications, Nat. Immunol. 15 (2014) 1009-1016. https://doi.org/10.1038/ni.3002.

[34] A. Mohr, R. Zwacka, The future of mesenchymal stem cell-based therapeutic approaches for cancer - From cells to ghosts, Cancer Lett. 414 (2018) 239-249. https://doi.org/10.1016/j.canlet.2017.11.025.

[35] K. Hozumi, K. Nakamura, H. Hori, M. Miyagi, R. Nagao, K. Takasaki, F. Katagiri, Y. Kikkawa, M. Nomizu, Mixed Fibronectin-Derived Peptides Conjugated to a Chitosan Matrix Effectively Promotes Biological Activities through Integrins, alpha4beta1, alpha-5beta1, alphavbeta3, and Syndecan, Biores Open Access. 5 (2016) 356-366. https://doi.org/10.1089/biores.2016.0037.

[36] D. Cox, M. Brennan, N. Moran, Integrins as therapeutic targets: lessons and opportunities, Nat. Rev. Drug Discov. 9 (2010) 804-820. https://doi.org/10.1038/nrd3266.

[37] P. Tomakidi, S. Schulz, S. Proksch, W. Weber, T. Steinberg,Focal adhesion kinase (FAK) perspectives in mechanobiology: implications for cell behaviour, Cell Tissue Res. 357 (2014) 515-526. https://doi.org/10.1007/s00441-014-1945-2.

[38] A. Hamadi, M. Bouali, M. Dontenwill, H. Stoeckel, K. Takeda, P. Ronde, Regulation of focal adhesion dynamics and disassembly by phosphorylation of FAK at tyrosine 397, J. Cell. Sci. 118 (2005) 4415-4425. https://doi.org/10.1242/jcs.02565.

[39] M. Bharadwaj, N. Strohmeyer, G.P. Colo, J. Helenius, N. Beerenwinkel, H.B. Schiller, R. Fassler, D.J. Muller, alphaV-class integrins exert dual roles on alpha5beta1 integrins to strengthen adhesion to fibronectin, Nat. Commun. 8 (2017) 14348. https://doi.org/10.1038/ncomms14348.

[40] A. Gonzalez-Pujana, E. Santos, G. Orive, J.L. Pedraz, R.M. Hernandez, Cell microencapsulation technology: Current vision of its therapeutic potential through the administration routes, Journal of Drug Delivery Science and Technology. (2017). https://doi.org/10.1016/j.jddst.2017.03.028.

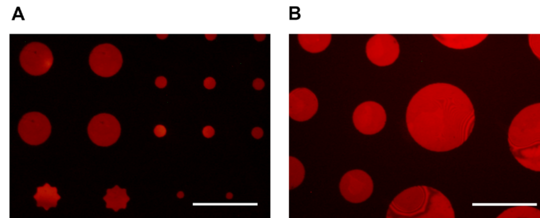
[41] A. Mardilovich, J.A. Craig, M.Q. McCammon, A. Garg, E. Kokkoli, Design of a novel fibronectin-mimetic peptide-amphiphile for functionalized biomaterials, Langmuir. 22 (2006) 3259-3264. https://doi.org/10.1021/la052756n.

[42] A. Sharma, J.A. Askari, M.J. Humphries, E.Y. Jones, D.I. Stuart, Crystal structure of a heparin- and integrin-binding segment of human fibronectin, EMBO J. 18 (1999) 1468-1479. https://doi.org/10.1093/emboj/18.6.1468.

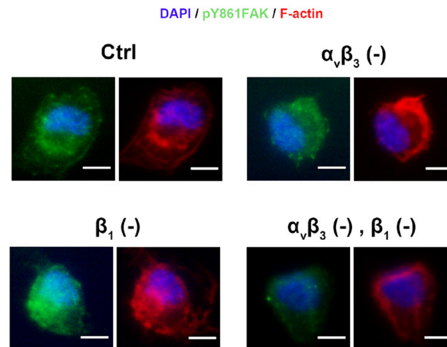
[43] R. Cruz-Acuna, A.J. Garcia, Synthetic hydrogels mimicking basement membrane matrices to promote cell-matrix interactions, Matrix Biol. 57-58 (2017) 324-333. https://doi.org/10.1016/j.matbio.2016.06.002.

[44] S.G. Ricoult, A.S. Nezhad, M. Knapp-Mohammady, T.E. Kennedy, D. Juncker, Humidified microcontact printing of proteins: universal patterning of proteins on both low and high energy surfaces, Langmuir. 30 (2014) 12002-12010. https://doi.org/10.1021/la502742r.

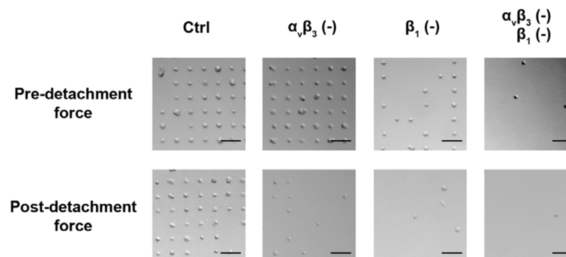
Supplementary figures and tables



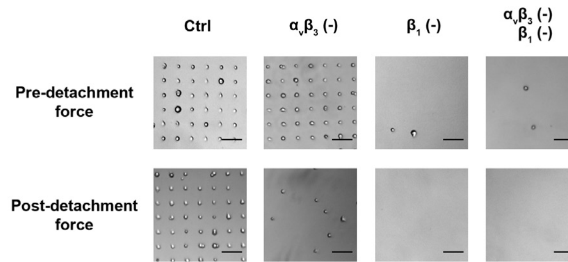
Supplementary Fig. 1. Pattern size and shape adaptation. The modulation of the pattern **(A)** shape and **(B)** size offers the possibility to adapt the method to specific cell morphologies and experimental procedures. Scale bars = 100 µm.



Supplementary Fig. 2. Cell-substrate interaction after integrin blocking. Focal adhesion determination by pFAK Y-861 fluorescence imaging in human adipose-derived mesenchymal stromal cells. DAPI in blue, pY397-FAK in green and F-actin in red. Scale bars = 100 µm.



Supplementary Fig. 3. Integrin blocking effects on cell adhesion strenghtening in hMSCa. Dot array ocupancy (DAO) prior and after the application of a detachment force. Scale bars = 100 µm. hMSCa: adipose-derived human mesenchymal stromal cells.



Supplementary Fig. 4. Integrin blocking effects on cell adhesion strenghtening in hDF. Dot array ocupancy (DAO) prior and after the application of a detachment force. Scale bars = 100 μm. hDF: human dermal fibroblasts.

Supplementary Table 1. Short peptide sequences employed in material biofunctionalization.

	Sequence	References
RGD	Arg-Gly-Asp	Santos *et al.*, 2014
PHSRN	Pro-His-Ser-Arg-Asn	Bharadwaj *et al.*, 2017
PRARI	Pro-Arg-Ala-Arg-Ile	Hozumi *et al.*, 2016
YIGSR	Tyr-Ile-Gly-Ser-Arg	Potter *et al.*, 2008
IKVAV	Ile-Lys-Val-Ala-Val	Farrukh *et al.*, 2017

Supplementary Table 2. CV of the dot array occupancy (DAO). For a certain sample, the DAO was analyzed in each replicate (n = 4) and the CV (%) was calculated among them. Samples from the different cell types employed in the study were evaluated.

CV (%)			
BHK	**C_2C_{12}**	**hMSCa**	**hDF**
4,77	4,39	5,16	6,77

Chapter 3

Multifunctional biomimetic hydrogel systems to boost the immunomodulatory potential of mesenchymal stromal cells

This chapter has been sumbitted to *Biomaterials* (Manuscript ID jbmt50283)

Multifunctional biomimetic hydrogel systems to boost the immunomodulatory potential of mesenchymal stromal cells

Ainhoa Gonzalez-Pujana[a,b*], Kyle H. Vining[c,d*], David K. Y. Zhang[c,d], Edorta Santos-Vizcaino[a,b], Manoli Igartua[a,b], Rosa Maria Hernandez[a,b #], David J. Mooney[c,d #]

[a] NanoBioCel Group, Laboratory of Pharmaceutics, School of Pharmacy, University of the Basque Country (UPV/EHU), Spain.

[b] Biomedical Research Networking Centre in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Spain.

[c] John A. Paulson School of Engineering and Applied Sciences, Harvard University, Cambridge, Massachusetts, USA

[d] The Wyss Institute for Biologically Inspired Engineering, Harvard University, Cambridge, Massachusetts, USA

*Authors contributed equally. # Corresponding authors: Rosa Maria Hernandez, David J. Mooney.

ABSTRACT

Mesenchymal stromal cells (MSCs) hold great therapeutic potential because of their unique immunomodulatory properties. However, their widespread application is limited because these properties can be transient and depend on multiple factors. Here, we developed a multifunctional hydrogel system to enhance the immunomodulatory properties of MSCs, based on the combination of sustained inflammatory licensing and three dimensional (3D) biomimetic culture in hydrogels with tunable mechanical properties. The immunomodulatory extracellular matrix hydrogels (iECM) consist of a bioinspired interpenetrating network of click functionalized-alginate and fibrillar collagen, in which interferon γ (IFN-γ) loaded heparin beads are incorporated. The 3D microenvironment significantly enhanced the expression of a wide panel of pivotal immunomodulatory genes in bone marrow derived primary human MSCs (hMSCs), in comparison to two-dimensional (2D) tissue culture. Moreover, the inclusion of IFN-γ loaded heparin beads provided a continuous inflammatory microenvironment which prolonged the expression of key regulatory genes dependent on licensing, including indoleamine 2,3-dioxygenase 1 (*IDO1*) and galectin-9 (*GAL9*). Further, iECM hydrogels were demonstrated to enable the correct hMSCs mediated immunomodulation. Co-culture of iECM encapsulated hMSCs and T cells resulted in suppressed proliferation of the latter, confirming the correct release and diffusion of the factors secreted by hMSCs, and hence, the functionality of the platform. Together, these results validate a novel multifunctional system for enhanced therapeutic potential of hMSCs.

1. Introduction

The unique immunomodulatory properties of mesenchymal stromal cells (MSCs) make them promising candidates for the treatment of inflammatory and immune disorders. MSCs are able to regulate both innate and adaptive immunity by suppressing basic functions such as maturation, proliferation or activation of different immune cells [1] including monocytes and macrophages [2,3], dendritic cells [4] or T, B and natural killer (NK) lymphocytes [5-7]. MSCs mediate their effects by a complex combination of different mechanisms including direct cell-cell contact and the release of bioactive soluble factors, such as interleukins, metabolic enzymes or growth factors. Interestingly, the paracrine effects of the released agents are considered to be mainly responsible for the immunomodulatory potential of MSCs [8,9].

However, important bottlenecks in the therapeutic application of MSCs include the transient nature of their immunomodulatory properties and their dependence on environmental factors. To address these issues and enhance the regulatory potential of MSCs, researchers have explored a number of approaches. Such is the case of MSCs licensing with inflammatory signals such as interferon γ (IFN-γ) or tumor necrosis factor α (TNF-α). This preconditioning strategy promotes the immunosuppressive phenotype of MSCs and enhances the secretion of bioactive factors, but these effects are transient [10-12]. The immunomodulatory potential of MSCs is also dependent on the culture conditions. Three-dimensional (3D) culture has been reported to improve the anti-inflammatory and immunomodulatory properties of MSCs [13]. Currently, the formation of cell spheroids is the most common 3D culture approach to boost the potential of MSCs [13-15] and this effect is believed to be mediated by enhanced cell-cell contact. Additionally, the mechanical properties of the MSC microenvironment have also been demonstrated to influence their biology, as MSC expression of immunomodulatory markers was differentially influenced by the stiffness and viscoelasticity of the matrix [16]. However, there is a necessity to further evaluate the effect of particular matrix configurations in the presence of other stimulatory conditions. Moreover, it is important to note that multiple studies are focused on a single immunomodulatory gene target or bioactive factor, failing to provide a deep insight on the overall regulatory potential of MSCs.

Here, we propose an integrated solution to enhance the immunomodulatory properties of MSCs, based on the combination of multiple strategies. In particular, we developed a bioinspired multifunctional system for the encapsulation of bone-marrow derived primary human MSCs (hMSCs), combining sustained inflammatory licensing and 3D biomimetic cell culture in hydrogels with tunable mechanical properties. The platform consisted of a biomimetic interpenetrating network of click-functionalized alginate and fibrillar collagen, intended to maintain robust cell viability and functionality by mimicking their natural niche, in which IFN-γ loaded heparin beads were incorporated. To provide broad insight on the effects of this combinatorial approach on the immunosuppressive properties of hMSCs, we assessed the expression of several immunomodulatory genes. In particular, we analyzed pivotal genes that are translated to bioactive factors acting at different levels of the regulatory response, and which are influenced by different stimuli, including indoleamine 2,3-dioxygenase 1 (*IDO1*), galectin 9 (*GAL9*), prostaglandin-endoperoxide synthase 2 / cyclooxygenase 2 (*PTGS2*), hepatocyte growth factor (*HGF*), interleukin-10 (*IL10*) and interleukin-1 receptor antagonist (*IL1RN*) [17-22]. The functionality of the system was validated with co-culture studies using T cells.

2. Materials and Methods

2.1 Cell isolation and culture

hMSCs were obtained from human bone marrow from donors less than 45 years old. After acquiring the fresh bone marrow (Lonza), hMSCs were isolated by means of a density gradient using Lymphoprep (StemCell Technologies) and the subsequent adherent culture to tissue culture plastic. hMSCs were grown in minimum essential medium α (α-MEM) (no nucleosides, + GlutaMax, Gibco) supplemented with 20% heat-inactivated fetal bovine serum (HIFBS) and 1% penicillin/streptomycin (P/S) (Thermo). Cells were maintained at 37°C in a 5% CO_2 / 95% air atmosphere and passaged every 3 - 7 days at 70 - 90% confluence. For experiments, cells were used at passage 2 - 4.

Primary human T cells were isolated from de-identified leukapheresis samples using Ficoll-paque separation and cultured in T cell media (RPMI 1640 supplemented with 10% HI-FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM beta-mercaptoethanol, 0.1 mM non-essential

amino acids, 10 mM N-2-hydroxyethylpiperazine- N-2-ethane sulfonic acid (HEPES), and 1% P/S) supplemented with 30 U ml^{-1} recombinant human interleukin-2 (IL-2), which was obtained from Biolegend. All antibodies were purchased from Biolegend.

2.2 Click alginate polymer synthesis

Low molecular weight (MW ~ 32 kDa), ultra-pure very low viscosity sodium alginates (UP-VLVG) (Pronova) were purchased from NovaMatrix and used either unmodified or click modified with norborene (Nb) or (4-(1,2,4,5-Tetrazin-3-yl)phenyl)methanamine - Trifluoroacetic acid (Tz). While Nb was commercially available (Nb Methanamine, TCI America), the synthesis of Tz was carried out as previously described [23]. Briefly, 50 mmol of 4-(aminomethyl)benzonitrile hydrochloride and 150 mmol formamidine acetate were stirred with 1 mol of anhydrous hydrazine at 80°C for 45 min and quenched with 0.5 mol of sodium nitrite. Subsequently, the product was isolated sequentially in 10% HCl and $NaHCO_3$ and extracted with dichloromethane (DCM). The final product was recovered by rotary evaporation and purified by high performance liquid chromatography (HPLC).

To obtain click-modified UP-VLVG, a covalent coupling of either Tz or Nb was performed [23]. Briefly, UP-VLVG alginate was dissolved in pH 6.5 buffer (0.1 M MES, 0.3 M NaCl) at 1% w/v. Next, N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimidehydrochloride (EDC) were added in 5X molar excess of the carboxylic acid groups of alginate. Finally, either Nb or Tz was incorporated at 1 mmol per gram of alginate. The coupling reaction was stirred at room temperature for 16 h and then quenched with hydroxylamine. The product was centrifuged and purified by means of tangential flow filtration using a 1 kDa molecular weight cut-off (MWCO) column (Spectrum Labs) against a decreasing salt gradient from 150 mM to 0 mM NaCl in de-ionized water. The purified Alg-Tz or Alg-Nb polymers were treated with activated charcoal, sterile filtered (0.22 µm), and freeze-dried for long-term storage. The resulting modified alginates present a 5% degree of substitution, which refers to 5% of available carboxylic acid groups modified with Nb or Tz per mol of low molecular weight alginate (MW = 32 kDa). All chemicals were obtained from Sigma-Aldrich.

2.3 Cell encapsulation in aECM or iECM hydrogels

Artificial extracellular matrix (aECM) hydrogels were fabricated with slight modifications of a previously described method [16]. Briefly, alginate and collagen solutions were prepared prior to gel manufacture. A stock solution of collagen (Rat tail telo-collagen, Type I 8–11 mg/mL, Corning) was mixed on ice with 10X Hank's balanced salt solution (HBSS) (without calcium and magnesium, with phenol red, Sigma-Aldrich), HEPES 20mM final concentration, (Gibco) and 1M sodium hydroxide (NaOH, ~ 1 % final concentration) to pH 5 - 6.5. UP-VLVG were used either unmodified (to obtain viscoelastic gels) or modified with Nb (Alg-Nb) or Tz (Alg-Tz) (to obtain elastic gels). Alginate solutions were prepared at a 5% concentration in a buffered salt solution (HBSS, 20 mM HEPES), adjusting pH to ~ 7 with 1 M NaOH. To obtain a calcium carbonate ($CaCO_3$) slurry (100 mg mL^{-1}) precipitated calcium carbonate nanoparticles (nano-PCC, Multifex-MM, Specialty Minerals) were suspended in sterile water for injection (Gibco) and ultrasonicated (70% amplitude, 15 s). On the other hand, hMSCs intended for encapsulation were overnight licensed (≈16 h) by supplementing the culture media with IFN-γ (20 ng mL^{-1}) and TNF-α (10 ng mL^{-1}). The day of encapsulation, cells were retrieved from culture, suspended at 40×10^6 cells mL-1 in HBSS/HEPES and maintained on ice.

The hydrogel fabrication was performed on ice with continuous mixing by a micro-stir bar to ensure a homogeneous distribution of all components. First, the calcium slurry was incorporated into the collagen solution, followed by the addition of the appropriate volume of stock cell solution for the desired final concentration (2×10^6 cells mL^{-1}). Next, alginate was included in the mixture. For viscoelastic hydrogels, only unmodified alginates employed, whereas for elastic hydrogels, click-modified alginates, Alg-Nb and Alg-Tz, were included as well. The ratio of Alg-Nb to Alg-Tz was adjusted depending on calcium condition. For these elastic gels, unmodified UP-VLVG and Alg-Nb were mixed with the collagen and calcium slurry, and Alg-Tz was reserved to add later. Once all the components were appropriately mixed, freshly dissolved glucono-delta-lactone (GDL) (EMD Millipore, 0.4 g mL^{-1} in HBSS/HEPES) was included. In the case of the elastic gels, the reserved amount of Alg-Tz was incorporated after GDL addition. For immunomodulatory extracellular matrix (iECM) hydrogels, agarose beads coated with heparin (BioRad) were added in the hydrogels as a final step. The beads were previously loaded with IFN-γ by incubating them in an 80 ng mL^{-1} solution of the cytokine for

1 h at 37°C. Final concentrations of each component of the hydrogels are detailed in Table 1. The resulting hydrogel mixture was rapidly pipetted to untreated 12 well plates and incubated 1 h at 37°C for initial gelation. Afterwards, 1 mL of buffered salt solution (HBSS, 20 mM HEPES) was added to each plate well and hydrogels were equilibrated for another hour at 37°C. The buffer pH was monitored to be replaced when dropping below pH 7. Finally, the buffered salt solution was replaced by fresh α-MEM supplemented with 10% HIFBS and 1% P/S and hydrogels were cultured at 37°C, 5% CO_2. Control, tissue culture plate (TCP) 2D seeded hMSCs were cultured under the same conditions.

2.4 Cell retrieval

For cell retrieval, hydrogels were incubated for 20 min at 37°C with 250 μL of a digestion solution containing 34 U mL^{-1} alginate lyase (Sigma-Aldrich) and 300 U mL^{-1} collagenase type I (Sigma-Adrich). After mechanical disruption of the hydrogels by gentle pipetting, extra 250 μL of fresh digestion mixture were added and gels were incubated at 37°C for additional 20 min. Next, 0.1 mL of wash buffer (DPBS w/o Ca/Mg, 2 mM EDTA, 0.5% BSA) was added and all the content was transferred from the culture plates to protein low bind Eppendorf tubes for centrifugation at 400 *g* for 5 min at 4°C. The supernatants were discarded and two additional washes were performed. The cell count and viability of the pellet was assessed by flow cytometry (MUSE), prior to adding RNA lysis buffer (Invitrogen), supplemented with 1% β-mercaptoethanol. After vortexing, samples were stored at - 80°C.

2.5 Relative gene expression measurement

RNA isolation and purification was performed by means of PureLink RNA Micro Kit (Invitrogen) following the manufacturer's instructions. Subsequently, RNA quantity and quality was assessed by NanoDrop spectrophotometer and cDNA was reverse-transcribed by iScript Advanced Reverse Transcription Supermix for real-time quantitative polymerase chain reaction (RT-qPCR) (Bio-Rad). Real time RT-qPCR was carried out using CFX96 (Bio-Rad). Samples were run in duplicate with 10 ng of cDNA, 2× AdvancedSSO SYBR Green Supermix (Bio-Rad) and Bio-Rad PrimePCR primers in each reaction. Relative gene expression was computed by the delta Ct method using a reference gene (*GAPDH*). The list of individual gene primers is shown in Supplementary Table 1.

2.6 Quantification of the heparin bead IFN-y load

To quantify the IFN-γ loaded in iECM hydrogels, the IFN-γ binding to heparin beads was determined. Heparin beads were incubated in an 80 ng ml^{-1} solution of IFN-γ for 1 h at 37°C. Afterwards, beads were decanted and the supernatant was collected to assess the amount of free IFN-γ remaining in the solution. To quantify the amount of unbound cytokine, a human IFN-γ enzyme-linked immunosorbent assay (ELISA) was performed (Biolegend, human IFN-γ ELISA MAXTM Set Deluxe) and results were compared to those of the equivalent amount of IFN-γ incubated without beads.

2.7 Primary T cell isolation and co-culture with hMSCs encapsulated in iECM

Primary human T cells were obtained from de-identified leukoreduction collars (Brigham and Women's Hospital Specimen Bank) and used within 24 h of initial collection (stored at room temperature). Peripheral blood mononuclear cells (PBMCs) were enriched from leukoreductions in a Ficoll gradient, then isolated using pan T cell MACS kits to obtain $CD3^+$ T cells for polyclonal expansion. Dynabeads (ThermoFisher Scientific) were used for T cell activation, according to the manufacturer-optimized protocol. T cells were initially seeded at $1x10^5$ cells in the starting culture with pre-washed Dynabeads at 1:1 bead-to-cell ratio in T cell media supplemented with 30 U/mL recombinant IL-2. Fresh IL-2-supplemented media was added throughout culture to bring the cell suspension to a density of 0.5 - $1x10^6$ cells/mL.

T cells were cultured for 7 days prior to staining with CFSE (1:10,000, ThermoFisher Scientific). Following CFSE-labeling, T cells were added at $2.5x10^5$ cells mL^{-1} to co-culture with $2x10^5$ hMSCs encapsulated in iECM hydrogels, in 12-well MatTek dishes (4 days after hMSCs encapsulation). T cells were retrieved 3 days after co-culture for flow cytometry staining and analysis. T cells were stained with dead cell stain (Thermo) prior to blocking with FcRx and staining with anti-CD3, anti-CD4, and anti-CD8. Flow analysis was performed on Fortessa LSRII with compensation.

2.8 Data analysis and statistics

All statistical computations were performed by means of SPSS 23 (IBM SPSS, Chicago, IL). The normal distribution of the data was checked by the Shapiro-Wilk test. For comparisons between two groups Student's t-test was performed, while one-way ANOVA was used for multiple comparisons. For the latter, the Levene test was used to determine the homogeneity of variances. If homogeneous, the Tukey post-hoc was applied and if non-homogeneous the Tamhane post-hoc was chosen. p values less than 0.05 were assumed to be significant in all analyses. The statistical studies of real time RT-qPCR data were performed employing the delta-Ct values.

3. Results

First, we used 2D tissue culture to determine the effects of inflammatory licensing on the expression of relevant immunomodulatory genes. Following the experimental procedure shown in Fig. 1A, hMSCs were overnight licensed with IFN-γ and TNF-α. Assessment of gene expression immediately after licensing (day 0), demonstrated that the pre-treatment significantly upregulated the expression of *IDO1*, *GAL9* and *PTGS2* in comparison to non-treated cells. *IL1RN* expression was also enhanced in licensed hMSCs, but to a lower extent. Conversely, *HGF* and *IL10* were not regulated by this cytokine stimulation (Fig. 1B). Therefore, we classified our gene panel into two groups: genes responsive to inflammatory licensing (*IDO1*, *GAL9*, *PTGS2*) and not responsive (*HGF*, *IL10*, *IL1RN*). However, after the cells were maintained in 2D culture for 3 days, the normalized expression of the upregulated genes was significantly reduced compared to the initially licensed levels (Fig. 1C).

We next tested the hypothesis that cell encapsulation in an appropriate 3D hydrogel would maintain the expression of immunomodulatory genes. hMSCs were encapsulated in a range of aECM hydrogels after overnight licensing (Fig. 2A). The matrix of aECM hydrogels consists of an interpenetrating network of alginate and fibrillar collagen type I, intended to mimic the architecture of native extracellular matrix (ECM) (Fig. 2B). Four types of aECM hydrogels were utilized: soft viscoelastic, stiff viscoelastic, soft elastic and stiff elastic (Table 1), which were previously developed and characterized [16]. Elasticity and stiffness were independently tuned by varying the mode and magnitude of alginate crosslinking, as indicated in Fig. 2C-D.

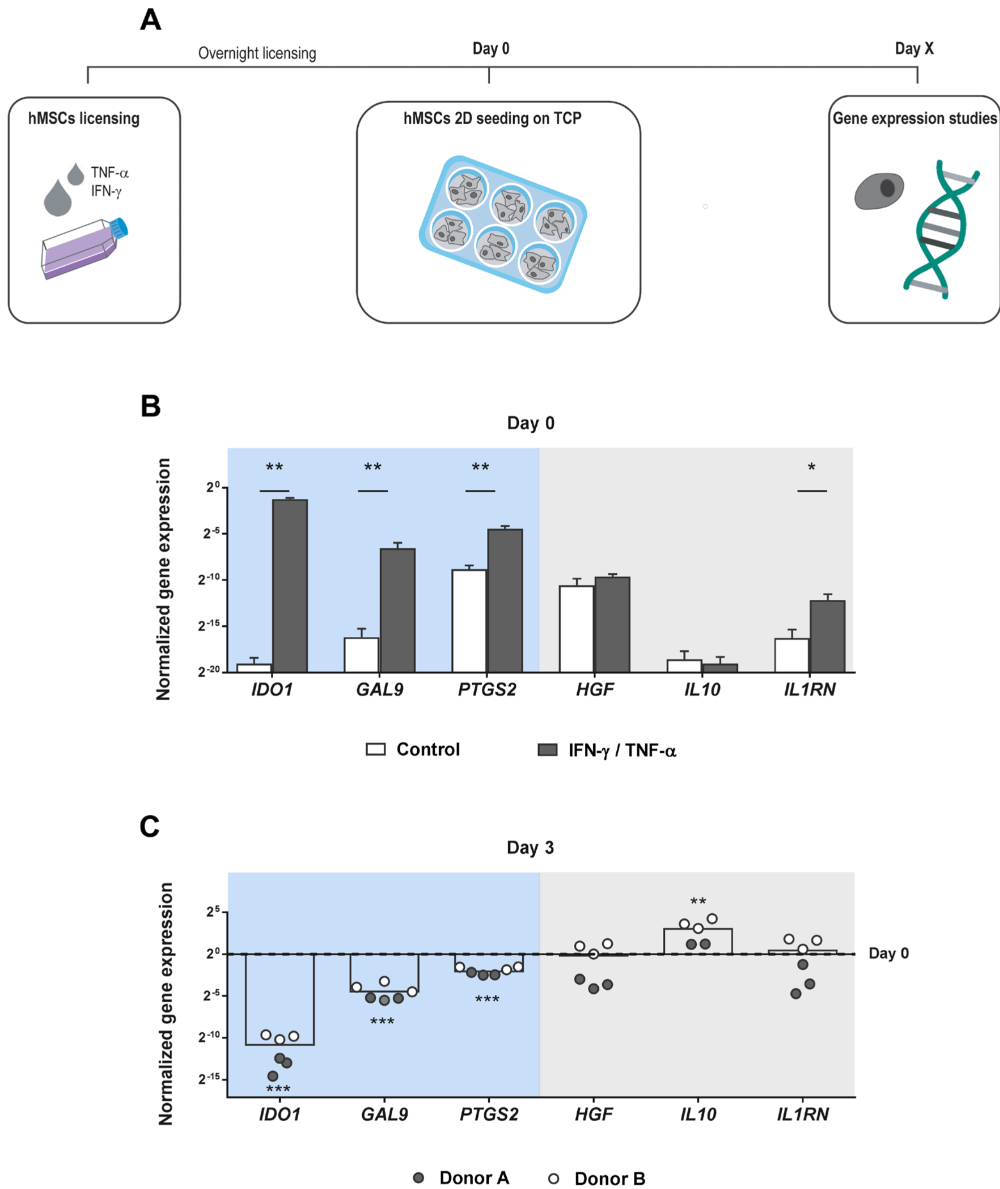


Fig. 1. Immunomodulatory gene expression by 2D tissue cultured hMSCs. (A) Schematic representation of the experimental procedure. After hMSC ≈ 16 h licensing with IFN-γ and TNF-α, cells were detached and 2D seeded on tissue culture plates (TCP). At different time-points, RNA was isolated from the cells for the subsequent real time RT-qPCR analyses. **(B)** Normalized gene expression of *IDO1*, *GAL9*, *PTGS2*, *HGF*, *IL10* and *IL1RN* by tissue culture hMSCs after overnight inflammatory licensing with IFN-γ and TNF-α compared to control untreated cells. Normalized to the housekeeping gene *GAPDH*. Statistical significance: *$p < 0.05$, **$p < 0.01$. **(C)** Normalized gene expression of *IDO1*, *GAL9*, *PTGS2*, *HGF*, *IL10* and *IL1RN* by hMSCs on tissue culture 3 days after licensing with IFN-γ and TNF-α. Normalized to *GAPDH* and day 0 expression. Statistical significance: ***$p < 0.001$, **$p < 0.01$ compared to day 0. hMSCs: human mesenchymal stromal cells. IFN-γ: interferon γ. TNF-α: tumor necrosis factor α.

Viscoelastic alginate hydrogels exhibit rapid stress-relaxation behavior from the reversible ionic crosslinks at blocks of guluronic acid-rich regions (i.e., G-blocks). The addition of permanent covalent crosslinking makes the network more elastic, and this is accomplished with the incorporation of norborene (Nb) and tetrazine (Tz) that undergo bio-orthogonal inverse electron demand Diels-Alder reactions to "click" together the existing G-block ionic crosslinks [16,23].

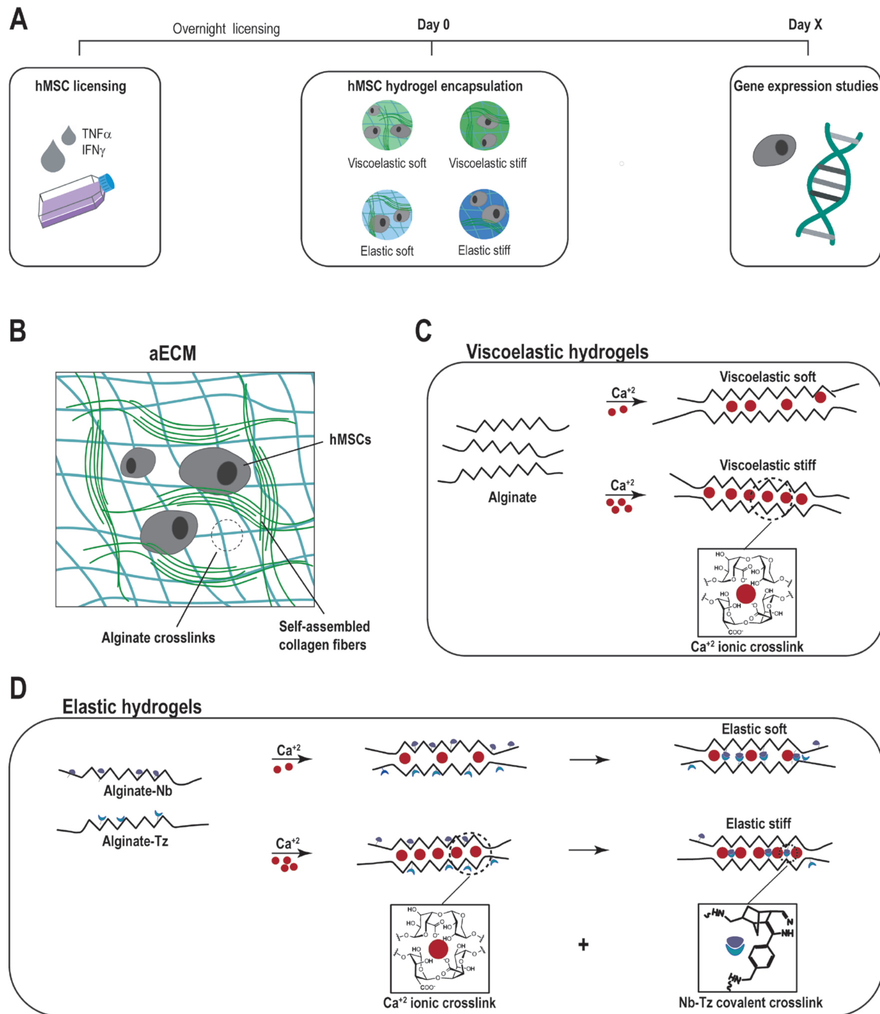


Fig. 2. aECM hydrogels. (A) Schematic representation of the experimental procedure. After hMSC ≈ 16 h licensing with IFN-γ and TNF-α, cells were detached and 3D encapsulated in aECM hydrogels. At different time-points, RNA was isolated from the cells for the subsequent real time RT-qPCR analyses. **(B)** aECM hydrogel system structure. **(C)** When alginates were crosslinked with calcium, viscoelastic hydrogels were obtained, **(D)** whereas the combination of ionic crosslinking and covalent crosslinking between the Nb and Tz groups led to elastic gels. aECM: artificial extracellular matrix hydrogels. Nb: norborene. Tz: tetrazine. hMSCs: human mesenchymal stromal cells.

After 3 days, the expression of the whole panel of immunomodulatory genes in 3D encapsulated cells was significantly higher than in tissue culture (2D), regardless of whether the individual genes were upregulated (Fig. 3 A-C) or not (Fig. 3 D-F) by inflammatory licensing. Confirming the effect of 3D biomimetic culture by itself, aECM encapsulation of non-licensed hMSCs also upregulated expression of immunomodulatory genes (Supplementary Fig. 1). Subsequent experiments were conducted with licensed encapsulated cells, since they expressed higher levels of the IFN-γ / TNFα induced genes than unlicensed hMSCs (Supplementary Fig. 2). No statistically significant differences in gene expression were observed between the aECM hydrogel types.

Next, the enhanced immunomodulatory gene expression in aECM encapsulated hMSCs was evaluated over a time-course of 7 days, and two different trends in the gene panel were noted. Genes that were strongly regulated by licensing, namely *IDO1*, *GAL9* and *PTGS2*, significantly reduced their expression after 7 days of aECM culture in all the hydrogels compared to day 3 (Fig. 4 A-C). Conversely, the genes that were not responsive to inflammatory licensing, *HGF* and *IL10*, increased in expression by day 3, and the increases were maintained at day 7 (Fig. 4 D-E). *IL1RN* (Fig. 4F), previously reported to be slightly influenced by licensing, presented the same tendency. Again, no statistically significant differences were observed among different aECM hydrogels. Therefore, stiff elastic hydrogels were selected for the following sets of experiments, as they were the most mechanically robust, and thus likely most relevant for future implantation studies.

Table 1. aECM hydrogel formulations.

Nb: norborene. Tz: tetrazine. VLVG: very low viscosity. GDL: glucono-delta-lactone.

	Collagen (mg/mL)	**VLVG alginate** (% w/v)	**Nb-alginate** (% w/v)	**Tz-alginate** (% w/v)	**Total alginate** (% w/v)	**$CaCO_3$** (% w/v)	**GDL** (mM)
Viscous soft	4	1.5	0	0	1.5	0.1	40
Viscous stiff	4	1.5	0	0	1.5	0.3	120
Elastic soft	4	0.5	0.5	0.5	1.5	0.1	40
Elastic stiff	4	0.5	0.5	0.5	1.5	0.3	120

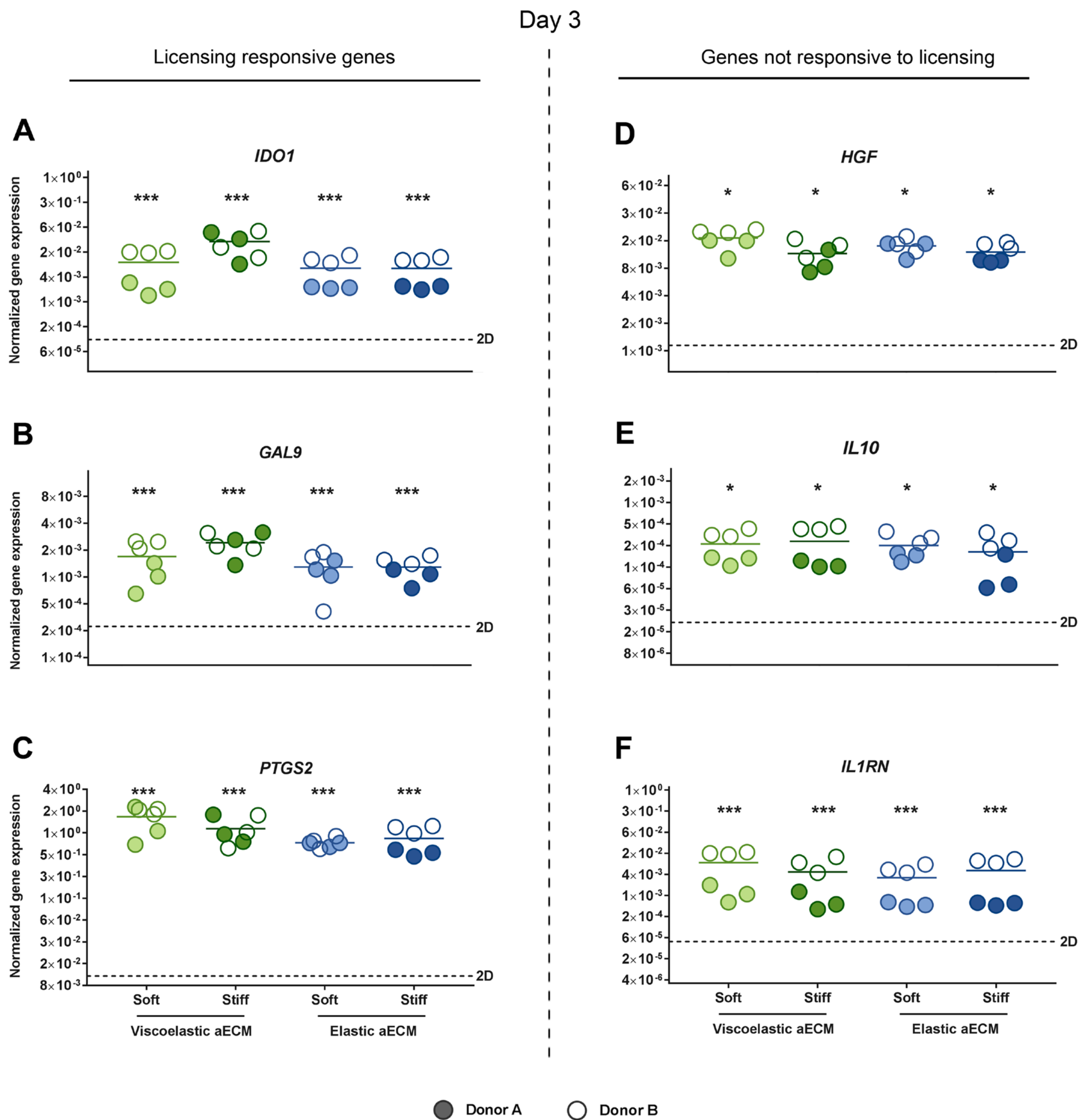


Fig. 3. Immunomodulatory gene expression by hMSCs 3D cultured in aECM hydrogels on day 3. Normalized gene expression of **(A)** *IDO1*, **(B)** *GAL9*, **(C)** *PTGS2*, **(D)** *HGF*, **(E)** *IL10* and **(F)** *IL1RN* by hMSCs encapsulated within aECM hydrogels 3 days after ≈ 16 h licensing with IFN-γ / TNF-α and subsequent encapsulation. Normalized to *GAPDH*. Statistical significance: *p < 0.05, **p < 0.01 and ***p < 0.001 compared to cells cultured 2D. aECM: artificial extracellular matrix hydrogels. hMSCs: human mesenchymal stromal cells. IFN-γ: interferon γ. TNF-α: tumor necrosis factor α.

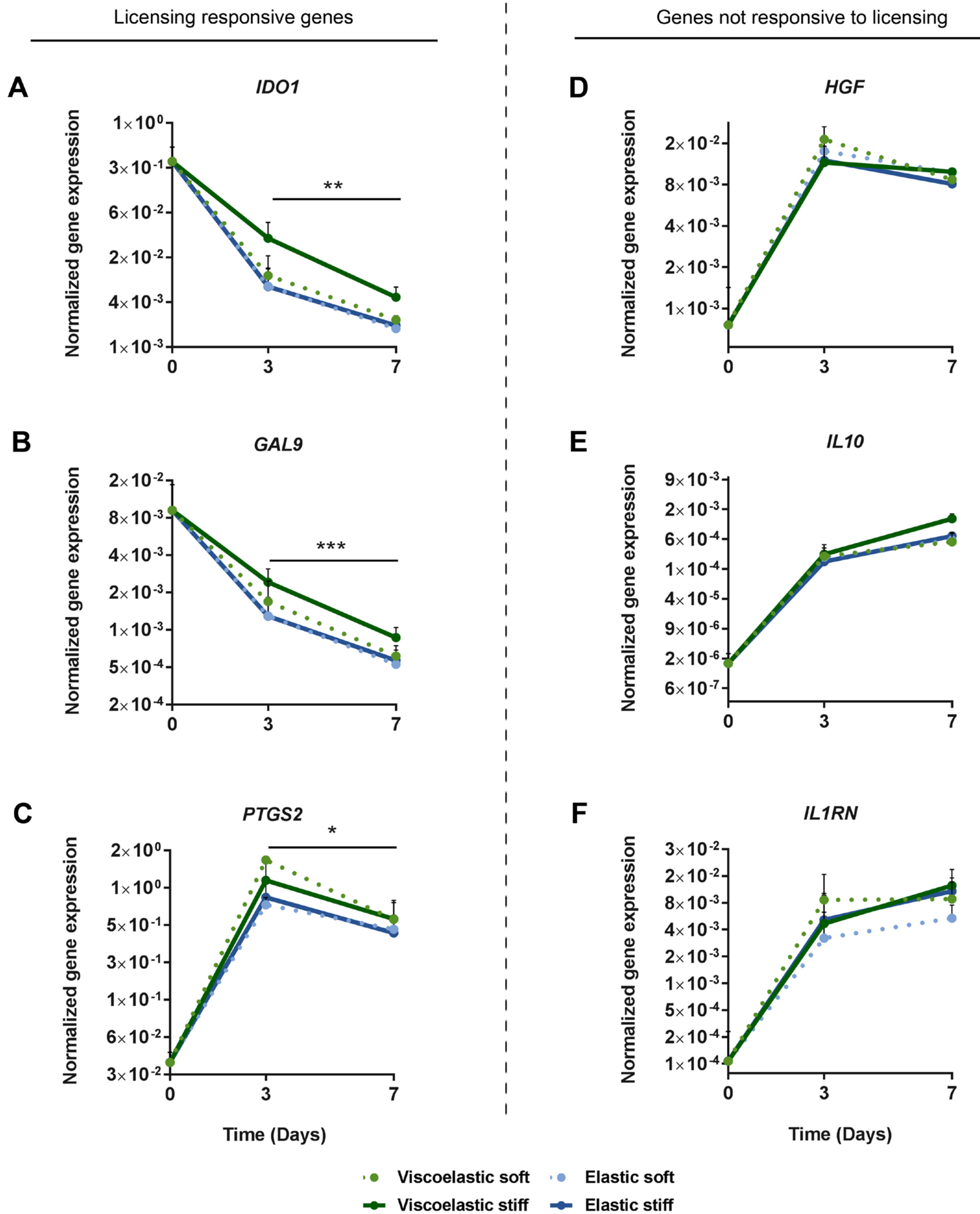


Fig. 4. Immunomodulatory gene expression by aECM encapsulated hMSCs over time. Normalized gene expression of **(A)** *IDO1*, **(B)** *GAL9*, **(C)** *PTGS2*, **(D)** *HGF*, **(E)** *IL10* and **(F)** *IL1RN* by aECM encapsulated hMSCs 0, 3 and 7 days after ≈ 16 h licensing with IFN-γ and TNF-α and subsequent encapsulation. Normalized to *GAPDH*. Statistical significance: *p < 0.05, **p < 0.01 and ***p < 0.00 for the comparison between days 3 and 7. hMSCs: human mesenchymal stromal cells. IFN-γ: interferon γ. TNF-α: tumor necrosis factor α. aECM: artificial extracellular matrix hydrogels.

Considering that *IDO1*, *PGE2* or *GAL9* encode for soluble factors that play key roles in the immunoregulatory effects of hMSCs, our next aim was to overcome the important decrease in expression observed by day 7 in aECM hydrogels. With that purpose, we developed an immunomodulatory extracellular matrix (iECM) hydrogel multifunctional system, combining biomimetic 3D culture and sustained inflammatory licensing. In particular, heparin-coated beads were incorporated in aECM elastic stiff hydrogels, to provide binding for IFN-γ [15]. We hypothesized that sustained exposure of IFN-γ within the hydrogel matrix would prolong

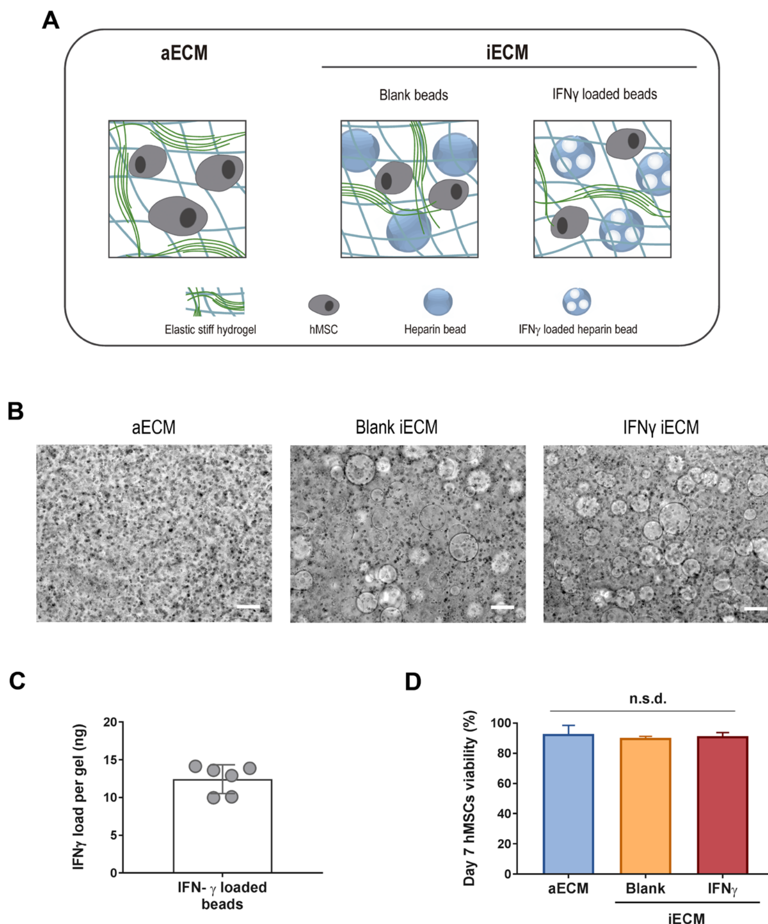


Fig. 5. Characterization of the new iECM materials. (A) Schematic representation of iECM hydrogel types. **(B)** Phase microscopy images of hMSCs encapsulated in aECM or iECM hydrogels. Scale bars = 200 μm. **(C)** Quantification of IFN-γ load in heparin beads. **(D)** hMSC viability in aECM and iECM hydrogels 7 days after ≈ 16 h licensing with IFN-γ and TNF-α and subsequent encapsulation. aECM: artificial extracellular matrix. iECM: immunomodulatory extracellular matrix. IFN-γ: interferon γ. hMSC: human mesenchymal stromal cells. n.s.d.: no significant differences.

hMSC licensing. Hydrogels were fabricated that contained heparin-coated beads loaded with IFN-γ (IFN-γ iECM), and compared to gels with beads that were not loaded with IFN-γ (blank iECM) (Fig. 5A). Bead incorporation did not significantly affect the cell encapsulation process, as shown by the homogeneity of cells and beads in phase microscopy images (Fig. 5B). IFN-γ loaded heparin beads were obtained by first determining the optimal loading conditions, which yielded 12.66 ± 2.33 ng of IFN-γ per 0.25 mL gel (Fig. 5C). The addition of blank heparin beads and IFN-γ beads had no significant effect on hMSC viability in iECM hydrogels after 7 days compared to aECM without heparin beads (Fig. 5D).

To determine whether heparin beads enhanced the expression of immunomodulatory genes, hMSCs were licensed overnight with IFN-γ / TNF-α, encapsulated in aECM or iECM hydrogels and maintained in culture for 7 days. The expression of *IDO1* and *GAL9*, the genes most regulated by inflammatory licensing, significantly increased in IFN-γ-iECM compared to blank iECM and aECM (Fig. 6 A-B). Blank iECM hydrogels enhanced the expression of the licensing responsive gene *PTGS2*, and the not responsive gene *HGF* (Fig. 6 C-D). No differences were observed in the expression of *IL10* and *IL1RN* (Fig. 6 E-F). Together, these data suggest the immunomodulatory gene expression of hMSCs was better maintained by day 7 in iECM hydrogels compared to hydrogels without heparin beads, especially for genes responsive to licensing.

Finally, the immunomodulatory function of hMSCs in iECM gels was analyzed. hMSCs were licensed and encapsulated in iECM hydrogels for 4 days, when they were co-cultured with CFSE-stained T cells. After 3 days of co-culture (hMSCs in culture in iECM hydrogels for a total of 7 days), the proliferation of T cells was examined via CFSE dilution. Histograms for the $CD3^+$ population showed the ability of iECM encapsulated hMSCs to suppress overall T cell proliferation (Fig. 7A-B) with $CD4^+$ T cell proliferation specifically being suppressed (Fig. 7C-D). These results suggest that, 7 days after licensing, hMSCs encapsulated in iECM hydrogels maintained their immunomodulatory function by inhibiting T cell proliferation.

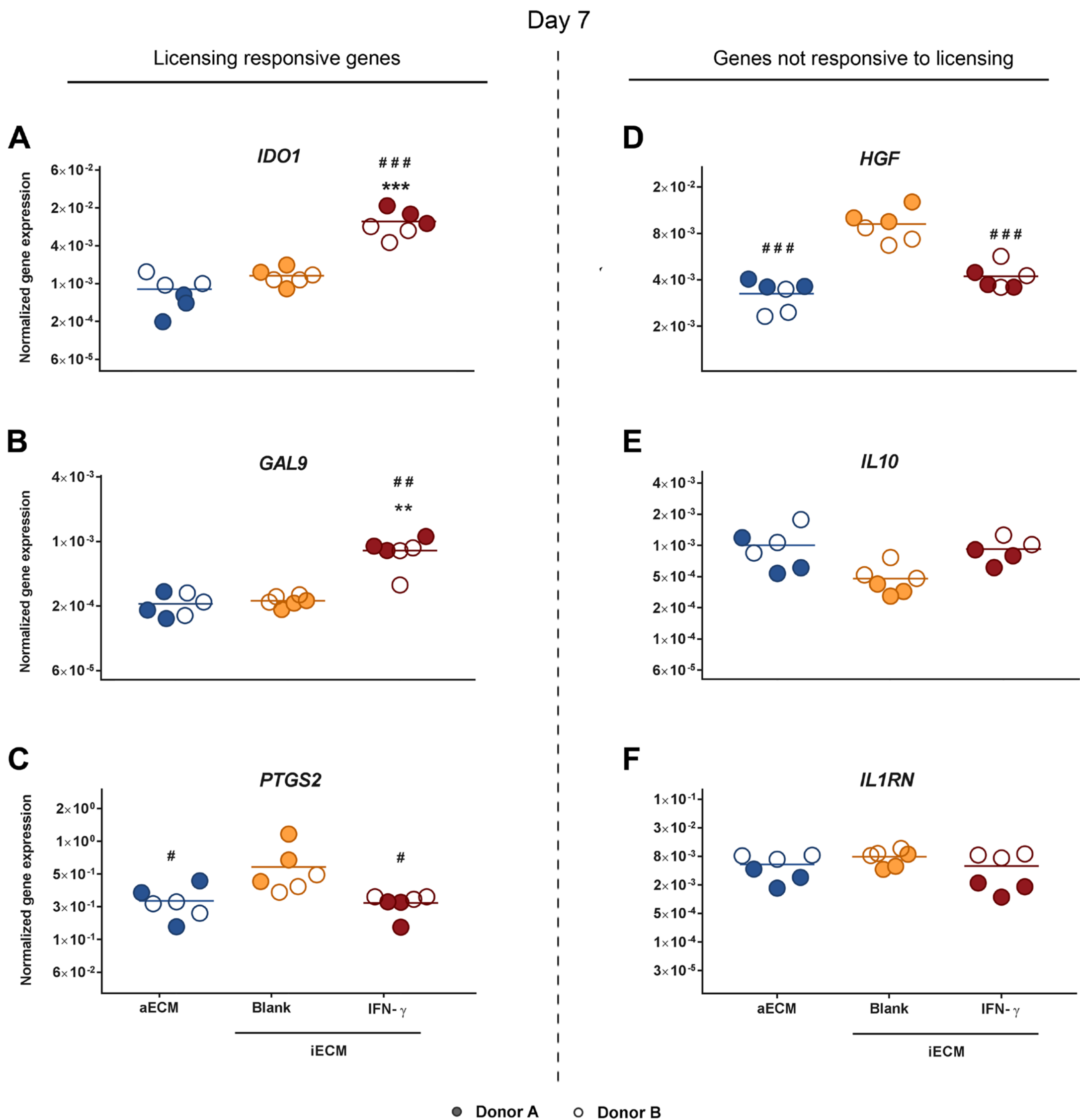


Fig. 6. Immunomodulatory gene expression by hMSCs encapsulated in iECM hydrogels on day 7. Normalized gene expression of **(A)** *IDO1*, **(B)** *GAL9*, **(C)** *PTGS2*, **(D)** *HGF*, **(E)** *IL10* and **(F)** *IL1RN* by hMSCs overnight licensed with IFN-γ / TNF-α and subsequently cultured in iECM hydrogels for 7 days. Normalized to *GAPDH*. Statistical significance: $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to aECM. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared to blank iECM. aECM: artificial extracellular matrix. iECM: immunomodulatory extracellular matrix. hMSC: human mesenchymal stromal cells. IFN-γ: interferon γ. TNF-α: tumor necrosis factor α.

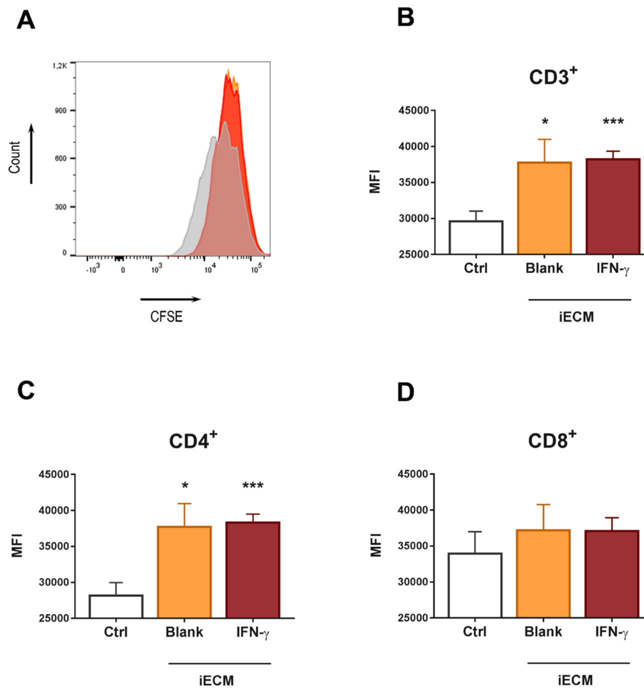


Fig. 7. Immunomodulatory effect of hMSCs encapsulated in iECM hydrogels. 4 days after hMSC encapsulation, iECM hydrogels were co-cultured with T cells stained with CFSE at 1:1.25 hMSC:T cell ratio. The co-culture was maintained for 3 days (hMSCs in culture in iECM hydrogels for a total of 7 days). The proliferation of T cells was examined by flow cytometry for dilution of CFSE. **(A)** Representative histograms for the $CD3^+$ population. **(B)** MFI determination on $CD3^+$ cells. Analysis of **(C)** $CD4^+$ and **(D)** $CD8^+$ populations. iECM: immunomodulatory extracellular matrix. hMSCs: human mesenchymal stromal cells. IFN-γ: interferon γ. Ctrl: control (no hMSC). MFI: mean fluorescence intensity.

4. Discussion

This study demonstrates a novel multifunctional system to enhance and prolong the immunomodulatory properties of hMSCs. In a single platform, we combined the effects of 3D biomimetic cell culture and sustained inflammatory licensing. To the best of our knowledge, this is the first study that explored the effects of such combinatorial approach on a broad panel of immunomodulatory genes that encode for pivotal regulatory factors acting at different levels of the immunosuppressive response.

The expression of *IDO1*, *PTGS2* and *GAL9* was found to be upregulated upon inflammatory licensing with IFN-γ and TNF-α. This increase in gene expression is consistent with previous studies that showed the combination of these two cytokines polarized hMSCs to an

immunosuppressive phenotype [12], inducing hMSCs to secrete regulatory enzymes and soluble factors such as IDO or cyclooxygenase 2 (COX2), which is encoded by the *PTGS2* gene [24]. However, here, the effect of the inflammatory pre-treatment was demonstrated to be transient, since after 3 days of 2D culture the expression of such genes significantly decreased. Considering these results, we classified our gene panel into two groups: genes responsive to inflammatory licensing (*IDO1*, *GAL9*, *PTGS2*) and not responsive (*HGF*, *IL-10*, *IL1RN*).

Encapsulation in a 3D hydrogel matrix had a significant impact on hMSC gene expression. In particular, aECM hydrogels were fabricated of interpenetrating networks of alginate and fibrillar collagen type I, being the latter intended to favor cell viability [25]. 3D encapsulation significantly promoted hMSC immunomodulatory potential over 3 days, as compared to 2D culture. Upregulation in expression was observed in the whole panel of immunomodulatory genes, regardless of whether the gene was influenced by exposure to IFN-γ / TNF-α or not. This indicates an important effect of the 3D biomimetic culture by itself, independent of licensing, as confirmed in Supplementary Fig. 1.

However, the expression of the licensing responsive genes *IDO1*, *PTGS2* and *GAL9* significantly decreased by day 7. Conversely, the expression of genes not regulated by IFN-γ / TNFα, namely *HGF*, *IL10* and *IL1RN*, was maintained over 7 days in aECM encapsulated hMSCs, indicating that the effect of the matrix persisted. Therefore, for licensing responsive genes, these data suggest an additive effect of inflammatory licensing and 3D biomimetic culture up to day 3, which was lost by day 7 due to the transient effect of the pre-treatment. Despite previous studies highlighting the impact of the mechanical properties of the matrix on MSC biology [26-28], no significant differences in hMSC immunomodulatory gene expression were detected among the four hydrogel types tested here. This may be due to the range of mechanical properties tested in this study, or an overwhelming impact of the inflammatory cytokines. Further work is required to determine the mechanism by which 3D encapsulation in aECM enhances immunomodulatory potential.

To prolong the expression of licensing-responsive immunomodulatory genes, heparin coated beads were incorporated in elastic stiff aECM, leading to multifunctional iECM hydrogels. The high affinity of heparin for IFN-γ (KD = 1 - 5 nM) [29] enabled loading the beads with the

cytokine (IFN-γ iECM), providing a continuous inflammatory microenvironment for hMSCs. Importantly, the binding of IFN-γ to heparin has been demonstrated to limit the extent of proteolytic degradation to one of its domains, which in turn, enhances the cytokine potency [29,30]. Moreover, since heparin can bind multiple growth factors and cytokines [29,31], we also tested iECM hydrogels that incorporated unloaded heparin beads (Blank iECM), to explore if the presence of such biomolecules in the hMSC microenvironment could boost their immunomodulatory properties.

The expression of licensing dependent genes *IDO1* and *GAL9* significantly increased in IFN-γ-iECM hydrogels, indicating a prolonged licensing effect, which acted synergistically with the effects of the matrix by day 7. Interestingly, *PTGS2* and *HGF* expression was promoted in blank iECM, suggesting that heparin may bind biomolecules that enhance their expression by hMSCs. In the case of *IL10* and *IL1RN*, not responsive to licensing, significant differences were not detected, likely because the effect of 3D culture was sufficient to maintain their expression. Overall, iECM hydrogels were demonstrated to prolong the expression of major immunomodulatory molecules by two main mechanisms: 3D biomimetic culture and sustained inflammatory licensing.

Finally, iECM hydrogels were demonstrated to enable the correct immunomodulatory function of hMSCs. Co-culture of iECM encapsulated hMSCs with T cells suppressed the proliferation of the latter, indicating that the system permitted the diffusion and release of hMSC-derived immunomodulatory factors. This is a key feature of the system, because biomaterial formulations have been reported to hamper the biomolecule diffusion by their relatively large volume [26]. Moreover, it was evidenced that heparin beads did not sequester all the factors secreted by the cells, nor did they physically impede their diffusion. Additionally, the presence of IFN-γ showed no negative effects, since we did not observe an exacerbated response on T cells when compared to blank iECM. This suggests that loaded IFN-γ likely was not released from the hydrogel, or at least, not in significant quantities. Together, our results indicate iECM hydrogels as a valuable multifunctional platform to enhance the immunomodulatory potential of hMSCs while enabling a correct diffusion of the secreted bioactive factors.

5. Conclusion

In the present study, we developed a bioinspired multifunctional system that combined 3D biomimetic cell culture and sustained inflammatory licensing as an integrated solution to enhance the immunomodulatory potential of hMSCs. 3D cell culture increased expression of key immunomodulatory genes that encode bioactive factors acting at different levels of the regulatory response, and the inclusion of heparin beads prolonged the expression of pivotal immunomodulatory genes dependent on licensing, including *IDO1* and *GAL9*. Functionality of the system was demonstrated when co-culture of iECM encapsulated hMSCs resulted in inhibition of T cell proliferation. Together, these findings have significant implications in MSCs therapies, since new strategies to boost their therapeutic potential are being extensively explored.

6. Acknowledgements

Authors thank for the projects SAF2017-82292-R (MINECO/AEI/FEDER, UE), ICTS "NANBIOSIS" (Drug Formulation Unit, U10), the National Institute of Dental & Craniofacial Research of the National Institutes of Health under Award Numbers R01DE013033 (DM), and the support from the Basque Country Government (Grupos Consolidados, No ref: IT907-16). A. Gonzalez-Pujana thanks the Basque Government (Department of Education, Universities and Research) for the PhD grant (PRE_2018_2_0133).

7. References

[1] F. Gao, S.M. Chiu, D.A. Motan, Z. Zhang, L. Chen, H.L. Ji, H.F. Tse, Q.L. Fu, Q. Lian, Mesenchymal stem cells and immunomodulation: current status and future prospects, Cell. Death Dis. 7 (2016) e2062. https://doi.org/10.1038/cddis.2015.327.

[2] M. Francois, R. Romieu-Mourez, M. Li, J. Galipeau, Human MSC suppression correlates with cytokine induction of indoleamine 2,3-dioxygenase and bystander M2 macrophage differentiation, Mol. Ther. 20 (2012) 187-195. https://doi.org/10.1038/mt.2011.189.

[3] J.H. Ylostalo, T.J. Bartosh, K. Coble, D.J. Prockop, Human mesenchymal stem/stromal cells cultured as spheroids are self-activated to produce prostaglandin E2 that directs stimulated macrophages into an anti-inflammatory phenotype, Stem Cells. 30 (2012) 2283-2296. https://doi.org/10.1002/stem.1191.

[4] M. Benkhoucha, M.L. Santiago-Raber, G. Schneiter, M. Chofflon, H. Funakoshi, T. Nakamura, P.H. Lalive, Hepatocyte growth factor inhibits CNS autoimmunity by inducing tolerogenic dendritic cells and CD25+Foxp3+ regulatory T cells, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 6424-6429. https://doi.org/10.1073/pnas.0912437107.

[5] G.M. Spaggiari, A. Capobianco, H. Abdelrazik, F. Becchetti, M.C. Mingari, L. Moretta, Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2, Blood. 111 (2008) 1327-1333. https://doi.org/10.1182/blood-2007-02-074997.

[6] P. Terness, T.M. Bauer, L. Rose, C. Dufter, A. Watzlik, H. Simon, G. Opelz, Inhibition of allogeneic T cell proliferation by indoleamine 2,3-dioxygenase-expressing dendritic cells: mediation of suppression by tryptophan metabolites, J. Exp. Med. 196 (2002) 447-457. https://doi.org/10.1084/jem.20020052.

[7] A. Corcione, F. Benvenuto, E. Ferretti, D. Giunti, V. Cappiello, F. Cazzanti, M. Risso, F. Gualandi, G.L. Mancardi, V. Pistoia, A. Uccelli, Human mesenchymal stem cells modulate B-cell functions, Blood. 107 (2006) 367-372. https://doi.org/10.1182/blood-2005-07-2657.

[8] J.R. Ferreira, G.Q. Teixeira, S.G. Santos, M.A. Barbosa, G. Almeida-Porada, R.M. Goncalves, Mesenchymal Stromal Cell Secretome: Influencing Therapeutic Potential by Cellular Pre-conditioning, Front. Immunol. 9 (2018) 2837. https://doi.org/10.3389/fimmu.2018.02837.

[9] F.J. Vizoso, N. Eiro, S. Cid, J. Schneider, R. Perez-Fernandez, Mesenchymal Stem Cell Secretome: Toward Cell-Free Therapeutic Strategies in Regenerative Medicine, Int. J. Mol. Sci. 18 (2017) 10.3390/ijms18091852. https://doi.org/10.3390/ijms18091852.

[10] M. Krampera, L. Cosmi, R. Angeli, A. Pasini, F. Liotta, A. Andreini, V. Santarlasci, B. Mazzinghi, G. Pizzolo, F. Vinante, P. Romagnani, E. Maggi, S. Romagnani, F. Annunziato, Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells, Stem Cells. 24 (2006) 386-398. https://doi.org/10.1634/stemcells.2005-0008.

[11] N. Murphy, O. Treacy, K. Lynch, M. Morcos, P. Lohan, L. Howard, G. Fahy, M.D. Griffin, A.E. Ryan, T. Ritter, TNF-alpha/IL-1beta-licensed mesenchymal stromal cells promote corneal allograft survival via myeloid cell-mediated induction of Foxp3(+) regulatory T cells in the lung, FASEB J. (2019) fj201900047R. https://doi.org/10.1096/fj.201900047R.

[12] P. Jin, Y. Zhao, H. Liu, J. Chen, J. Ren, J. Jin, D. Bedognetti, S. Liu, E. Wang, F. Marincola, D. Stroncek, Interferon-gamma and Tumor Necrosis Factor-alpha Polarize Bone Marrow Stromal Cells Uniformly to a Th1 Phenotype, Sci. Rep. 6 (2016) 26345. https://doi.org/10.1038/srep26345.

[13] T.J. Bartosh, J.H. Ylostalo, A. Mohammadipoor, N. Bazhanov, K. Coble, K. Claypool, R.H. Lee, H. Choi, D.J. Prockop, Aggregation of human mesenchymal stromal cells (MSCs) into 3D spheroids enhances their antiinflammatory properties, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 13724-13729. https://doi.org/10.1073/pnas.1008117107.

[14] Y. Petrenko, E. Sykova, S. Kubinova, The therapeutic potential of three-dimensional multipotent mesenchymal stromal cell spheroids, Stem Cell. Res. Ther. 8 (2017) 94-017-0558-6. https://doi.org/10.1186/s13287-017-0558-6.

[15] J.A. Zimmermann, M.H. Hettiaratchi, T.C. McDevitt, Enhanced Immunosuppression of T Cells by Sustained Presentation of Bioactive Interferon-gamma Within Three-Dimensional Mesenchymal Stem Cell Constructs, Stem Cells Transl. Med. 6 (2017) 223-237. https://doi.org/10.5966/sctm.2016-0044.

[16] K.H. Vining, A. Stafford, D.J. Mooney, Sequential modes of crosslinking tune viscoelasticity of cell-instructive hydrogels, Biomaterials. 188 (2019) 187-197. https://doi.org/10.1016/j.biomaterials.2018.10.013.

[17] A. Naji, M. Eitoku, B. Favier, F. Deschaseaux, N. Rouas-Freiss, N. Suganuma, Biological functions of mesenchymal stem cells and clinical implications, Cell Mol. Life Sci. (2019). https://doi.org/10.1007/s00018-019-03125-1.

[18] R. Duffin, R.A. O'Connor, S. Crittenden, T. Forster, C. Yu, X. Zheng, D. Smyth, C.T. Robb, F. Rossi, C. Skouras, S. Tang, J. Richards, A. Pellicoro, R.B. Weller, R.M. Breyer, D.J. Mole, J.P. Iredale, S.M. Anderton, S. Narumiya, R.M. Maizels, P. Ghazal, S.E. Howie, A.G. Rossi, C. Yao, Prostaglandin E(2) constrains systemic inflammation through an innate lymphoid cell-IL-22 axis, Science. 351 (2016) 1333-1338. https://doi.org/10.1126/science.aad9903.

[19] J. Fan, X. Tang, Q. Wang, Z. Zhang, S. Wu, W. Li, S. Liu, G. Yao, H. Chen, L. Sun, Mesenchymal stem cells alleviate experimental autoimmune cholangitis through immunosuppression and cytoprotective function mediated by galectin-9, Stem Cell. Res. Ther. 9 (2018) 237-018-0979-x. https://doi.org/10.1186/s13287-018-0979-x.

[20] J.Y. Lim, K.I. Im, E.S. Lee, N. Kim, Y.S. Nam, Y.W. Jeon, S.G. Cho, Enhanced immunoregulation of mesenchymal stem cells by IL-10-producing type 1 regulatory T cells in collagen-induced arthritis, Sci. Rep. 6 (2016) 26851. https://doi.org/10.1038/srep26851.

[21] J.C. Mbongue, D.A. Nicholas, T.W. Torrez, N.S. Kim, A.F. Firek, W.H. Langridge, The Role of Indoleamine 2, 3-Dioxygenase in Immune Suppression and Autoimmunity, Vaccines (Basel). 3 (2015) 703-729. https://doi.org/10.3390/vaccines3030703.

[22] L.A. Ortiz, M. Dutreil, C. Fattman, A.C. Pandey, G. Torres, K. Go, D.G. Phinney, Interleukin 1 receptor antagonist mediates the antiinflammatory and antifibrotic effect of mesenchymal stem cells during lung injury, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 11002-11007. https://doi.org/10.1073/pnas.0704421104.

[23] R.M. Desai, S.T. Koshy, S.A. Hilderbrand, D.J. Mooney, N.S. Joshi, Versatile click alginate hydrogels crosslinked via tetrazine-norbornene chemistry, Biomaterials. 50 (2015) 30-37. https://doi.org/10.1016/j.biomaterials.2015.01.048.

[24] F. Gao, S.M. Chiu, D.A. Motan, Z. Zhang, L. Chen, H.L. Ji, H.F. Tse, Q.L. Fu, Q. Lian, Mesenchymal stem cells and immunomodulation: current status and future prospects, Cell. Death Dis. 7 (2016) e2062. https://doi.org/10.1038/cddis.2015.327.

[25] C. Popov, T. Radic, F. Haasters, W.C. Prall, A. Aszodi, D. Gullberg, M. Schieker, D. Docheva, Integrins alpha2beta1 and alpha-11beta1 regulate the survival of mesenchymal stem cells on collagen I, Cell. Death Dis. 2 (2011) e186. https://doi.org/10.1038/cddis.2011.71.

[26] N. Huebsch, P.R. Arany, A.S. Mao, D. Shvartsman, O.A. Ali, S.A. Bencherif, J. Rivera-Feliciano, D.J. Mooney, Harnessing traction-mediated manipulation of the cell/matrix interface to control stem-cell fate, Nat. Mater. 9 (2010) 518-526. https://doi.org/10.1038/nmat2732.

[27] O. Chaudhuri, L. Gu, D. Klumpers, M. Darnell, S.A. Bencherif, J.C. Weaver, N. Huebsch, H.P. Lee, E. Lippens, G.N. Duda, D.J. Mooney, Hydrogels with tunable stress relaxation regulate stem cell fate and activity, Nat. Mater. 15 (2016) 326-334. https://doi.org/10.1038/nmat4489.

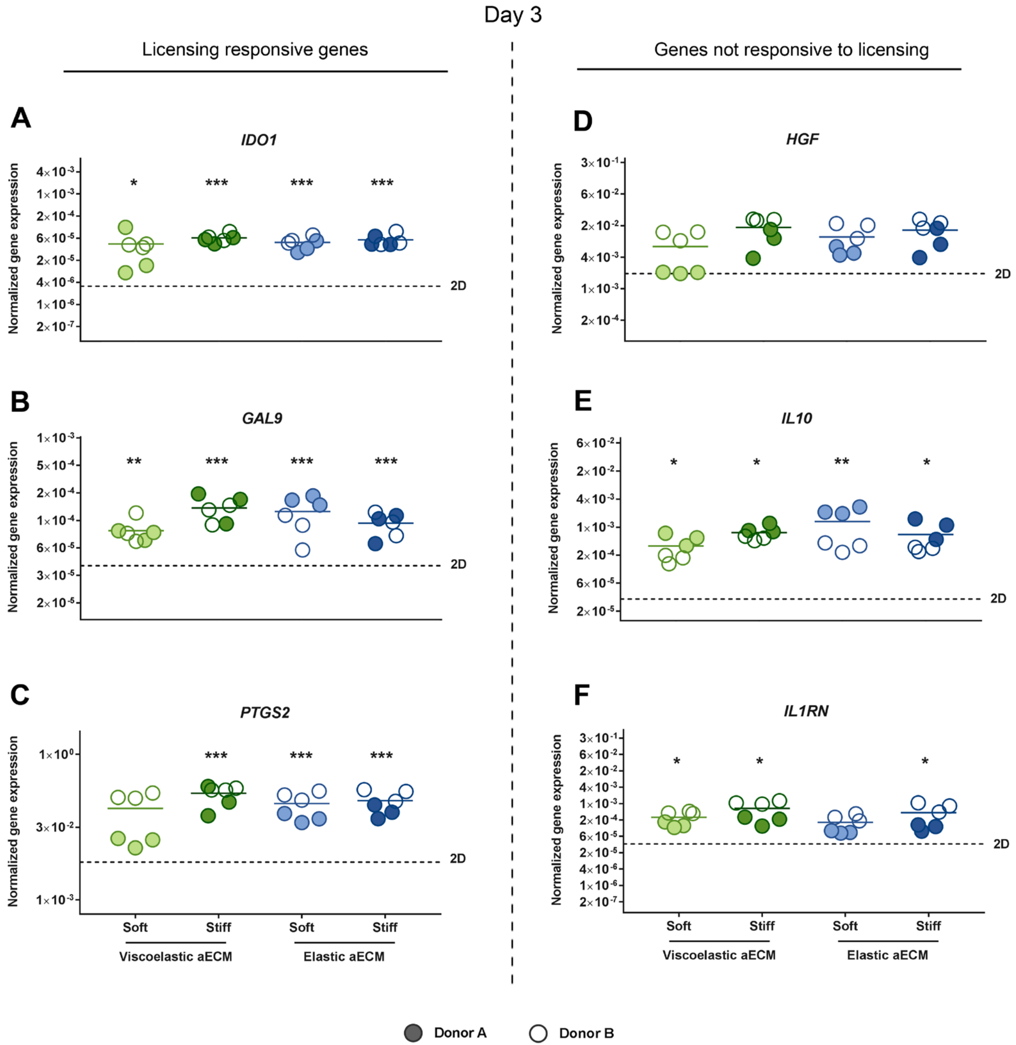
[28] A. Gonzalez-Pujana, A. Rementeria, F.J. Blanco, M. Igartua, J.L. Pedraz, E. Santos-Vizcaino, R.M. Hernandez, The role of osmolarity adjusting agents in the regulation of encapsulated cell behavior to provide a safer and more predictable delivery of therapeutics, Drug Deliv. 24 (2017) 1654-1666. https://doi.org/10.1080/10717544.2017.1391894.

[29] E. Saesen, S. Sarrazin, C. Laguri, R. Sadir, D. Maurin, A. Thomas, A. Imberty, H. Lortat-Jacob, Insights into the mechanism by which interferon-gamma basic amino acid clusters mediate protein binding to heparan sulfate, J. Am. Chem. Soc. 135 (2013) 9384-9390. https://doi.org/10.1021/ja4000867.

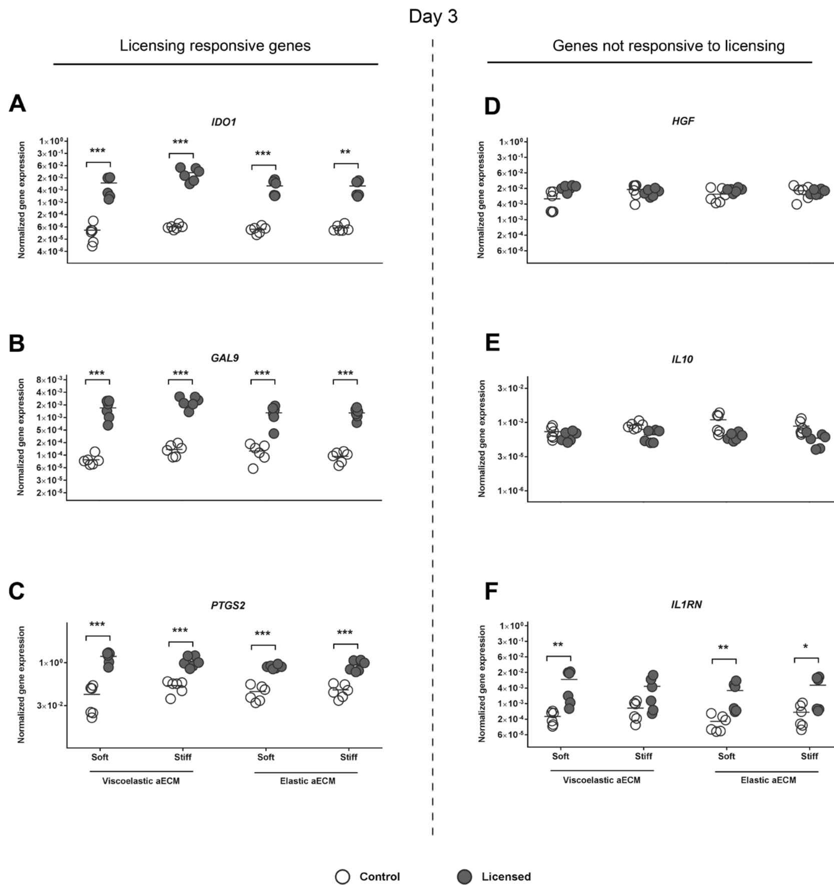
[30] H. Lortat-Jacob, F. Baltzer, J.A. Grimaud, Heparin decreases the blood clearance of interferon-gamma and increases its activity by limiting the processing of its carboxyl-terminal sequence, J. Biol. Chem. 271 (1996) 16139-16143. https://doi.org/10.1074/jbc.271.27.16139.

[31] S.N. Bolten, U. Rinas, T. Scheper, Heparin: role in protein purification and substitution with animal-component free material, Appl. Microbiol. Biotechnol. 102 (2018) 8647-8660. https://doi.org/10.1007/s00253-018-9263-3.

Supplementary figures and tables



Supplementary Fig. 1. Immunomodulatory gene expression by not licensed hMSCs 3D cultured in aECM hydrogels on day 3. Normalized gene expression of **(A)** *IDO1*, **(B)** *GAL9*, **(C)** *PTGS2*, **(D)** *HGF*, **(E)** *IL10* and **(F)** *IL1RN* by not licensed hMSCs 3 days after aECM hydrogel encapsulation. Normalized to *GAPDH*. Statistical significance: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to cells cultured 2D. hMSCs: human mesenchymal stromal cells. aECM: artificial extracellular matrix.



Supplementary Fig. 2. Comparison of immunomodulatory gene expression between licensed and not licensed hMSCs 3D cultured in aECM hydrogels. Normalized gene expression of **(A)** *IDO1*, **(B)** *GAL9*, **(C)** *PTGS2*, **(D)** *HGF*, **(E)** *IL10* and **(F)** *IL1RN* by licensed and not licensed hMSCs 3 days after aECM encapsulation. Normalized to *GAPDH*. Statistical significance: *p < 0.05, **p < 0.01 and ***p < 0.001. hMSCs: human mesenchymal stromal cells. aECM: artificial extracellular matrix.

Supplementary Table 1. Bio-Rad PrimePCR primers for RT-qPCR

Gene symbol	Gene Name	PrimePCR ID
IDO1	Indoleamine 2,3-dioxygenase 1	qHsaCED0044371
LGALS9 (GAL9)	Galectin-9	qHsaCID0014464
PTGS2	Prostaglandin-endoperoxide synthase 2 (COX2)	qHsaCED0042341
HGF	Hepatocyte growth factor	qHsaCID0011441
IL10	Interleukin-10	qHsaCED0044704
IL1RN	Interleukin-1 receptor antagonist	qHsaCED0044706
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	qHsaCED0038674

Chapter 4

Three-dimensional encapsulation and inflammatory licensing alter mesenchymal stromal cell reference gene expression in real-time RT-qPCR

Three-dimensional encapsulation and inflammatory licensing alter mesenchymal stromal cell reference gene expression in real time RT-qPCR

Ainhoa Gonzalez-Pujana[a,b*], Irene de Lázaro[c,d*], Edorta Santos-Vizcaino[a,b], Manoli Igartua[a,b], Rosa Maria Hernandez[a,b #], David J. Mooney[c,d #]

[a] NanoBioCel Group, Laboratory of Pharmaceutics, School of Pharmacy, University of the Basque Country (UPV/EHU), Spain.
[b] Biomedical Research Networking Centre in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Spain.
[c] John A. Paulson School of Engineering and Applied Sciences, Harvard University, Cambridge, Massachusetts, USA
[d] The Wyss Institute for Biologically Inspired Engineering, Harvard University, Cambridge, Massachusetts, USA
* Authors contributed equally. # Corresponding authors: Rosa Maria Hernandez, David J. Mooney.

ABSTRACT

Human mesenchymal stromal cells (hMSCs) hold great therapeutic potential in the treatment of inflammatory and immune diseases, due to their immunomodulatory capacity. The levels of expression of immunomodulatory genes such as indoleamine 2,3-dioxygenase 1 (*IDO1*) are often used to assess the latter, and real-time RT-qPCR is most predominantly the method of choice due to its high sensitivity and relative simplicity. However, the need to validate the expression of so-called housekeeping genes that are used to normalize the data under specific experimental conditions is often ignored and can lead to inaccurate results and flawed conclusions. Here, we have systematically investigated how 3D encapsulation, the mechanical properties of the 3D matrix and inflammatory licensing impact the expression of common reference genes in hMSCs. These conditions have been extensively explored to enhance the immunomodulatory capacity of hMSCs. Our results show that both, 3D encapsulation and inflammatory licensing significantly alter the expression of *ACTB* and *UBC* (which encode for β-actin and ubiquitin C, respectively), among other commonly used reference genes. We demonstrate that normalization with these reference genes introduces significant errors in the assessment of *IDO1* mRNA levels, leading to over or underestimation of hMSCs' therapeutic potential. In contrast, the range of mechanical properties of the matrix encapsulating the cells tested here did not significantly affect the expression of any of the reference genes studied. Our results highlight the absolute need to validate the expression of commonly used reference genes in order to obtain reliable gene expression data by real-time RT-qPCR.

1. Introduction

Mesenchymal stromal cells (MSCs) hold great clinical promise. Owing to their ability to differentiate into various mesodermal cell lineages (osteogenic, chondrogenic and adipogenic) [1,2], they have been extensively explored for tissue regeneration applications [3-5]. In addition, MSCs are also promising candidates for the treatment of inflammatory and immune disorders, since they regulate innate and adaptive immunity via direct cell-to-cell contact, or by the production of soluble factors, such as indoleamine 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2) and interleukin-6 (IL-6), which mediate a paracrine immunosuppressive effect [6,7].

The MSC secretome is highly dependent on the local microenvironment, since cells adopt an immunosuppressive phenotype in the presence of inflammatory conditions [8]. Therefore, MSC licensing with inflammatory cytokines has been explored to enhance their immunomodulatory effects and ultimately, therapeutic potential [9]. In particular, treatment with interferon γ (IFN-γ) or tumor necrosis factor α (TNF-α) [10,11] has been widely employed, and the combination of both cytokines has been proven to induce a synergistic effect [12]. Three-dimensional (3D) culture has also been suggested as a valuable strategy to increase the regulatory potential of MSCs [13-15], as the natural microenvironment of a tissue is more closely mimicked than in 2D culture [16]. Furthermore, the mechanical properties of hydrogels in which MSCs are encapsulated regulate intracellular pathways [17-19] and may also influence MSC mediated immunomodulation.

The therapeutic potential of MSCs is usually assessed by exploring immunomodulatory gene expression including *IDO1* or prostaglandin-endoperoxide synthase 2 (*PTGS2*). Real-time, reverse transcription, quantitative polymerase chain reaction (RT-qPCR) is widely employed for mRNA detection and quantitative gene expression analysis, because of its high sensitivity and specificity [20]. However, variations in the amount of starting material, RNA recovery and integrity, efficiency of cDNA synthesis or reverse transcription may lead to inaccurate results [21]. To minimize the impact of these possible errors, data is normalized to the so-called reference genes, under the assumption that they are constitutively expressed [22,23]. However, multiple studies highlight the variability in the expression of many traditionally used reference genes under several experimental conditions, which in the particular case of MSCs include treatment with growth factors such as vascular endothelial growth factor (VEGF) [24], culture

under differentiation conditions [25] or obtaining cells from different species [26,27] or tissues [24,28,29]. Such variability can lead to inaccurate results in real-time RT-qPCR analyses and flawed conclusions [30]. In 2009, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines were published, which advise to validate reference genes to each experimental set-up in order to produce reliable real-time RT-qPCR data [31]. Along with these guidelines, multiple software tools have been developed to analyze the stability of candidate reference genes in different experimental conditions [32-34].

In the present study, our aim was to determine if inflammatory licensing of MSCs together with 3D culture in collagen-alginate hydrogels interfered in the stability of 10 widely employed reference genes (*HMBS*, *UBC*, *GAPDH*, *OAZ1*, *RPL27*, *RPL30*, *RPS13*, *TBP*, *MAPK1* and *ACTB*). Moreover, the influence of the mechanical properties of the hydrogels was also explored, by tuning gel viscoelasticity and stiffness. This stability assessment was performed by means of BestKeeper (BK), NormFinder (NF) and geNorm (GN) algorithms. The expression of the target gene *IDO1* was normalized to the most dysregulated reference genes to detect possible misleading results due to incorrect normalizations, and the expression of these dysregulated housekeeping genes was further analyzed to evaluate the actual impact of cytokine stimulation, 3D encapsulation and mechanical properties on their stability.

2. Methods

2. 1 Primary cell isolation and culture

Primary human MSCs (hMSCs) were obtained from fresh bone marrow (Lonza) of donors under 45 years of age. Cells were isolated by a density gradient employing Lymphoprep (StemCell Technologies) followed by adherent culture to tissue culture plastic. For hMSC culture, minimum essential medium α (α-MEM, no nucleosides, + GlutaMax, Gibco) was supplemented with 20% heat-inactivated fetal bovine serum (HIFBS) and 1% penicillin/streptomycin (P/S) (Thermo). Cells were grown at 37°C in a 5% CO_2 / 95% air atmosphere and passaged at 70 - 90% confluence. Passage 2 - 4 hMSCs were employed for the experiments included in this work.

2.2 hMSC inflammatory licensing and experimental conditions

Control (unstimulated) hMSCs were cultured in α-MEM (20% HIFBS, 1% P/S). Cells were detached from the culture flasks and either encapsulated in collagen-alginate artificial extracellular matrix (aECM) hydrogels (3D), as described below, or seeded on tissue culture plates (TCP) at a density of $2.5x10^5$ cells per well (2D), and maintained in culture for 3 days in α-MEM (10% HIFBS, 1% P/S).

Stimulated hMSCs were licensed overnight by supplementing α-MEM (20% HIFBS, 1% P/S) with IFN-γ (20 ng mL^{-1}) and TNF-α (10 ng mL^{-1}). After ≈16 h, cells were retrieved from the culture flaks and either encapsulated in aECM hydrogels (3D) or seeded on TCP at a density of $2.5x10^5$ cells per well (2D), and maintained in culture for 3 days in α-MEM (10% HIFBS, 1% P/S).

2.3 hMSC encapsulation in aECM hydrogels

aECM fabrication was performed as previously described [35]. In brief, a collagen stock solution (Rat tail telo-collagen, Type I 8–11 mg mL-1, Corning) was incorporated in a buffer consisting of Hanks' balanced salt solution (HBSS) (without calcium and magnesium, with phenol red, Sigma-Aldrich), supplemented with N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) (Gibco) (20mM final concentration). 1M NaOH (~1 % final concentration) was incorporated to achieve a pH of 5 - 6.5. The same buffer (HBSS, 20 mM HEPES) was employed to prepare ultra-pure very low viscosity sodium alginates (UP-VLVG) solutions at a 5% concentration. pH was adjusted to 7 with 1M NaOH. In the case of viscoelastic hydrogels, unmodified alginates were used, whereas for elastic gels a mixture of unmodified and norbornene and tetrazine modified alginates (Alg-Nb, Alg-Tz) were employed. The latter were modified as described in [36]. Finally, a $CaCO_3$ slurry (100 mg mL^{-1}) was prepared by suspending precipitated calcium carbonate nanoparticles (nano-PCC, Multifex-MM, Specialty Minerals) in water for injection (Gibco). The resulting suspension was ultra-sonicated (70% amplitude, 15 s) immediately prior to gel manufacture. Finally, cells were retrieved from culture and suspended at $40x10^6$ cells mL^{-1} in the buffered salt solution (HBSS, 20 mM HEPES).

The process of hydrogel fabrication was carried out on ice and all the components were continuously mixed with micro-stir bars. As a first step, the calcium slurry was added to the collagen solution. Next, the appropriate volume of stock cell solution to obtain a final concentration of $2x10^6$ cells mL^{-1} was included. Subsequently, alginates were incorporated into the mixture. In the case of viscoelastic hydrogels, the unmodified alginate solution was added, whereas for elastic hydrogels, Alg-Nb was included too (Alg-Tz was reserved to be added as a final step). Next, freshly dissolved glucono-delta-lactone (GDL) (EMD Millipore. 0.4 g mL^{-1} in HBSS/HEPES) was incorporated to cause the rupture of the nanoparticles and release of calcium for gelation purposes. For the elastic gels, the reserved amount of Alg-Tz was incorporated as a final step. Final concentrations of each component in the hydrogels are detailed in Table 1. Hydrogel solutions were quickly transferred to non tissue culture treated 12 well plates and incubated for 1 h at 37°C for initial gelation. After gelation, hydrogels were covered with 1 mL of buffered salt solution (HBSS, 20 mM HEPES) for equilibration, and incubated for an additional hour at 37 °C during which the buffer pH was monitored to be replaced when dropping below 7. The buffer was then replaced by fresh culture media (α-MEM 10% HIFBS, 1% P/S) and gels were cultured in a 5% CO_2 atmosphere at 37°C for 3 days.

2.4. Compliance with MIQE guidelines

All gene expression analyses in this work adhered to the MIQE guidelines [31], which promote transparency and ensure result reliability. The MIQE checklist is detailed in Supplementary Table 1. Experimental procedures were carried out in the investigators' laboratory, with the exception of the RNA quality assessment, which was performed with Agilent TapeStation 4200 at the Bauer Core (Harvard University).

2.5 RNA extraction, RNA quality assessment and cDNA synthesis

After 3 days of culture, cells were retrieved for RNA extraction. For encapsulated hMSCs, α-MEM was replaced by 500 µL of a solution containing 34 U mL^{-1} alginate lyase (Sigma-Aldrich) and 300 U mL^{-1} collagenase type I (Sigma-Aldrich) and incubated for 40 minutes at 37°C, when the remaining hydrogel was triturated with a pipet until total fragmentation. TCP seeded hMSCs were treated with accutase (Thermo) for 15 min at 37°C. The total cell content was transferred to RNA-se free low bind eppendorf tubes and centrifuged at 400 *g* for 5 min

at 4°C. The supernatant was discarded and a set of 3 washes was performed with cold wash buffer (Dulbecco's phosphate-buffered saline (DPBS) without Ca^{+2}/Mg^{+2}, 2mM ethylenediaminetetraacetic acid (EDTA) , 0.5% bovine serum albumin), followed by centrifugation to obtain a cell pellet.

For RNA isolation and purification, PureLink RNA Micro Kit (Cat. 12183-016, Invitrogen) was employed following the manufacturer's indications. Cell pellets were lysed in 300 μl lysis buffer provided in the kit, previously supplemented with 1% β-mercaptoethanol (M6250, Sigma). DNAse treatment was carried out on-column by means of Purelink DNA-se set (12185010, Invitrogen). RNA was eluted in 15 μl nuclease-free water. Samples were stored at - 80ºC and used within a month. For preliminary RNA yield and quality assessment, NanoDrop spectrophotometer was employed. RNA concentrations and A260/280 and A260/230 ratios are shown in Supplementary Table 2. RNA integrity was further analyzed in Agilent 4200 Tape Station (Agilent Genomics). In brief, samples were diluted to a range of 30 - 500 ng μl^{-1}. 1 μl of the resulting dilution was incorporated in 5 μl RNA Screen Tape Sample Buffer (5067-5577, Agilent Genomics) and denatured for 3 min at 72ºC. After cooling for 2 min on ice, samples were run in RNA Screen Tape (5067-5576, Agilent Genomics). 28S/18S ratios and RNA integrity number (RIN) scores are reported in Supplementary Table 2. For cDNA synthesis, RNA was defrosted on ice, and immediately reverse-transcribed by means of iScript Advanced Reverse Transcription Supermix for real-time RT-qPCR (172-5038 Bio-Rad). Reverse transcription (20 μl volume) was performed according to the following steps: 46ºC for 20 min, 95ºC for 1 min, cool down to 4ºC. cDNA was stored at - 20ºC until real-time RT-qPCR analyses.

2.6 Primer design

Primers sequences for reference genes employed in this study are detailed in Supplementary Table 3. We utilized the Primer Basic Local Alignment Search Tool (Primer BLAST) to design primer sequences that met the following criteria: amplicon size 75-200 bp, GC content 50 - 65%, ≤3 G or C repetitions, ≤4 base repetitions, melting temperature (Tm) 55 - 65°C. When gene targets had several splicing variants (including predicted variants), primer pairs were designed to amplify all of them at the same product length. Each primer pair was verified with Blast Tool (NCBI) to confirm its specificity for the desired target. Primers were synthetized and purchased from Sigma. To detect *IDO1*, we used qHsaCED0044371 primer pair from BioRad.

2.7 Real-time RT-qPCR analyses

For each sample reaction, 10 ng of cDNA were mixed with 2 × AdvancedSSO SYBR Green Supermix (172-5274, Bio-Rad) and 0.5 μM of primers to a total volume of 20 μl. Reactions were loaded in duplicate on low profile, unskirted, clear 96-well plates (MLL9601, Bio-Rad) and run on a CFX96 Touch real-time RT-qPCR detection system (BioRad) according to the following protocol: 2 min at 50°C, 2 min at 95°C, (15 sec at 95°C and 1 min at 60°C) x 40 cycles. Assessment of each gene was carried out in the same run for the totality of the samples to avoid inter-run variability. Moreover, a melt curve analysis was performed to confirm the single-product amplification. No amplification was detected in non-template (NTC) and non-reverse transcription (NRT) controls. Cq values were determined with the Single Threshold mode in the CFX Manager software (BioRad). To determine primer efficiency (E), the slope of a linear regression of the Cq values obtained from a dilution series of the starting cDNA was employed and applied in the following equation: $E = 10^{(-\frac{1}{slope})}$.

2.8 Candidate reference gene stability assessment

The BestKeeper (BK) algorithm provides descriptive statistics of Cq values. By means of the BK Excel tool, a pair-wise correlation of raw Cq values for each sample was performed, obtaining standard deviation (SD) and coefficient of variance (CV) values. The most stable reference genes are those with the lowest SD and CV. The latter was calculated as the percentage of the Cq SD to the Cq mean. For data normalization, the algorithm provides the BK index: the geometric mean of the Cq values of all candidate reference genes that presented a SD < 1 [32].

NormFinder (NF) is an analysis of variance (ANOVA)-based model that provides each candidate reference gene with a stability value, considering both, intra and intergroup variation, and ranks them based on this parameter [33]. For this analysis, Cq values were transformed to relative quantities by means of the following formula: E (lowest Cq - Cq), which considers E and uses the lowest Cq as a calibrator. The resulting relative quantities were employed as input data in NF to calculate stability values for the 10 candidate reference genes under analysis. The lowest stability value represents the lowest variation, and thus, the highest stability. The software also provides the best combination of 2 reference genes for data normalization.

The geNorm (GN) algorithm is based on the principle that the expression ratio of two ideal reference genes is identical in all samples, regardless of the experimental condition [34]. Therefore, differences on ratios of two housekeeping genes means that one, or both, are not constantly expressed. For analysis of candidate reference genes with GN, the qbase+ software was employed. Each candidate reference gene was scored with the stability value M, which is based on the average pairwise variation of a particular gene with all other control genes. The lower the M value, the higher the reference gene stability. The software also provided the combination of the two housekeeping genes with the most stable expression for data normalization purposes. Moreover, GN also generates a V value, which refers to the suitability of employing a particular number of reference genes in a study.

2.9 Relative gene expression analyses

The Livak method [37] was performed to calculate relative gene expression. As calibrator, either the BK index or the geometric mean of Cq values of two reference genes calculated by NF or GN algorithms was employed. Error was propagated by means of the formula:

$$\boldsymbol{Error}(\boldsymbol{a}+\boldsymbol{b}) = \sqrt{\boldsymbol{Error}(\boldsymbol{a})^2 + \boldsymbol{Error}(\boldsymbol{b})^2}$$

2.10 Statistical analysis of relative gene expression data

For statistical analyses, ΔΔCq values were employed to determine differences among the different candidate reference genes and ΔCq data for the rest of studies. The normal distribution of the data was confirmed by the Shapiro-Wilk test. To detect statistically significant differences between two groups, a two-tailed t-test was performed. For multiple comparisons, one-way ANOVA was employed. In this case, the Levene test was used to determine the homogeneity of variances. If homogeneous, the Bonferroni post-hoc was applied and if non-homogeneous, the Tamhane test was selected. p values < 0.05 were considered significant. All statistical computations were performed with SPSS 23 (IBM SPSS, Chicago, IL).

3. Results

3.1 RNA quality and expression levels of candidate reference genes

Primary hMSCs were isolated from fresh bone marrow of human donors by means of a density gradient. In order to study the influence of hMSC licensing and encapsulation on the expression of candidate reference genes, we used non-stimulated control hMSCs or overnight IFN-γ / TNF-α stimulated hMSCs as starting materials, and either seeded in TCP (2D) or encapsulated in collagen-alginate artificial extracellular matrix (aECM) hydrogels (3D) (Fig. 1A). Furthermore, to investigate the effect of the mechanical properties (viscoelasticity and stiffness) of the local microenvironment on gene expression, we encapsulated hMSCs in four different types of hydrogels: viscoelastic soft, viscoelastic stiff, elastic soft and elastic stiff. Viscoelasticity and stiffness were tuned by varying the mode and magnitude of alginate crosslinking, as indicated in Fig. 1B and 1C. Formulations of aECM hydrogels are detailed in Table 1. Full characterization of the hydrogel system has been previously reported in [35]. Gene expression was assessed by real-time RT-qPCR 3 days after hMSCs TCP seeding or encapsulation. A schematic representation of the experimental design is shown in Fig. 1D. To ensure reproducibility and reliability of the results, all experiments were performed in strict compliance with MIQE guidelines [31] (see checklist provided in Supplementary Table 1).

Table 1. Formulation of the different types of aECM hydrogels. aECM: artificial extracellular matrix. Nb: norborene. Tz: tetrazine. VLVG: very low viscosity. GDL: glucono-delta-lactone.

	VLVG alginate (% w/v)	**Nb-alginate** (% w/v)	**Tz-alginate** (% w/v)	**Total alginate** (% w/v)	**Collagen** (mg/mL)	**$CaCO_3$** (% w/v)	**GDL** (mM)
Viscoelastic soft	1.5	0	0	1.5	4	0.1	40
Viscoelastic stiff	1.5	0	0	1.5	4	0.3	120
Elastic soft	0.5	0.5	0.5	1.5	4	0.1	40
Elastic stiff	0.5	0.5	0.5	1.5	4	0.3	120

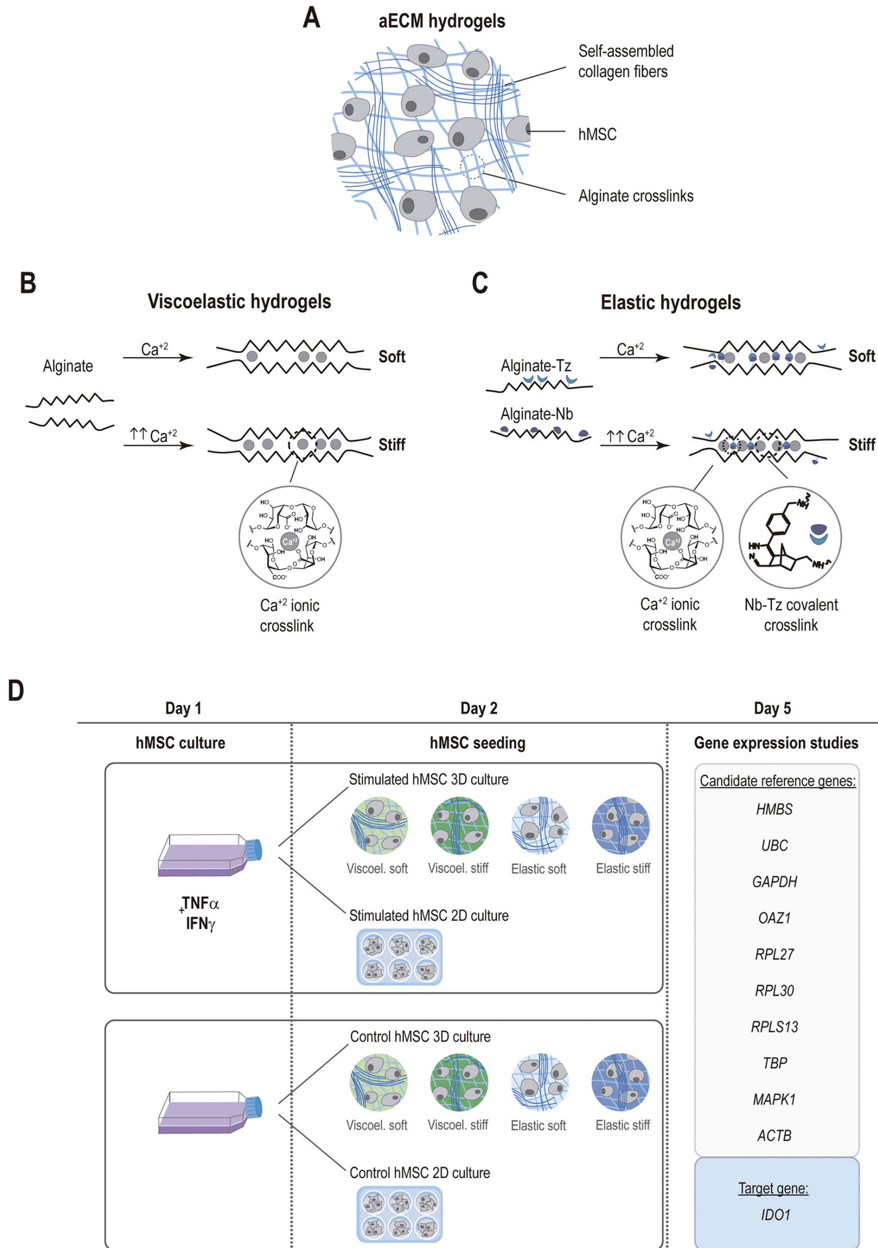


Fig. 1. hMSC encapsulation in aECM hydrogels. (A) Structure and major components of aECM hydrogels. **(B)** In viscoelastic hydrogels, alginates are ionically crosslinked with calcium, whereas in elastic hydrogels **(C)** the ionic crosslinking is combined with covalent crosslinking between norborene (Nb) and tetrazine (Tz) groups. **(D)** Schematic representation of the experimental procedure. Human primary mesenchymal stromal cells (hMSCs) were stimulated overnight with interferon γ (IFN-γ) and tumor necrosis factor α (TNF-α). Subsequently, hMSCs were detached and encapsulated in four hydrogels with different mechanical properties (3D) or seeded in tissue culture plates (2D). The same procedure was followed with unstimulated control hMSCs. After 3 days of culture, RNA was extracted from the cells and real time RT-qPCR analyses of 10 different reference genes and the target gene *IDO1* were performed.

The samples included in the study met RNA quality criteria and a detailed list of RNA amount, quality and integrity (RIN values, 28S/18S, A260/280 and A260/230 ratios and RNA concentrations) is displayed in Supplementary Table 2. Our selection of candidate reference genes is shown in Table 2. We included ten of the most frequently used housekeeping genes in real-time RT-qPCR normalization [38] taking special care to include candidates with distinct cellular functions to minimize possible bias caused by co-regulated genes. As previously reported [39], ideal reference genes are expressed at relatively high and stable levels. Among our 10 candidate genes, the expression levels ranged between 18.65 ± 0.29 (*GAPDH*) to 27.88 ± 1.00 (*UBC*) as shown in Supplementary Fig. 1.

The primer pairs employed in the study were designed in house and details are provided in Supplementary Table 3. All the real-time RT-qPCR reactions produced single amplicons. The efficiency of each primer pair was determined by serial dilution of the cDNA samples. Primer pairs demonstrated E values between 1.93 – 2.05 with correlation coefficients > 0.99.

Table 2. Selection of candidate reference genes for stability assessment in primary human mesenchymal stromal cells (hMSCs).

Protein function	Gene ID	Gene symbol	Gene name
Metabolic enzyme	3145	*HMBS*	Hydroxymethylbilane synthase
	7316	*UBC*	Ubiquitin C
	2597	*GAPDH*	Glyceraldehyde-3 phosphate dehydrogenase
	4946	*OAZ1*	Ornithine decarboxylase antizyme 1
Translation	6155	*RPL27*	Ribosomal protein L27
	852853	*RPL30*	Ribosomal protein L30
	6207	*RPLS13*	Ribosomal protein S13
Transcription	6908	*TBP*	TATA-box binding protein
Signalling	5594	*MAPK1*	Mitogen-activated protein kinase 1
Structural	60	*ACTB*	β-actin

3.2 Stability assessment of the candidate reference genes

We first analyzed candidate reference gene stability with BK (Fig. 2A). According to this algorithm, genes with Cq values showing a SD > 1 should be considered unacceptable for real-time RT-qPCR normalization and excluded from further analysis. Among our selection, all genes showed an acceptable range of variation (SD < 1). Therefore, the BK index, the normalization index the algorithm provides to normalize each sample, was calculated as the geometric mean of Cq values of all the 10 genes. *GAPDH* scored as the most stable reference gene, with the lowest SD and CV values (0.24 and 1.3, respectively). It was followed by *OAZ1* (CV = 1.88) and *TBP* (CV = 2.26). On the contrary, *ACTB* (CV = 2.49), *RPL30* (CV = 2.61), *UBC* (CV = 2.79) and *MAPK1* (CV = 3.03) were the least stable candidate reference genes. SD and CV values for each gene are reported in Supplementary Table 4.

NF analysis is an ANOVA-based model that assigns each candidate reference gene a stability value, considering both intra and intergroup variation. According to this parameter, the algorithm provides a precise ranking from the most stable (presenting the lowest stability value) to the most variable candidate reference gene (with the highest stability value) (Fig. 2B). Here, *RPL30* was ranked as the most stable housekeeping gene, with a stability value of 0.175. In accordance with the results obtained with BK, *ACTB* and *UBC* were defined as the least stable (with stability values of 0.35 and 0.36, respectively). All stability values are detailed in Supplementary Table 5. Moreover, the analysis also determines the combination of two reference genes that provides a lower stability value than any obtained for a single candidate. The combination of *RPS13* and *TBP* provided a lower stability value (0.12) than *RLP30* (0.17). Consequently, the optimal data normalization factor by this analysis would be calculated as the geometric mean of *RPS13* and *TBP*.

GN analysis also provides a ranking of the most stable candidate reference genes, but in this case, it is based on the M values that the algorithm assigns to each. The lower the M value, the most stable expression. As shown in Fig. 2C, in the present study, the most stable housekeeping gene was *RPS13* (M = 0.27), closely followed by *RPL30* (M = 0.271). Similar to NF, GN also provides a combination of two reference genes to obtain the best normalization factor. However, here, the suggested combination of *RPS13* and *RPL30* scored an M value of 0.27, the same stability value as *RPS13* alone. The least stable candidate reference

genes, *UBC* (M = 0.54) and *ACTB* (M = 0.60) scored the highest M values, in agreement with the results obtained with BK and NF. M values of reference genes determined by geNorm are shown in Supplementary Table 6. GN also calculates the pairwise variation (V), which provides information regarding the optimal number of reference genes to employ in a study. Starting with the combination of 2 genes, the algorithm provides V, a ratio based on the normalization factor values (normalization factor obtained with *n* reference genes / normalization factor obtained with *n* + 1 reference genes). If the obtained V factor is below the threshold of 0.15, *n* represents a sufficient number of housekeeping genes. In this case, the inclusion of 2 reference genes would be enough to obtain an optimal normalization factor (Fig. 2D). In sum, both NF and GN ranked *UBC* and *ACTB* as the least stable candidate reference genes, and these also scored poor stability values in BK.

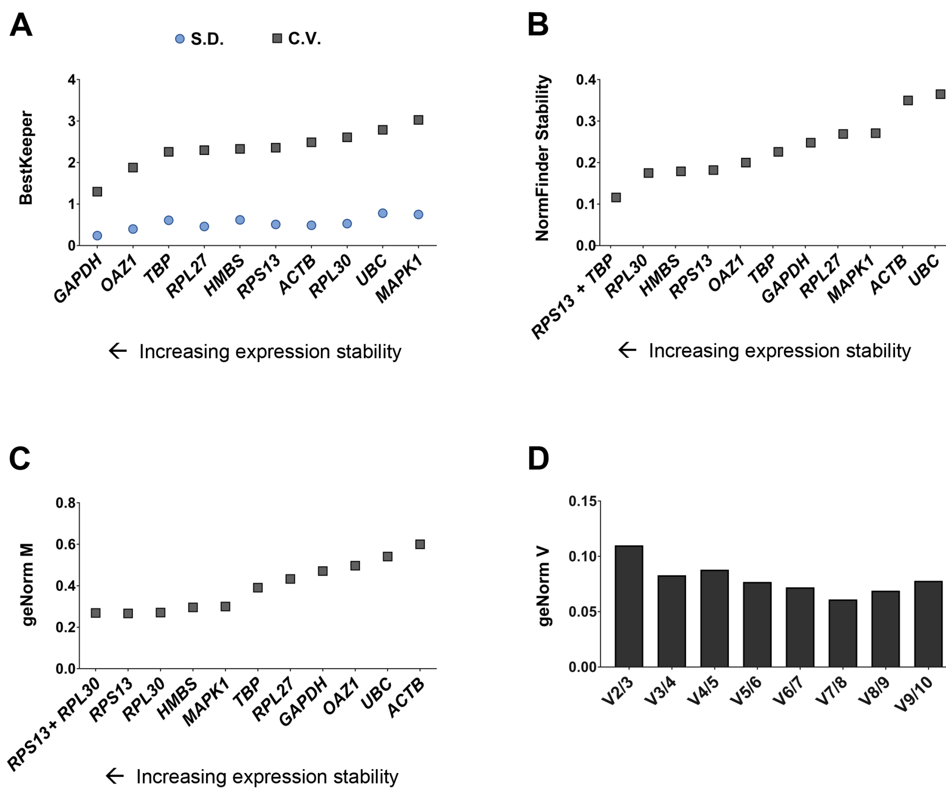


Fig. 2. Reference gene stability determination upon 3D encapsulation in aECM hydrogels with differing mechanical properties and inflammatory licensing. (A) C.V. and S.D. values determined by BestKeeper. **(B)** NormFinder stability values. **(C)** Average expression stability of reference targets determined by geNorm. **(D)** Determination of the optimal number of reference targets by geNorm. n = 3 samples per experimental condition.

3.3 Effect of hMSC encapsulation on the expression of candidate reference genes

Once we assessed the stability of all selected candidate reference genes, we investigated whether the use of those with the least stable expression as real-time RT-qPCR calibrators (namely, *UBC* and *ACTB*) would lead to misleading expression levels of a target gene, under our specific experimental conditions. *IDO1* was chosen as target gene as it is an important marker of hMSC immunomodulatory potential and it is widely employed in a wealth of studies regarding hMSC therapy in inflammatory and immune diseases [12,14].

Real-time RT-qPCR data was analyzed following the $2^{-\Delta\Delta CT}$ method, also known as the Livak method [37]. First, the Cq of the target gene was normalized to that of the reference gene, obtaining the ΔCT. Next, the ΔCT of the sample group was normalized to the ΔCT of the calibrator group (ΔCT sample group - ΔCT calibrator group), obtaining the ΔΔCT, and finally, the normalized expression ratio was calculated ($2^{-\Delta\Delta CT}$).

First, to explore if hMSC encapsulation had an impact on the expression of reference genes, we normalized *IDO1* Cq values to the Cq of different reference candidates: the BK index, the combination of reference genes suggested by NF (*RPS13* + *TBP*), the combination of reference genes proposed by GN (*RPS13* + *RPL30*), *ACTB* or *UBC*. Next, using *IDO1* ΔCT values of 3D encapsulated hMSCs as the sample group, and *IDO1* ΔCT values of 2D cultured hMSCs as the calibrator group, we calculated the normalized expression ratio. As expected, the levels of *IDO1* expression did not change when GN, NF or BK were used to normalize the data. However, when normalizing the data with *ACTB* o *UBC*, statistically different results were obtained in the four hydrogel types (Fig. 3 A-D). In particular, we determined an overestimation of *IDO1* expression when normalizing to *ACTB*, versus an underestimation of *IDO1* expression when normalizing to *UBC*.

To confirm that *ACTB* and *UBC* expression varied depending on hMSC 2D or 3D culture, we used the most stable combination of reference genes proposed by NF and normalized *ACTB* or *UBC* expression in 3D cultured cells (sample group) to their expression in 2D cultured cells (calibrator group). We performed the analysis in parallel with control and stimulated cells. Confirming our previous observations, *ACTB* was significantly downregulated in 3D encapsulated hMSCs (Fig. 3 E-F). *UBC*, on the contrary, was significantly upregulated in encapsulated

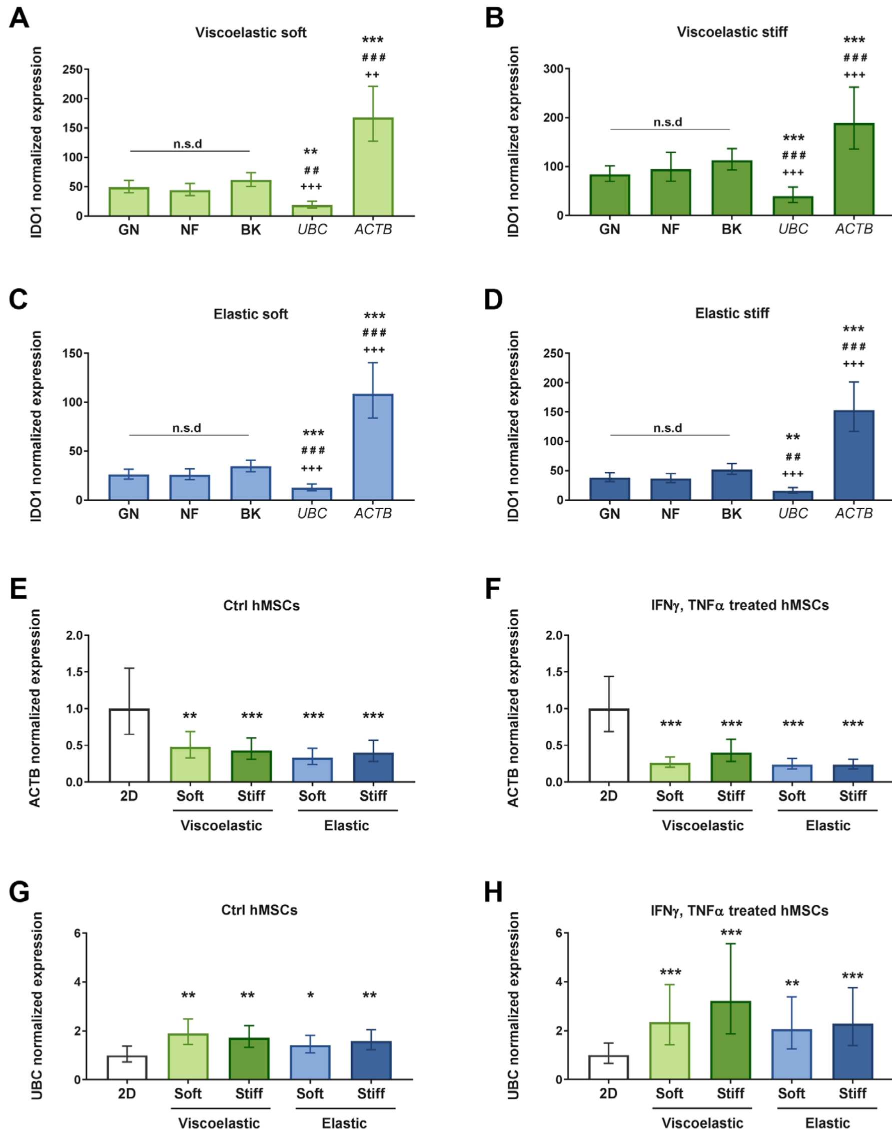


Fig. 3. Effect of 3D encapsulation on reference gene stability. *IDO1* expression in cells encapsulated in **(A)** soft viscoelastic **(B)** stiff viscoelastic **(C)** soft elastic and **(D)** stiff elastic gels was normalized to 2D cultured cells, using the reference gene combinations provided by GN and NF, the BK index or the reference genes *UBC* or *ACTB*. Values represent mean ± S.E. (n = 3 samples per experimental condition). Statistical significance: one-way ANOVA with Bonferroni multiple comparisons test: $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to GN $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared to NF and $^{++}p < 0.01$ and $^{+++}p < 0.001$ compared to BK. NF: NormFinder. GN: geNorm. BK: BestKeeper. n.s.d.: no significant difference. Evaluation of *ACTB* expression in all encapsulated conditions: **(E)** unstimulated cells and **(F)** stimulated cells, all normalized to their 2D controls. Evaluation of *UBC* expression in all encapsulated conditions: **(G)** non-stimulated cells and **(H)** stimulated cells, all normalized to their 2D controls. The reference gene combination employed was that recommended by NormFinder. Values represent mean ± S.E. (n = 3 samples per experimental condition). Statistical significance: one-way ANOVA with Bonferroni multiple comparisons test: $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to 2D.

hMSCs (Fig. 3 G-H). These results explained the over and underestimation of *IDO1* observed when these genes were used as the reference gene (Fig. 3 A-D). Taken together, these results highlight the inadequacy of *ACTB* and *UBC* as reference genes when gene expression in 2D and 3D cultured cells is investigated.

3.4 Effect of the mechanical properties of the matrix on the expression of candidate reference genes

Next, we evaluated the impact of the mechanical properties of the matrix in which hMSCs were encapsulated on reference gene expression. To do so, the effect of both viscoelasticity and stiffness was analyzed. To determine the effect of viscoelasticity, we used *IDO1* ΔCT values of hMSCs encapsulated in viscoelastic hydrogels as the sample group, and the *IDO1* ΔCT values of hMSCs encapsulated in elastic hydrogels as the calibrator group (Fig. 4 A-B). To analyze the influence of matrix stiffness, we employed *IDO1* ΔCT values of hMSCs

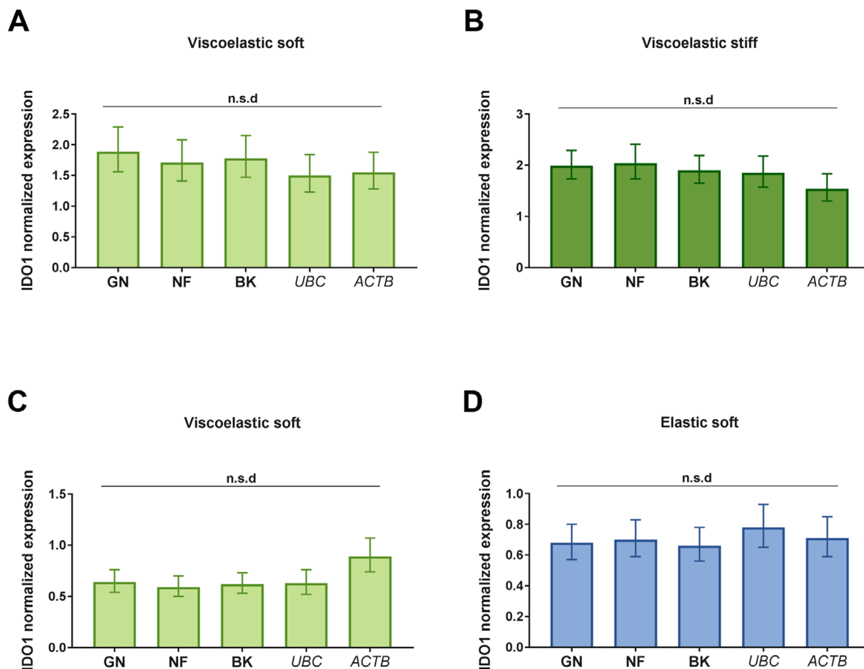


Fig. 4. Effect of the mechanical properties of aECM hydrogels on reference gene stability. *IDO1* expression in cells encapsulated in **(A)** soft viscoelastic and **(B)** stiff viscoelastic gels normalized to their elastic controls using the reference gene combinations. *IDO1* expression when normalizing soft viscoelastic **(C)** and soft elastic **(D)** gels to their stiff controls. Error bars mean ± S.E. (n = 3 samples per experimental condition). Statistical significance: one-way ANOVA. NF: NormFinder. GN: geNorm. BK: BestKeeper. n.s.d: no significant difference.

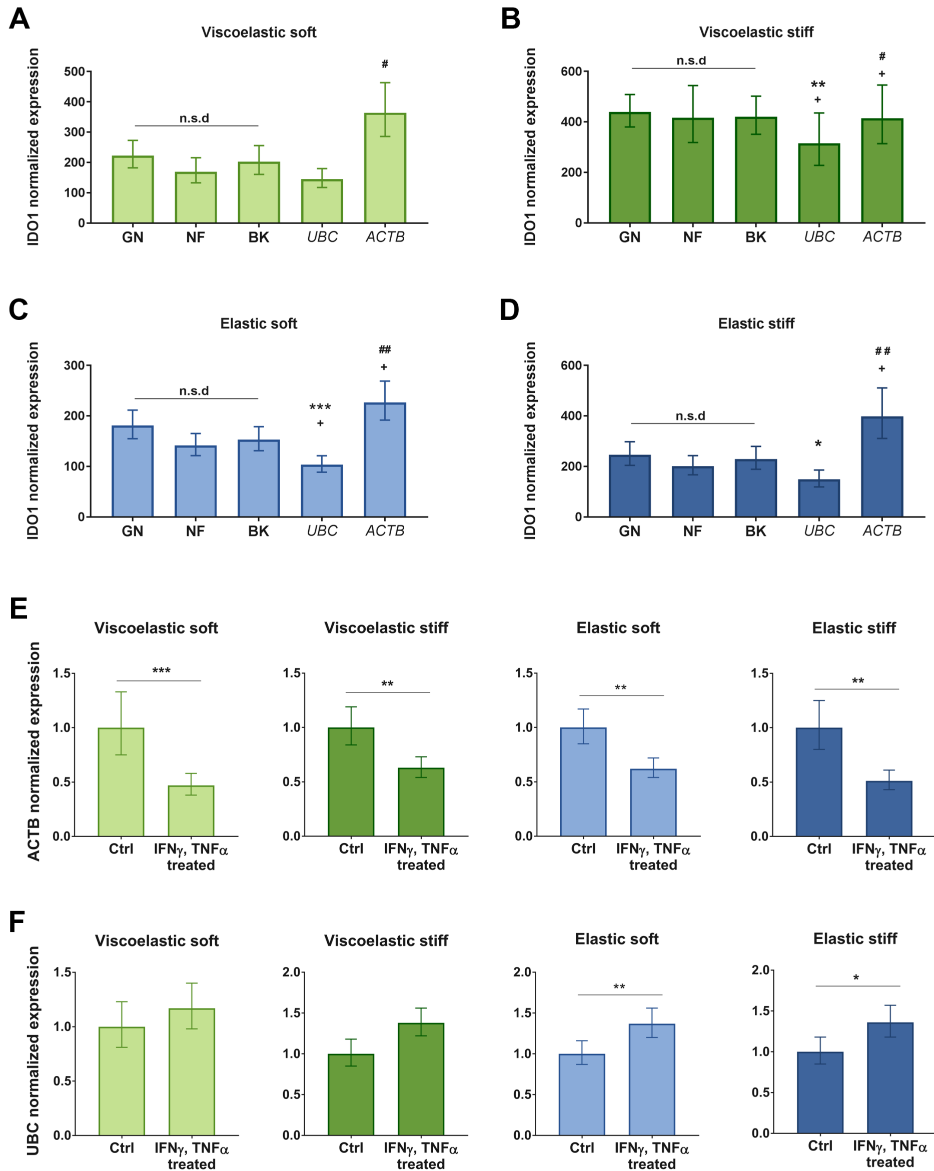


Fig. 5. Effect of inflammatory licensing on reference gene stability. *IDO1* expression in stimulated cells encapsulated in **(A)** soft viscoelastic, **(B)** stiff viscoelastic, **(C)** soft elastic and **(D)** stiff viscoelastic gels was normalized to the non-stimulated controls using the reference gene combinations provided by GN and NF, the BK index or the reference genes *UBC* or *ACTB*. Values represent mean ± S.E. (n = 3 samples per experimental condition). Statistical significance: one-way ANOVA with Bonferroni multiple comparisons test: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to GN $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to NF and $^{+}p < 0.05$ compared to BK. NF: NormFinder. GN: geNorm. BK: BestKeeper. n.s.d.: no significant difference. *ACTB* **(E)** and *UBC* **(F)** expression in stimulated cells in the four gel types, all normalized to their non-stimulated controls. The reference gene combination employed was that recommended by NormFinder. Values represent mean ± S.E. (n = 3 samples per experimental condition). Statistical significance: Student's t-test: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to the non-stimulated control. Ctrl: control.

encapsulated in soft hydrogels (sample group) and *IDO1* ΔCT values of hMSCs encapsulated in stiff hydrogels (calibrator group) (Fig. 4 C-D). In both cases, data normalization with all the different reference genes led to the same *IDO1* expression results, indicating that their expression remained stable within the specific mechanical variations tested here.

3.5 Effect of hMSCs IFN-γ / TNF-α stimulation on the expression of candidate reference genes

Finally, following the same analysis, we explored the influence of hMSC overnight stimulation with IFN-γ/TNF-α. In this case, we used the *IDO1* ΔCT values of IFN-γ / TNF-α stimulated hMSCs as the sample group, and the *IDO1* ΔCT values of control hMSCs as the calibrator group. Once again, data normalization with the reference genes proposed by the 3 different algorithms led to equal *IDO1* expression values. On the contrary, statistically different results were obtained when normalizing the data with *ACTB* or *UBC*, for all the four hydrogel types (Fig. 5 A-D). As in the 2D *versus* 3D comparison, *IDO1* was over and underestimated when normalized to *ACTB* and *UBC*, respectively. However, the differences in mRNA levels were less striking than in the previous comparison. To confirm the observations above, we normalized *ACTB* and *UBC* expression in stimulated hMSCs with NF. As shown in Fig. 5 E, *ACTB* expression was downregulated in IFN-γ and TNF-α stimulated hMSCs, leading to an overestimation of target gene expression if used as a reference gene under these experimental conditions (Fig. 5 A-D). On the other hand, *UBC* upregulation was observed (Fig. 5 F), explaining why when used as a reference gene, target gene expression resulted in an underestimation (Fig. 5 A-D).

4. Discussion

Our results demonstrate that experimental conditions intended to promote the immunomodulatory properties of hMSCs induce significant changes in the stability of commonly employed housekeeping genes. Here, we explored the combination of both hMSC inflammatory licensing with IFN-γ and TNF-γ, and encapsulation in four different types of alginate-collagen hydrogels with differing viscoelasticity and stiffness. To the best of our knowledge, this is the first study that evaluates reference gene stability in hMSCs across a pool of licensed or

non-licensed control cells either in 2D or 3D culture in hydrogels with different mechanical properties. This gap needed to be filled considering the vast number of studies aiming to precondition hMSCs to enhance their immunomodulatory potential.

The results obtained with BK, NF and GN algorithms revealed the ribosomal proteins *RPS13* and *RPL30* as two of the most stable reference genes. This matches the results obtained in a meta-analysis conducted by de Jonge *et al.* [38], where *RPS13* and *RPL30* ranked the first and fourth reference genes, respectively, to present enhanced stability among multiple cell types and a multitude of experimental conditions. Indeed, in our study, GN proposed the combination of *RPS13* and *RPL30* as the most stable, whereas NF suggested combining *RPS13* and *TBP*. Regarding the latter, *TBP* has been proposed as a stable housekeeping gene in previous studies evaluating MSC 3D culture in cancellous bone cube [40] and fibrinogen or fibrinogen-alginate scaffolds [41]. In our case, *TBP* was ranked as the third most stable gene by BK, and was positioned in the middle by NF and GN. Despite not scoring as the most stable it still presented adequate stability values. Importantly, one should consider, taking NF as an example, that stability values from the 1st to the 8th position only varied from 0.17 to 0.27 (*TBP* scored 0.23). On the contrary, the last two candidates, namely *ACTB* and *UBC*, presented stability values of 0.35 and 0.36, differing significantly from the rest of housekeeping genes. BK and GN also ranked *UBC* and *ACTB* among the least stable candidates. Although *ACTB* has been reported to be among the 12 most widely used reference genes [38], in agreement with our results, its instability upon different experimental conditions has previously been demonstrated in multiple publications [25,40,42]. The differences we detected within the rankings provided by BK, NF and GN were expected, since each one of these tools is based on a different algorithm. Indeed, discrepancies among them have been previously reported [42]. However, we demonstrated that choosing either one of them for *IDO1* normalization resulted in the same relative expression values (Fig. 3 A-D, Fig. 4 A-D, Fig. 5 A-D), supporting the significance of the results reported in this study.

The poor stability of *ACTB* and *UBC* led to misleading results when studying the expression of the target gene *IDO1* in these experiments. We observed important differences in the expression of these two candidate reference genes when comparing 2D to 3D cultured hMSCs. Normalization to *ACTB* resulted in an overestimation of *IDO1*, whereas when employing *UBC*,

IDO1 expression was underestimated. This was caused by a downregulation of *ACTB* and an upregulation of *UBC* in 3D cultured hMSCs, when compared to 2D cultured cells. These results are consistent with previous studies, where geNorm and NormFinder analyses identified *ACTB* among the three least stable reference genes in 3D cultivated bone marrow MSCs [40]. In addition, Liu *et al*. ranked *ACTB* as the least stable candidate housekeeping gene in MSCs under dynamic hydrostatic pressure and concluded that *ACTB* is not a suitable internal control gene for mRNA assay in mechanobiology studies [43]. While the rigidity of the microenvironment [18,44] and the matrix stress-relaxation [17] have been reported to regulate intracellular pathways, the expression of *ACTB* and *UBC* was not significantly altered in hMSCs encapsulated in aECM hydrogels with varying viscoelasticity and stiffness.

Significant differences were noted in *ACTB* and *UBC* expression when comparing IFN-γ / TNF-α licensed hMSCs to control, non-stimulated cells. *ACTB* expression was downregulated and *UBC* upregulated in cytokine stimulated hMSCs, in comparison to control, non-stimulated cells, although these effects were not as drastic as the observed when comparing 3D *versus* 2D expression. In agreement with our results, a recent publication demonstrated the poor stability of some miRNA reference genes extensively employed to quantify the nucleic acid content of extracellular vesicles produced by MSCs, upon cell inflammatory licensing with IFN-γ [28]. Together, these results indicate that the utilization of *ACTB* and *UBC* is not advisable in studies that explore promoting the immunomodulatory potential of hMSCs in 3D culture or via inflammatory licensing.

5. Conclusion

Here, we have shown that the expression of some of the most widely employed reference genes, including *UBC* and *ACTB*, was importantly altered upon hMSC 3D encapsulation in collagen-alginate hydrogels, as well as upon inflammatory licensing with IFN-γ / TNF-α. Their utilization as housekeeping genes can lead to significant over and underestimation of target gene mRNA levels in real-time RT-qPCR studies. Together, these results highlight the importance of reference gene validation when using hMSC pre-conditioning strategies intended to enhance their immunomodulatory potential.

6. Acknowledgements

Authors thank for the projects SAF2017-82292-R (MINECO/AEI/FEDER, UE), ICTS “NANBIOSIS” (Drug Formulation Unit, U10), the National Institute of Dental & Craniofacial Research of the National Institutes of Health under Award Numbers R01DE013033 (DM), and the support from the Basque Country Government (Grupos Consolidados, No ref: IT907-16). A. Gonzalez-Pujana thanks the Basque Government for the PhD grant (PRE_2018_2_0133).

7. References

[1] A.J. Friedenstein, U.F. Deriglasova, N.N. Kulagina, A.F. Panasuk, S.F. Rudakowa, E.A. Luria, I.A. Ruadkow, Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method, Exp. Hematol. 2 (1974) 83-92.

[2] D.G. Phinney, G. Kopen, R.L. Isaacson, D.J. Prockop, Plastic adherent stromal cells from the bone marrow of commonly used strains of inbred mice: variations in yield, growth, and differentiation, J. Cell. Biochem. 72 (1999) 570-585.

[3] Y. Zhang, M. Chen, J. Tian, P. Gu, H. Cao, X. Fan, W. Zhang, In situ bone regeneration enabled by a biodegradable hybrid double-network hydrogel, Biomater. Sci. (2019). https://doi.org/10.1039/c9bm00561g.

[4] V.P. Ribeiro, A. da Silva Morais, F.R. Maia, R.F. Canadas, J.B. Costa, A.L. Oliveira, J.M. Oliveira, R.L. Reis, Combinatory approach for developing silk fibroin scaffolds for cartilage regeneration, Acta Biomater. 72 (2018) 167-181. https://doi.org/10.1016/j.actbio.2018.03.047.

[5] L. Jiang, D. Su, S. Ding, Q. Zhang, Z. Li, F. Chen, W. Ding, S. Zhang, J. Dong, Salt-Assisted Toughening of Protein Hydrogel with Controlled Degradation for Bone Regeneration, Adv. Funct. Mater. 0 (2019) 1901314. https://doi.org/10.1002/adfm.201901314.

[6] J.R. Ferreira, G.Q. Teixeira, S.G. Santos, M.A. Barbosa, G. Almeida-Porada, R.M. Goncalves, Mesenchymal Stromal Cell Secretome: Influencing Therapeutic Potential by Cellular Pre-conditioning, Front. Immunol. 9 (2018) 2837. https://doi.org/10.3389/fimmu.2018.02837.

[7] F. Gao, S.M. Chiu, D.A. Motan, Z. Zhang, L. Chen, H.L. Ji, H.F. Tse, Q.L. Fu, Q. Lian, Mesenchymal stem cells and immunomodulation: current status and future prospects, Cell. Death Dis. 7 (2016) e2062. https://doi.org/10.1038/cddis.2015.327.

[8] M.E. Bernardo, W.E. Fibbe, Mesenchymal stromal cells: sensors and switchers of inflammation, Cell. Stem Cell. 13 (2013) 392-402. https://doi.org/10.1016/j.stem.2013.09.006.

[9] Y. Shi, J. Su, A.I. Roberts, P. Shou, A.B. Rabson, G. Ren, How mesenchymal stem cells interact with tissue immune responses, Trends Immunol. 33 (2012) 136-143. https://doi.org/10.1016/j.it.2011.11.004.

[10] M. Krampera, L. Cosmi, R. Angeli, A. Pasini, F. Liotta, A. Andreini, V. Santarlasci, B. Mazzinghi, G. Pizzolo, F. Vinante, P. Romagnani, E. Maggi, S. Romagnani, F. Annunziato, Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells, Stem Cells. 24 (2006) 386-398. https://doi.org/10.1634/stemcells.2005-0008.

[11] N. Murphy, O. Treacy, K. Lynch, M. Morcos, P. Lohan, L. Howard, G. Fahy, M.D. Griffin, A.E. Ryan, T. Ritter, TNF-alpha/IL-1beta-licensed mesenchymal stromal cells promote corneal allograft survival via myeloid cell-mediated induction of Foxp3(+) regulatory T cells in the lung, FASEB J. (2019) fj201900047R. https://doi.org/10.1096/fj.201900047R.

[12] P. Jin, Y. Zhao, H. Liu, J. Chen, J. Ren, J. Jin, D. Bedognetti, S. Liu, E. Wang, F. Marincola, D. Stroncek, Interferon-gamma and Tumor Necrosis Factor-alpha Polarize Bone Marrow Stromal Cells Uniformly to a Th1 Phenotype, Sci. Rep. 6 (2016) 26345. https://doi.org/10.1038/srep26345.

[13] T.J. Bartosh, J.H. Ylostalo, A. Mohammadipoor, N. Bazhanov, K. Coble, K. Claypool, R.H. Lee, H. Choi, D.J. Prockop, Aggregation of human mesenchymal stromal cells (MSCs) into 3D spheroids enhances their antiinflammatory properties, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 13724-13729. https://doi.org/10.1073/pnas.1008117107.

[14] M.J. Leijs, E. Villafuertes, J.C. Haeck, W.J. Koevoet, B. Fernandez-Gutierrez, M.J. Hoogduijn, J.A. Verhaar, M.R. Bernsen, G.M. van Buul, G.J. van Osch, Encapsulation of allogeneic mesenchymal stem cells in alginate extends local presence and therapeutic function, Eur. Cell. Mater. 33 (2017) 43-58. https://doi.org/10.22203/eCM.v033a04.

[15] A. Goren, N. Dahan, E. Goren, L. Baruch, M. Machluf, Encapsulated human mesenchymal stem cells: a unique hypoimmunogenic platform for long-term cellular therapy, FASEB J. 24 (2010) 22-31. https://doi.org/10.1096/fj.09-131888.

[16] Y. Petrenko, E. Sykova, S. Kubinova, The therapeutic potential of three-dimensional multipotent mesenchymal stromal cell spheroids, Stem Cell. Res. Ther. 8 (2017) 94-017-0558-6. https://doi.org/10.1186/s13287-017-0558-6.

[17] O. Chaudhuri, L. Gu, D. Klumpers, M. Darnell, S.A. Bencherif, J.C. Weaver, N. Huebsch, H.P. Lee, E. Lippens, G.N. Duda, D.J. Mooney, Hydrogels with tunable stress relaxation regulate stem cell fate and activity, Nat. Mater. 15 (2016) 326-334. https://doi.org/10.1038/nmat4489.

[18] A. Gonzalez-Pujana, A. Rementeria, F.J. Blanco, M. Igartua, J.L. Pedraz, E. Santos-Vizcaino, R.M. Hernandez, The role of osmolarity adjusting agents in the regulation of encapsulated cell behavior to provide a safer and more predictable delivery of therapeutics, Drug Deliv. 24 (2017) 1654-1666. https://doi.org/10.1080/10717544.2017.1391894.

[19] N. Huebsch, P.R. Arany, A.S. Mao, D. Shvartsman, O.A. Ali, S.A. Bencherif, J. Rivera-Feliciano, D.J. Mooney, Harnessing traction-mediated manipulation of the cell/matrix interface to control stem-cell fate, Nat. Mater. 9 (2010) 518-526. https://doi.org/10.1038/nmat2732.

[20] H.D. VanGuilder, K.E. Vrana, W.M. Freeman, Twenty-five years of quantitative PCR for gene expression analysis, BioTechniques. 44 (2008) 619-626. https://doi.org/10.2144/000112776.

[21] I. Chervoneva, Y. Li, S. Schulz, S. Croker, C. Wilson, S.A. Waldman, T. Hyslop, Selection of optimal reference genes for normalization in quantitative RT-PCR, BMC Bioinformatics. 11 (2010) 253-2105-11-253. https://doi.org/10.1186/1471-2105-11-253.

[22] T. Nolan, R.E. Hands, S.A. Bustin, Quantification of mRNA using real-time RT-PCR, Nat. Protoc. 1 (2006) 1559-1582. https://doi.org/10.1038/nprot.2006.236.

[23] B. Kozera, M. Rapacz, Reference genes in real-time PCR, J. Appl. Genet. 54 (2013) 391-406. https://doi.org/10.1007/s13353-013-0173-x.

[24] J. Tratwal, B. Follin, A. Ekblond, J. Kastrup, M. Haack-Sorensen, Identification of a common reference gene pair for qPCR in human mesenchymal stromal cells from different tissue sources treated with VEGF, BMC Mol. Biol. 15 (2014) 11-2199-15-11. https://doi.org/10.1186/1471-2199-15-11.

[25] A. Jacobi, J. Rauh, P. Bernstein, C. Liebers, X. Zou, M. Stiehler, Comparative analysis of reference gene stability in human mesenchymal stromal cells during osteogenic differentiation, Biotechnol. Prog. 29 (2013) 1034-1042. https://doi.org/10.1002/btpr.1747.

[26] F. Schulze, D. Malhan, T. El Khassawna, C. Heiss, A. Seckinger, D. Hose, A. Rosen-Wolff, A tissue-based approach to selection of reference genes for quantitative real-time PCR in a sheep osteoporosis model, BMC Genomics. 18 (2017) 975-017-4356-4. https://doi.org/10.1186/s12864-017-4356-4.

[27] W.J. Lee, R.H. Jeon, S.J. Jang, J.S. Park, S.C. Lee, R. Baregundi Subbarao, S.L. Lee, B.W. Park, W.A. King, G.J. Rho, Selection of Reference Genes for Quantitative Gene Expression in Porcine Mesenchymal Stem Cells Derived from Various Sources along with Differentiation into Multilineages, Stem Cells Int. 2015 (2015) 235192. https://doi.org/10.1155/2015/235192.

[28] E. Ragni, P. De Luca, C. Perucca Orfei, A. Colombini, M. Vigano, G. Lugano, V. Bollati, L. de Girolamo, Insights into Inflammatory Priming of Adipose-Derived Mesenchymal Stem Cells: Validation of Extracellular Vesicles-Embedded miRNA Reference Genes as A Crucial Step for Donor Selection, Cells. 8 (2019) 10.3390/cells8040369. https://doi.org/10.3390/cells8040369.

[29] F. Banfi, A. Colombini, C. Perucca Orfei, V. Parazzi, E. Ragni, Validation of reference and identity-defining genes in human mesenchymal stem cells cultured under unrelated fetal bovine serum batches for basic science and clinical application, Stem Cell. Rev. 14 (2018) 837-846. https://doi.org/10.1007/s12015-018-9822-0.

[30] K. Dheda, J.F. Huggett, J.S. Chang, L.U. Kim, S.A. Bustin, M.A. Johnson, G.A. Rook, A. Zumla, The implications of using an inappropriate reference gene for real-time reverse transcription PCR data normalization, Anal. Biochem. 344 (2005) 141-143. https://doi.org/10.1016/j.ab.2005.05.022.

[31] S.A. Bustin, V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M.W. Pfaffl, G.L. Shipley, J. Vandesompele, C.T. Wittwer, The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments, Clin. Chem. 55 (2009) 611-622. https://doi.org/10.1373/clinchem.2008.112797.

[32] M.W. Pfaffl, A. Tichopad, C. Prgomet, T.P. Neuvians, Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations, Biotechnol. Lett. 26 (2004) 509-515.

[33] C.L. Andersen, J.L. Jensen, T.F. Orntoft, Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets, Cancer Res. 64 (2004) 5245-5250. https://doi.org/10.1158/0008-5472.CAN-04-0496.

[34] J. Vandesompele, K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, F. Speleman, Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes, Genome Biol. 3 (2002) RESEARCH0034. https://doi.org/10.1186/gb-2002-3-7-research0034.

[35] K.H. Vining, A. Stafford, D.J. Mooney, Sequential modes of crosslinking tune viscoelasticity of cell-instructive hydrogels, Biomaterials. 188 (2019) 187-197. https://doi.org/10.1016/j.biomaterials.2018.10.013.

[36] R.M. Desai, S.T. Koshy, S.A. Hilderbrand, D.J. Mooney, N.S. Joshi, Versatile click alginate hydrogels crosslinked via tetrazine-norbornene chemistry, Biomaterials. 50 (2015) 30-37. https://doi.org/10.1016/j.biomaterials.2015.01.048.

[37] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods. 25 (2001) 402-408. https://doi.org/10.1006/meth.2001.1262.

[38] H.J. de Jonge, R.S. Fehrmann, E.S. de Bont, R.M. Hofstra, F. Gerbens, W.A. Kamps, E.G. de Vries, A.G. van der Zee, G.J. te Meerman, A. ter Elst, Evidence based selection of housekeeping genes, PLoS One. 2 (2007) e898. https://doi.org/10.1371/journal.pone.0000898.

[39] J. Huggett, K. Dheda, S. Bustin, A. Zumla, Real-time RT-PCR normalisation; strategies and considerations, Genes Immun. 6 (2005) 279-284. https://doi.org/10.1038/sj.gene.6364190.

[40] J. Rauh, A. Jacobi, M. Stiehler, Identification of stable reference genes for gene expression analysis of three-dimensional cultivated human bone marrow-derived mesenchymal stromal cells for bone tissue engineering, Tissue Eng. Part C. Methods. 21 (2015) 192-206. https://doi.org/10.1089/ten.TEC.2014.0230.

[41] B. Brinkhof, H. Jia, B. Zhang, Z. Cui, H. Ye, H. Wang, Improving characterisation of human Multipotent Stromal Cells cultured in 2D and 3D: Design and evaluation of primer sets for accurate gene expression normalisation, PLoS One. 13 (2018) e0209772. https://doi.org/10.1371/journal.pone.0209772.

[42] I. de Lázaro, K. Kostarelos, Exposure to graphene oxide sheets alters the expression of reference genes used for real-time RT-qPCR normalization, Scientific Reports. 9 (2019) 12520. https://doi.org/10.1038/s41598-019-48970-z.

[43] J. Liu, L. Zou, J. Wang, Z. Zhao, Validation of beta-actin used as endogenous control for gene expression analysis in mechanobiology studies, Stem Cells. 27 (2009) 2371-2372. https://doi.org/10.1002/stem.160.

[44] N. Huebsch, P.R. Arany, A.S. Mao, D. Shvartsman, O.A. Ali, S.A. Bencherif, J. Rivera-Feliciano, D.J. Mooney, Harnessing traction-mediated manipulation of the cell/matrix interface to control stem-cell fate, Nat. Mater. 9 (2010) 518-526. https://doi.org/10.1038/nmat2732.

Supplementary figures and tables

Supplementary Table 1. MIQUE guideline checklist.
E: essential information. D: desirable information. Y: yes (included in the manuscript). N: no (not inlcuded). N/A: not applicable. * Propietary information, not provided by the manufacturer.

ITEM TO CHECK	IMPORTANCE	INCLUDED?	COMMENTS
EXPERIMENTAL DESIGN			
Definition of experimental and control groups	**E**	**Y**	See Methods
Number within each group	**E**	**Y**	
Assay carried out by core lab or investigator's lab?	D	Y	
Acknowledgement of authors' contributions	D	Y	See Acknowledgedments
SAMPLE			
Description	**E**	Y	See Methods
Volume/mass of sample processed	D	Y	
Microdissection or macrodissection	**E**	N/A	
Processing procedure	**E**	Y	
If frozen - how and how quickly?	**E**	N/A	
If fixed - with what, how quickly?	**E**	N/A	
Sample storage conditions and duration	**E**	Y	
NUCLEIC ACID EXTRACTION			
Procedure and/or instrumentation	**E**	Y	See Methods
Name of kit and details of any modifications	**E**	Y	
Source of additional reagents used	D	Y	
Details of DNase or RNAse treatment	**E**	Y	
Contamination assessment (DNA or RNA)	**E**	Y	
Nucleic acid quantification	**E**	Y	
Instrument and method	**E**	Y	
Purity (A260/A280)	D	Y	Table S2
Yield	D	Y	
RNA integrity method/instrument	**E**	Y	See Methods
RIN/RQI or Cq of 3' and 5' transcripts	**E**	Y	Table S2
Electrophoresis traces	D	Y	Table S2
Inhibition testing (Cq dilutions, spike or other)	**E**	Y	See Methods
REVERSE TRANSCRIPTION			
Complete reaction conditions	**E**	Y	See Methods
Amount of RNA and reaction volume	**E**	Y	
Priming oligonucleotide (if using GSP) and concentration	**E**	N/A	
Reverse transcriptase and concentration	**E**	Y	
Temperature and time	**E**	Y	
Manufacturer of reagents and catalogue numbers	D	Y	
Cqs with and without RT	D*	Y	
Storage conditions of cDNA	D	Y	

Supplementary Table 1. MIQUE guidelines (continued)

ITEM TO CHECK	IMPORTANCE	INCLUDED?	COMMENTS
qPCR TARGET INFORMATION			
If multiplex, efficiency and LOD of each assay.	**E**	N/A	See Methods and Table S3
Sequence accession number	**E**	Y	
Location of amplicon	D	N	
Amplicon length	**E**	Y	
In silico specificity screen (BLAST, etc)	**E**	Y	
Pseudogenes, retropseudogenes or other homologs?	D	Y	
Sequence alignment	D	N	
Secondary structure analysis of amplicon	D	N	
Location of each primer by exon or intron (if applicable)	**E**	N/A	
What splice variants are targeted?	**E**	Y	
qPCR OLIGONUCLEOTIDES			
Primer sequences	**E**	Y	See Methods and Table S3
RTPrimerDB Identification Number	D	N/A	
Probe sequences	D**	N/A	
Location and identity of any modifications	**E**	N/A	
Manufacturer of oligonucleotides	D	Y	
Purification method	D	Y	
qPCR PROTOCOL			
Complete reaction conditions	**E**	Y	See Methods
Reaction volume and amount of cDNA/DNA	**E**	Y	
Primer, (probe), Mg++ and dNTP concentrations	**E**	N*	
Polymerase identity and concentration	**E**	Y	
Buffer/kit identity and manufacturer	**E**	Y	
Exact chemical constitution of the buffer	D	N	
Additives (SYBR Green I, DMSO, etc.)	**E**	Y	
Manufacturer of plates/tubes and catalog number	D	Y	
Complete thermocycling parameters	**E**	Y	
Reaction setup (manual/robotic)	D	Y	
Manufacturer of qPCR instrument	**E**	Y	
qPCR VALIDATION			
Evidence of optimisation (from gradients)	D	N	
Specificity (gel, sequence, melt, or digest)	**E**	Y	See Methods
For SYBR Green I, Cq of the NTC	**E**	Y	
Standard curves with slope and y-intercept	**E**	Y	See Methods and Tables S3
PCR efficiency calculated from slope	**E**	Y	
Confidence interval for PCR efficiency or standard error	D	N	
r2 of standard curve	**E**	Y	
Linear dynamic range	**E**	Y	
Cq variation at lower limit	**E**	N	
Confidence intervals throughout range	D	N	
Evidence for limit of detection	**E**	Y	See Methods
If multiplex, efficiency and LOD of each assay.	**E**	N/A	

Supplementary Table 1. MIQUE guidelines (continued)

ITEM TO CHECK	IMPORTANCE	INCLUDED?	COMMENTS
DATA ANALYSIS			
qPCR analysis program (source, version)	**E**	Y	See Methods
Cq method determination	**E**	Y	
Outlier identification and disposition	**E**	N/A	
Results of NTCs	**E**	Y	See Methods
Justification of number and choice of reference genes	**E**	Y	See Introduction
Description of normalisation method	**E**	Y	See Methods
Number and concordance of biological replicates	D	Y	
Number and stage (RT or qPCR) of technical replicates	**E**	Y	
Repeatability (intra-assay variation)	E	N	
Reproducibility (inter-assay variation, %CV)	D	N	
Power analysis	D	N	
Statistical methods for result significance	**E**	Y	See Methods
Software (source, version)	E	Y	
Cq or raw data submission using RDML	**D**	N	

Supplementary Table 2. RNA yield and integrity determination. RIN: RNA integrity number. TCP: tissue culture plate (2D). Ctrl: control. Stim: stimulated. VE: viscoelastic. EL: elastic.

Sample ID	RIN	28S/18S (Area)	$A_{260/280}$	$A_{260/230}$	RNA Conc. (ng μl^{-1})
1. TCP Ctrl_1	8.3	1.2	2.05	1.94	206
2. TCP Ctrl_2	8.7	1.3	2.06	2.02	161
3. TCP Ctrl _3	8.2	1.1	2.07	2.31	282
4. TCP Stim_1	8.1	1.5	2.04	2.34	203
5. TCP Stim_2	8.4	1.6	2.06	1.2	221
6. TCP Stim_3	8.1	1.3	2.03	2.32	156
7. VE soft Ctrl_1	8.9	2.0	2.05	0.39	58.4
8. VE soft Ctrl_2	8.7	1.8	1.97	1.6	62.7
9. VE soft Ctrl_3	9.2	2.3	1.99	2.32	78.9
10. VE stiff Ctrl_1	8.8	2.0	2	1.12	61.5
11. VE stiff Ctrl_2	8.9	2.0	1.93	2.55	75.9
12. VE stiff Ctrl_3	9.1	2.2	2.03	2.19	85.6
13. EL soft Ctrl_1	9.2	2.1	2.05	1.55	126
14. EL soft Ctrl_2	8.9	1.9	2	2.24	159
15. EL soft Ctrl_3	9.0	2.5	1.98	1.58	96.5
16. EL stiff Ctrl_1	9.0	2.2	1.98	1.98	94.6
17. EL stiff Ctrl_2	9.2	1.8	1.94	2.91	60.2
18. EL stiff Ctrl_3	8.9	1.8	1.98	2.4	144
19. VE soft Stim_1	9.2	2.1	2.07	1.3	62.7
20. VE soft Stim_2	9.0	2.2	2.05	2.26	99.6
21. VE soft Stim_3	9.0	2.1	2.07	1.3	93.1
22. VE stiff Stim_1	6.7	1.1	1.92	0.97	17.9
23. VE stiff Stim_2	8.6	1.8	1.99	0.93	34.7
24. VE stiff Stim_3	8.7	1.8	2.05	0.9	42.1
25. EL soft Stim_1	9.3	2.2	2.05	1.64	107
26. EL soft Stim_2	9.2	1.9	2.03	1.71	86.2
27. EL soft Stim_3	9.2	2.3	2.01	1.54	56.5
28. EL stiff Stim_1	9.1	2.0	2.05	1.22	47.1
29. EL stiff Stim_2	9.0	2.0	2.04	1.96	55.0
30. EL stiff Stim_3	9.0	1.8	2.09	0.58	40.0

Supplementary Table 3. Primer pairs employed for the reference gene study. Primer pairs amplified all transcription variants with equal amplicon length. E: efficiency of qPCR reaction. R^2: coefficient of determination from linear regression of Cq values (cDNA serial dilution).

Gene	Accession number (mRNA)	Primer sequences	Amplicon size (bp)	Amplification factor (E)	R^2
RPS13	NM_001017.2	**Fwd** CGCTCTCCTTTCGTTGCCT **Rv** CGCTGCGTCGATAGGGTAAA	96	1.97	0.9975
RPL27	NM_000988.3	**Fwd** ATCGCCAAGAGATCAAAGATAA **Rv** TCTGAAGACATCCTTATTGACG	123	1.97	0.999
RPL30	NM_000989.3	**Fwd** ACAGCATGCGGAAAATACTAC **Rv** AAAGGAAAATTTTGCAGGTTT	158	1.95	0.9977
OAZ1	NM_004152.3 NM_001301020.1	**Fwd** CTCCACTGCTGTAGTAACCCG Rv GATCCCTCTGACTATTCCCTCG	104	1.97	0.9993
ACTB	NM_001101.3	**Fwd** AGCACAGAGCCTCGCCTTT **Rv** GAGCGCGGCGATATCATCA	82	1.97	0.9993
GAPDH	NM_001289746.1 NM_001289745.1 NM_001256799.2 NM_002046.5	**Fwd** CCACATGGCCTCCAAGGAGTAAGAC **Rv** AGGAGGGGAGATTCAGTGTGGTGGG	131	1.96	0.9998
MAPK1	NM_002745.4 NM_138957.3	**Fwd** TCCCAAATGCTGACTCCAAAG **Rv** CATGTCGAACTTGAATGGTGC	164	1.98	0.9994
UBC	NM_021009.6	**Fwd** GCCTTAGAACCCCAGTATCAG **Rv** AAGAAAACCAGTGCCCTAGAG	74	2.05	0.9999
HMBS	NM_000190.3 NM_001024382.1 NM_001258208.1 NM_001258209.1 XM_017017629.1 XM_005271531.1 XM_005271532.1 XM_005271533.3 XM_011542796.1	**Fwd** AGCTTGCTCGCATACAGACG **Rv** AGCTCCTTGGTAAACAGGCTT	157	1.93	0.9973
TBP	NM_003194.4 NM_001172085.1	**Fwd** CCACTCACAGACTCTCACAAC **Rv** CTGCGGTACAATCCCAGAACT	127	1.94	0,9987

Supplementary Table 4. Cq values determined by Bestkeeper algorithm. Geo: geometric. SD: standard deviation. CV: coefficient of variance.

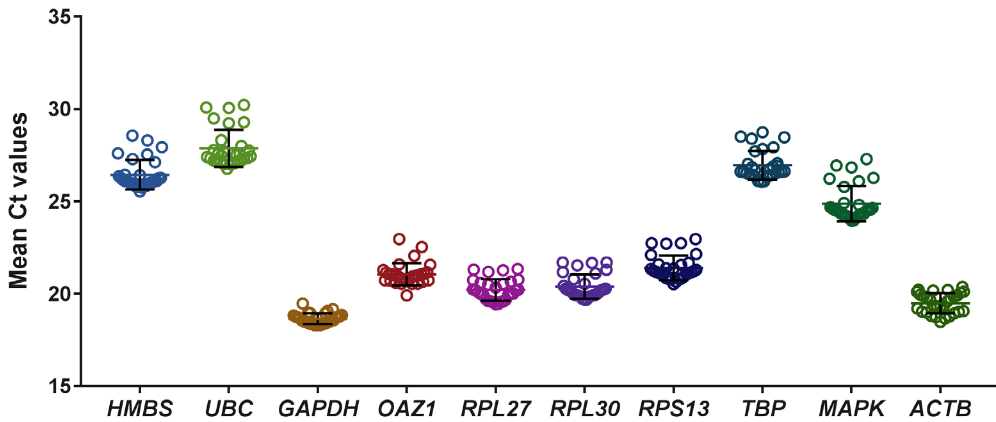
Gene	Geo. Mean [Cq]	SD [± Cq]	CV [% Cq]
GAPDH	18.64	0.24	1.30
OAZ1	21.05	0.40	1.88
TBP	26.94	0.61	2.26
RPL27	20.20	0.46	2.30
HMBS	26.44	0.62	2.33
RPS13	21.39	0.51	2.36
ACTB	19.48	0.49	2.49
RPL30	20.38	0.53	2.61
UBC	27.86	0.78	2.79
MAPK1	24.87	0.75	3.03

Supplementary Table 5. Stability values of reference genes determined by NormFinder algorithm.

Gene	Stability value
RPS13 + TBP	0.116
RPL30	0.175
HMBS	0.179
RPS13	0.182
OAZ1	0.200
TBP	0.226
GAPDH	0.248
RPL27	0.269
MAPK1	0.271
ACTB	0.350

Supplementary Table 6. Stability values of reference genes determined by geNorm.

Gene	Stability value (M)
RPS13 + RPL30	0.268
RPS13	0.267
RPL30	0.271
HMBS	0.296
MAPK1	0.3
TBP	0.391
RPL27	0.433
GAPDH	0.471
OAZ1	0.497
UBC	0.541
ACTB	0.6



Supplementary Fig. 1. Expression levels of candidate reference genes.

Discussion

Mesenchymal stromal cells (MSCs) present characteristics of great value for cell-based therapies. In addition to their low immunogenicity and their capacity for trilineage mesenchymal differentiation, MSCs are potent regulators of immune and inflammatory responses. Indeed, MSCs have been demonstrated to regulate both innate and adaptive immunity by modulating basic functions such as activation, maturation, proliferation or cytotoxic activity of multiple immune cells [1] including T, B and natural killer (NK) lymphocytes [2-4], dendritic cells [5], monocytes and macrophages [6,7]. Despite MSC-mediated immunomodulation is the result of a complex combination of different mechanisms, the paracrine effects of bioactive factors they release are considered to play a major role [8,9]. The MSC secretome includes a plethora of bioactive molecules such as interleukins, metabolic enzymes, growth factors and chemokines, which are applicable in multiple disorders. In particular, MSCs are able to directly release such soluble factors, or to transfer them by means of extracellular vesicles (Evs) (Fig. 1).

Cell encapsulation in three-dimensional (3D) hydrogel systems represents a valuable alternative for MSC administration. This strategy overcomes the important limitations of nude cell injection, such as the rapid clearance and low retention [10], enabling the sustained release of bioactive factors produced *de novo* by MSCs. Moreover, the possibility to tune multiple aspects of the hydrogel matrix makes cell encapsulation a versatile and promising strategy to enhance cell viability and functionality. However, the field of cell encapsulation still faces several challenges that hinder the progress of the technology towards clinical translation.

The immobilization of cells within alginate-poly-L-lysine-alginate (APA) microcapsules has been widely explored as a strategy to protect the implant from host immune response and therefore, enable a continuous release of therapeutic factors produced by the cells [11]. However, considering the performance of the system is directly dependent on cell responses, the impossibility to control encapsulated cell behavior importantly limits the implant functionality and safety [12,13]. Among the erratic cell responses, an excessive proliferation rate represents one of the major hurdles, since it results in the formation of giant cell-aggregates. The limited diffusion of nutrients and oxygen to the core of the aggregates causes inner cell death [14] and leads to the secretion of alarm molecules that evoke important immune and inflammatory responses [15]. Further, protruding cells can leak out of the matrix, which represents a major safety concern [16].

Moreover, to maintain a robust cell viability and functionality it is fundamental to develop 3D matrices that mimic the mechanical and biological properties of the natural extracellular matrix (ECM). However, the recreation of such a complex microenvironment remains a challenge since it is necessary to simultaneously imitate multiple scenarios that impact cell survival and immunomodulatory capacity. Some of these scenarios have been widely explored in isolation. Such is the case of matrix biofunctionalization, which consists of the inclusion of peptides or proteins naturally present in the ECM. Among other mechanisms, cells are able

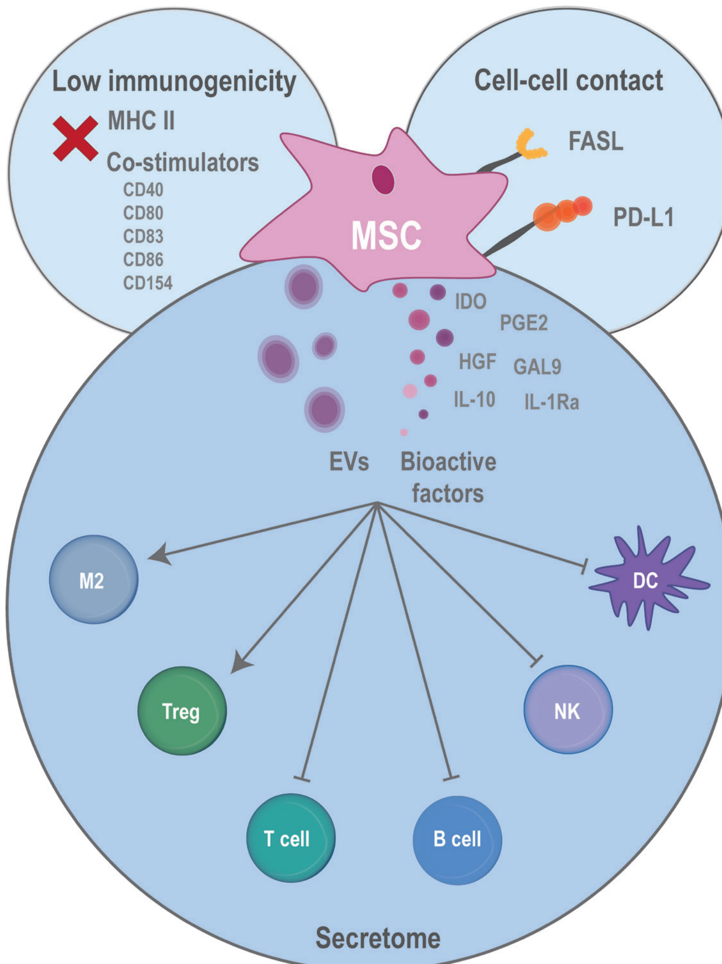


Fig.1 Immunomodulatory properties of MSCs. MSCs present low immunogenicity and mediate their immunosuppressive effects by ligand-receptor cell-cell interactions and the secretion of soluble bioactive factors or extracellular vesicles (EVs). MSCs: mesenchymal stromal cells.

to recognize these cues through transmembrane receptors known as integrins. Thus, the presence of these motifs enables cells to interact with otherwise inert biomaterials, triggering a series of intracellular pathways that contribute to a correct cell viability. Despite multiple studies highlight the potential of matrices with mechano-adhesive properties, the affinity of each integrin class for the ECM cues is different. Hence, for the design of effective functionalization strategies, it is necessary to determine the integrin classes that direct adhesion in the specific cell employed. Currently, it is possible to study the integrin expression via complex techniques such as flow cytometry, however, simple methods that evaluate not only the expression, but also the contribution of integrins to the whole adhesion process are still required.

Another of these scenarios is the local cytokine milieu, which has been demonstrated to directly influence the immunomodulatory capacity of MSCs. In particular, pre-stimulation of MSCs with inflammatory signals such as interferon γ (IFN-γ) or tumor necrosis factor α (TNF-α) is known to promote the immunosuppressive phenotype of the cells, enhancing the secretion of regulatory bioactive factors. However, this cell preconditioning approach presents a transient effect, importantly limiting its use in applications requiring a long-term modulatory effect [17-19]. The modulatory capacity of MSCs is often assessed by analyzing the expression of immunoregulatory genes such as indoleamine 2,3-dioxygenase 1 (*IDO1*) by real-time RT-qPCR. In that regard, it is important to note that reference gene validation upon specific experimental conditions is key to obtain accurate data and avoid flawed conclusions. Therefore, considering that nowadays multiple strategies are being explored to boost the immunomodulatory potential of MSCs, there is a necessity to evaluate their effect on broadly used reference genes.

Considering all the above mentioned, we hypothesized that the development of a biomimetic multifunctional system consisting of a hydrogel incorporating APA-microencapsulated MSCs, an adequate biofunctionalization strategy and the continuous presence of IFN-γ, would represent an integrated solution to promote the biosafety of the implant, by protecting the graft from the host immune response and controlling cell behavior, as well as to enhance the viability and immunomodulatory properties of encapsulated MSCs.

However, for the development of such a complex approach, a previous validation of the effectivity of each individual component is fundamental. Therefore, the goal of the present doctoral thesis was to optimize each one of the three main aspects in the multifunctional system: the APA microcapsules, the matrix biofunctionalization and the strategy to boost the immunomodulatory properties of MSCs based on the sustained exposure to of IFN-γ and the 3D cell culture itself (Fig. 2).

In a first step, we aimed at optimizing APA microcapsules to regulate uncontrolled cell responses. The process of cell microencapsulation requires the dissolution of the employed biomaterials. Therefore, it is important that such solutions present physiological osmolarity values so that they meet the standards of cell culture [20]. With that purpose, osmolarity adjusting agents are incorporated in the solutions, which can be classified as electrolyte or inert agents. Since APA microcapsule formation occurs by electrostatic interactions, this process is directly influenced by the presence of electrolytes, especially divalent cations, in the surrounding media [21,22]. Therefore, we hypothesized that the choice of different types of osmolarity adjusting agents may interfere in hydrogel formation and hence, in the mechanical properties of the matrix, which have been previously reported to influence cell responses [23].

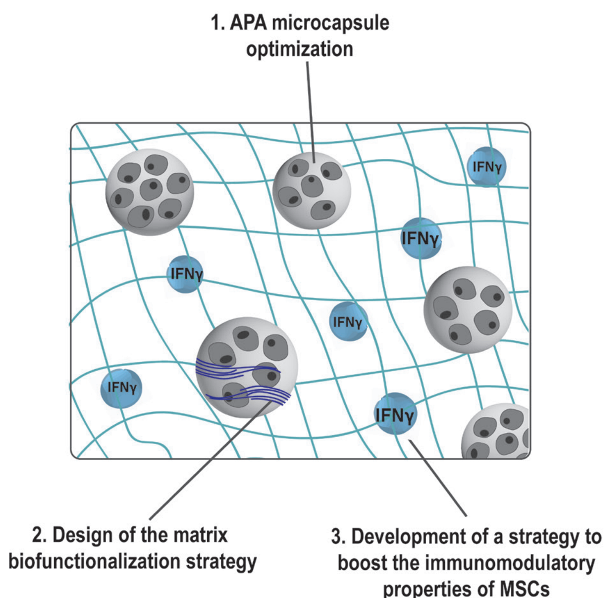


Fig. 2. Schematic representation of the objectives of the present doctoral thesis. APA: alginate-poly-L-lysine-alginate. MSCs: mesenchymal stromal cells.

To evaluate such effect, we designed two sets of solutions, each one including all the solutions required for the elaboration of microcapsules (1.5% alginate, 0.05% poly-L-lysine (PLL), 0.1% alginate and washing solutions). For the Biological set of solutions, electrolytes such as sodium, potassium, calcium or phosphates were chosen, whereas, mannitol was included as an inert agent in the Technological set of solutions. The resulting sets were used to encapsulate D1 MSCs, which were previously genetically modified with the lentiviral vector pSIN-EF2-Epo-Pur to produce erythropoietin (D1-MSCs-EPO) [24], obtaining Biological or Technological microcapsules. This cell line was selected because of its proliferative capacity [24,25], which makes it a suitable model for studying the control that the mechanical configuration of the matrix exerts on cell behavior.

In vitro analyses determined important differences between the two groups. The formation of giant cell-aggregates in Technological microcapsules demonstrated a significantly higher proliferation, which was confirmed by a BrdU uptake assay, an analogous of the DNA precursor thymidine (Fig. 3). These results could explain the so different increase-tendencies that cells repeatedly followed for viability, metabolism and erythropoietin (EPO) secretion studies: linear for Biological and exponential for Technological microcapsules. Interestingly, a dramatic decrease of proliferation was observed in the Technological group by day 45, probably caused by a decrease in viability, as the enormous aggregates can collapse the pores of the system [26] and limit oxygen and nutrient diffusion to the inner core.

To evaluate if these differences were originated by distinct mechanical configurations, structural studies were carried out. The binding between alginate and the crosslinker, in this case calcium, is responsible for conferring stiffness to the matrix. Therefore, microcapsules were treated with alginate lyase to cause their rupture and release the calcium forming the matrix, which was quantified by a colorimetric calcium detection kit. Higher calcium levels revealed a higher crosslinking degree in Biological microcapsules, suggesting a stiffer, less permissive matrix. According to these results, during the coating process, the calcium loss was lower when beads were put in contact with solutions that presented it, probably due to the gradient stabilization that could have prevented the leakage. Consequently, Biological microcapsules retained more calcium, forming a more restrictive matrix that established a control in cell division [27,28]. On the contrary, the Technological group presented a higher leakage of the

ion, resulting in a more permissive matrix [29,30]. This property allowed an aggressive proliferation, leading to the formation of huge cell-aggregates. Hence, it was not the direct effect of osmolarity adjusting agents on cells which evoked so contrasting behavior, but the effect this agents have in the capsule formation process and thus, in the final crosslinking degree of the alginate matrix. Interestingly, Technological microcapsules presented a higher osmotic resistance, indicating an enhanced interaction between the alginate core and the PLL coating. This was likely because the lower core crosslinking left available more alginate chains to interact with the PLL, and additionally, the absence of electrolytes in the coating process may facilitate the binding between both elements. Regarding permeability, no statistical differences were observed in the diffusion of fluorescein isothiocyanate (FITC)-dextrans of distinct molecular weights, with both groups maintaining an adequate molecular weight cut-off (MWCO) to fulfill the objectives of the technology.

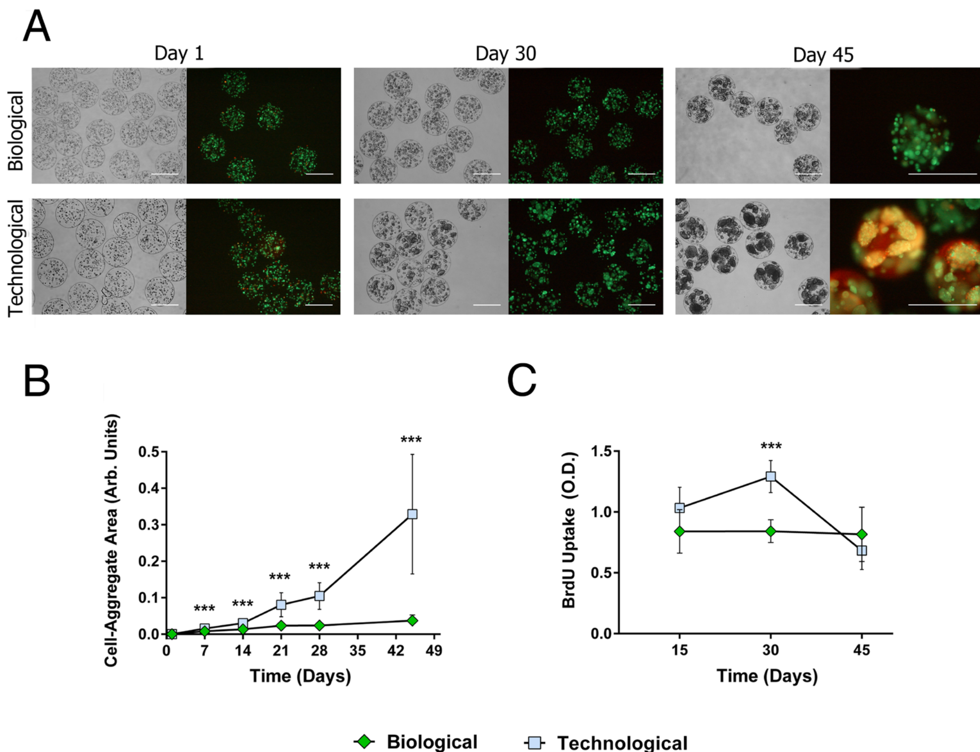


Fig. 3. APA microcapsules formulated with different types of osmolarity adjusting agents led to contrasting cell behavior *in vitro*. (A) Brightfield and LIVE/DEAD fluorescence micrographs taken at different time points after cell encapsulation revealed diverging cell behavior in each group (scale bars = 400 μm). **(B)** Subsequent quantification of cell-aggregate areas demonstrated statistically significant differences. **(C)** Bromodeoxyuridine (BrdU) uptake studies confirmed these data by showing an important increase in proliferation in the Technological group by day 30. Line graphs symbolize mean ± SD. Statistical significance: ***$p < 0.001$.

Subsequently, we analyzed in depth the effect of the matrix crosslinking degree in cell division. D1-MSCs-EPO were incubated with BrdU for 48 h, which incorporates into newly synthesized DNA, and subsequently stained with 7-aminoactinomycin D (7-AAD), which binds to the total DNA and resolves cell cycle phases in our populations: G0/1 (resting phase) or S/G2/M (DNA synthesis and division). When analyzing samples by flow cytometry, we observed that the proliferation rate was remarkably higher in Technological microcapsules by day 30. Despite the majority of Biological (55.3%) and Technological cells (57.9%) presented a G0/1 state, interesting proliferative differences were detected in the remaining cells. In particular, a significant number of cells in the Biological group were not labeled with BrDU indicating that they remained the 48 h after BrdU addition in the same S/G2/M state they were before inclusion of the thymidine analog. Contrarily, cells in the Technological group were able to duplicate their genetic material and complete the cycle, returning to G0/1 phase with the BrdU label incorporated in the DNA. These results indicate that the Technological softer matrices allowed a significantly faster cell division [26].

We next performed *in vivo* studies to determine if the obtained data remained significant from a therapeutic standpoint (Fig. 4). Microcapsules were subcutaneously aloimplanted in C57BL/6 immunocompetent mice and at different time points, blood samples were collected by facial vein puncture to determine hematocrit levels. D1-MSCs-EPO encapsulated in Biological microcapsules were able to progressively increase the hematocrit profiles for every mouse in the group. On the contrary, by day 30, two of the Technological implants had already failed to increase the hematocrit levels, and the remaining showed important data dispersion. Moreover, when morphologically analyzing the explants after 45 days of study, in the Technological group important cell-aggregates were observed and the majority of capsules were broken, allowing the cell content to be released to the surrounding tissue. That was probably originated by the enormous aggregates whose aggressive growth triggered an increment in pressure that was not tolerated by the membranes of the system. Conversely, Biological microcapsules remained spherical and maintained their integrity. Histological analyses supported these results by showing a significantly higher capsule rupture in the Technological group, which resulted in tissue invasion and tumor-like mass formation, as well as to a severe inflammatory response.

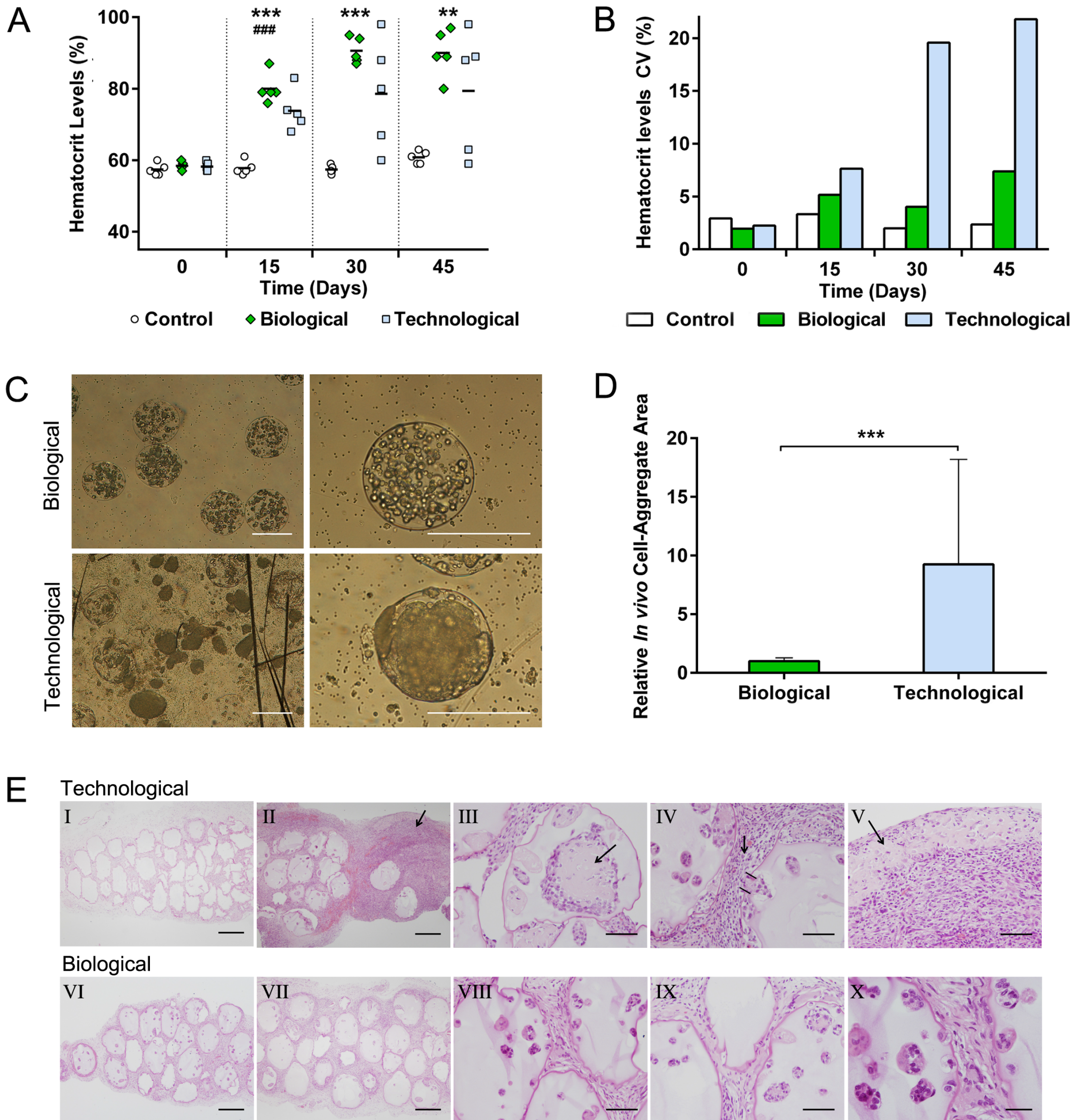


Fig. 4. *In vivo* studies exhibited divergent therapeutic profiles. (A-B) Hematocrit levels progressively increased in every mouse with Biological implants, whereas erratic profiles and big data dispersion were observed in mice with Technological implants. For the control group sole DPBS was administered. Statistical significance: between Control and Biological groups: **p < 0.01 and ***p < 0.001. Between Control and Technological groups: ### p < 0.001 **(C)** Morphological characterization after graft explantation revealed enormous cell-aggregates and broken capsules in the Technological group. Scale bars = 400 µm. **(D)** Cell-aggregate area quantification proved statistically significant differences between the groups ***p < 0.001. **(E)** Histological studies showed empty microcapsules for the Technological implants that failed (I). The analyses also confirmed that Technological implants presented an intense inflammatory response (II), cell-aggregates causing microtumors (III), capsule rupture (IV) and the consequent extra-capsular tumor formation (V). Such behavior was not observed in Biological implants, where a lower inflammation (VI-VII) and capsule integrity with no cell extravasation (VIII-X) were observed. Scale bars I,II, VI, VII = 400 µm. III, IV, V, VIII, IX = 100 µm. X = 50 µm. Graphs show mean ± SD. (n = 5 for animal studies, n = 30 for diameter quantification).

Finally, a microarray study determined that gene expression was considerably influenced by the mechanical characteristics of the matrix in which cells were encapsulated (Fig. 5). Important proliferative pathways were significantly activated in cells enclosed within Technological microcapsules, such as the PI3K/Akt/mTor route or the Ras/Raf/MAPK pathway. Interestingly, it has been reported that when these two pathways are mutated or amplified, proliferation and survival signals are constitutively activated and, ultimately, lead to tumorigenesis [31]. Supporting these results, genes involved in the reorganization of the cytoskeleton were upregulated, while tumor suppressor genes were down-regulated, indicating D1-MSCs-EPO enclosed in Technological microcapsules found less restriction to develop a tumor-like behavior. Therefore, our research is in agreement with literature pointing out to a mechanosensing process in the absence of integrin binding domains [16,32]. Further research should focus on the mechanism by which it occurs in order to gain knowledge over the factors involved in cell-microenvironment interactions.

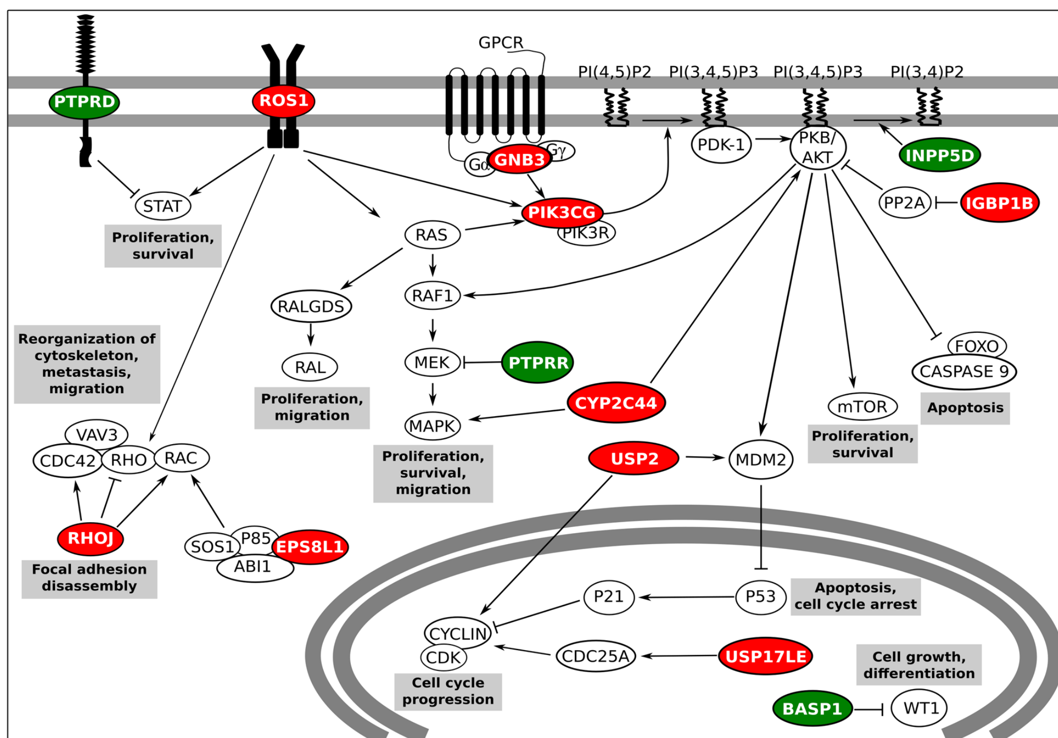


Fig. 5. Gene expression studies proved that the behavioral differences were caused at a genic level. Schematic representation of signaling pathways in which gene expression presented a fold change > 3 or < -3 in cells within Technological microcapsules, in comparison to the Biological group. Red color indicates up-regulated genes and green color down-regulated genes.

Overall, we proved the employment of osmolarity adjusting agents as a useful tool to modify the mechanical configuration of the matrix, with no need of altering the biomaterial or cross-linker type or proportion. Such mechanical properties influenced gene expression and resulted in a contrasting cell behavior both, *in vitro* and *in vivo*. The matrix resulting from the use of Biological solutions restricted cell proliferation, avoiding the enormous cell-aggregate formation, the risk of cell protrusion and the intense inflammatory response. Together, our data demonstrated that, by only tuning the osmolarity adjusting agents, we were able to control D1-MSCs-EPO behavior and thus improve the safety of the therapy and obtain a predictable and sustained *de novo* release of therapeutics.

Having explored how to control erratic cell responses in APA microcapsules, we next focused on the second element to optimize: the biofunctionalization strategy to promote cell viability. In particular, in this second step, our aim was to develop a biosensor that provides essential information to design adequate biofunctionalization strategies considering the particular substrate and cell type being studied. Thus, we developed a sensitive fibronectin (FN) dot array biosensor, with single cell resolution, to explore the integrin profile and characterize the cell-biomaterial dynamic interactions.

To fabricate the sensor, micro-contact printing was adapted to print FN dot arrays on tissue culture plate (TCP) wells. In particular, a patterned stamp master was obtained by photolithography and used to fabricate PDMS stamps presenting circular pillars of 20 µm. The stamps were incubated with a PBS solution containing FN and TAMRA-labeled bovine serum albumin (BSA) to obtain a positive control for protein printing. Subsequently, the inked PDMS was stamped on TCP wells, resulting in a homogeneous printing of 20 µm FN dot arrays, which provided single cell adhesion for the cells employed in the study (Fig. 6). In this regard, to demonstrate the real applicability of the platform, cells with differing characteristics were tested in the biosensor. In particular, cell lines including baby hamster kidney fibroblasts (BHK) and C_2C_{12} mouse myoblasts (C_2C_{12}), and primary cells such as adipose-derived human mesenchymal stromal cells (hMSCa) and human dermal fibroblasts (hDF) were employed.

We first studied the adhesion kinetics by incubating the cells in a FN dot array substrate for two hours and determining the dot array occupancy (DAO) at different time points. For every

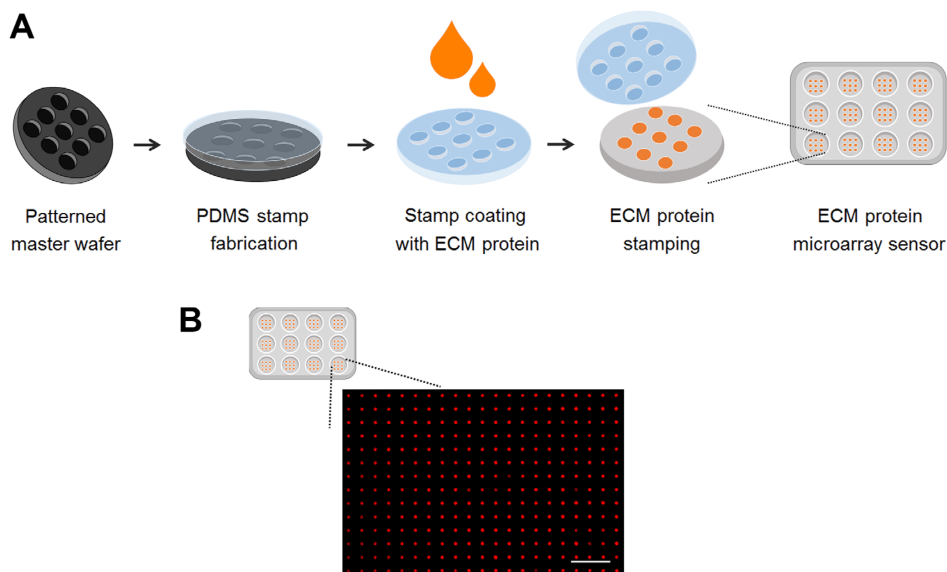


Fig. 6. Protein microarray biosensor development. (A) Scheme of the fabrication of the ECM protein microarray platform. **(B)** Homogeneous distribution of the fluorescently labeled fibronectin-BSA-TAMRA pattern (scale bar = 200 μm). PDMS: polydimethylsiloxane. ECM: extracellular matrix.

cell, we observed how the DAO increased over time until reaching a plateau. The adhesion curves were fitted to a first order kinetics, providing values for the rate constant (k) and the t_{50}, defined as the time point at which the 50% of the dot arrays are occupied. With these parameters, we were able to identify two different kinetics: presenting significantly higher k and lower t_{50} values, C_2C_{12} and hMSCa adhered significantly faster to the substrate than BHK and hDF, indicating that the cell type was the major determinant. Since cell-matrix interactions are mainly mediated by integrins [33], we hypothesized that the differences observed in the adhesion kinetics may be given by a distinct integrin expression profile in each cell type.

To prove such hypothesis, we next studied the integrin contribution to cell attachment (Fig. 7) focused on hMSCa and hDF, for presenting contrasting kinetics and for being the most attractive cells from a therapeutic standpoint [34-36]. More importantly, considering the multifunctional system showed in Fig. 2, these cells were specially interesting, since we wanted to explore in depth the biofunctinalization requirements for MSCs as our therapeutic target; and also for hDF, since in the event that the system was subcutaneously implanted and the outer hydrogel was degradable, it would be interesting to enhance the invasion of host fibroblasts

to restore the natural tissue. We first incubated the cells with anti-integrin antibodies, specifically blocking either the $\alpha_v\beta_3$ integrin, or all the integrins presenting the β_1 subunit. These integrins were selected for being primary FN binding receptors in both cell types [37,38]. Subsequently, cells were seeded in the FN dot array platform and the DAO was quantified. For both, hMSCa and hDF, DAO was not significantly affected when blocking the $\alpha_v\beta_3$ integrin, but it was when blocking all the integrins presenting the β_1 subunit. Interestingly, when normalizing the data to directly compare the two cell types, a significantly greater impact of β_1 blocking was observed in hDF. In particular, when blocking β_1 subunit presenting integrins, around a 40% of the hMSCa were able to attach to the substrate, but only around a 2% of hDF. These results suggest a different integrin profile, indicating that whereas in hDF β_1 integrin expression is major, in hMSCa, their influence is balanced up to a certain point by other integrins, such as α_v-class integrins. Moreover, this assay proved the sensitivity of the platform, which was able to accurately detect differences in DAO when blocking a single integrin, ($\alpha_v\beta_3$) *versus* blocking the β_1 domain, present in multiple integrins [39].

Further, the biosensor was able to determine the contribution of each integrin class to cell adhesion consolidation. Prior to using our platform, we confirmed that the commonly used immunocytochemistry failed to determine the actual contribution of each integrin to the achievement of a functional adhesion and did not discern between blocking the $\alpha_v\beta_3$ integrin or all the β_1 integrins. Contrarily, a detachment assay performed in our biosensor provided a deeper insight into cell-substrate interaction. After blocking $\alpha_v\beta_3$ or β_1-presenting integrins, cells were incubated in the FN dot array platform and the DAO was quantified. Subsequently, a detachment force was applied by liquid aspiration and the DAO was quantified again. Despite we previously detected that $\alpha_v\beta_3$ blocking did not alter the DAO, this assay indicated that adhered cells in this group presented less resistance to detachment forces than the control group, for both cell types. The crosstalk that exists between α_v and β_1 integrins may explain this phenomenon, since it has been reported that once engaged, α_v induce the clustering of integrins such as $\alpha_5\beta_1$, strengthening cell adhesion [40]. Consequently, blocking $\alpha_v\beta_3$ integrins would impair the crosstalk, weakening the cell-substrate interaction. Additionally, β_1 blocking also debilitated the cell binding, because of the absence of β_1-class integrin clusters which are responsible for providing a stronger attachment. As above mentioned, both cell types showed this behavior. However, for hDF, the β_1 blocking prevented cell

adhesion strengthening more importantly than $\alpha_v\beta_3$ blocking, which did not occur in hMSCa. This observation indicates a greater impact of β_1 integrins in hDF in comparison to hMSCa, confirming the results previously obtained for cell adhesion and in agreement with the literature highlighting the vital role of $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins in hDF [37].

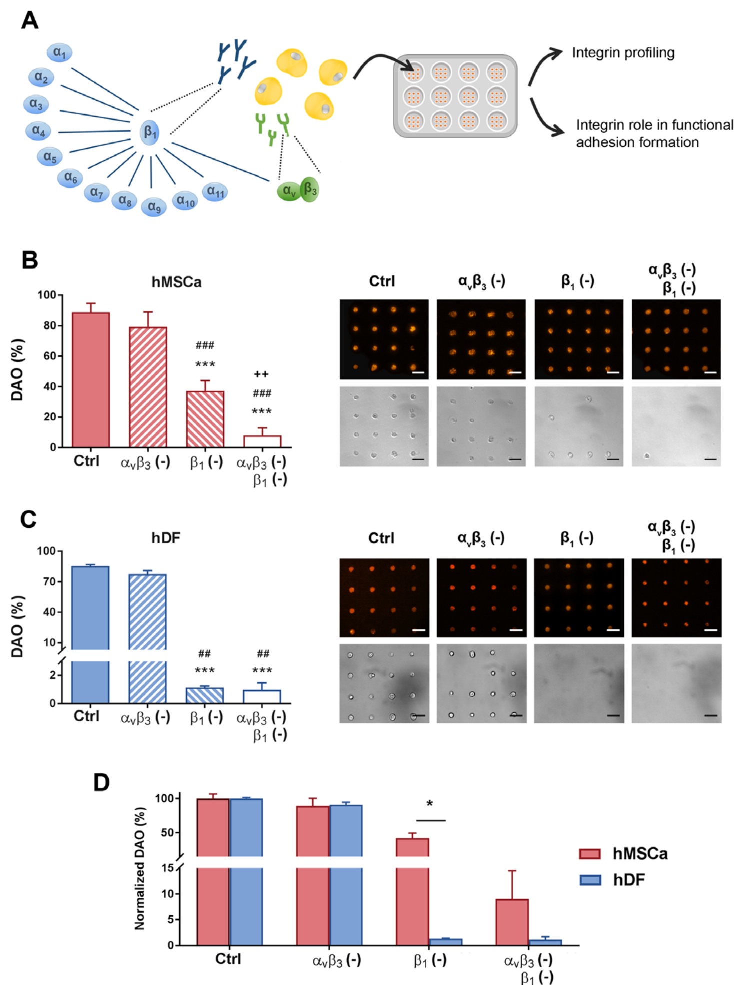


Fig. 7. hMSCa and hDF presented different integrin expression balance. (A) Experimental procedure followed for analyzing integrin profile and integrin contribution to adhesion strengthening. Influence of blocking $\alpha_v\beta_3$ and/or β_1-subunit presenting integrins on cell adhesion for **(B)** hMSCa and **(C)** hDF. Error bars mean ± SD (n = 4 samples per experimental condition). Statistical significance: one way ANOVA with Bonferroni multiple comparisons test for hMSCa, one-way ANOVA with Tamhane multiple comparisons test for hDF: ***p < 0.001 compared to Ctrl. ##p < 0.01 and ###p < 0.001 compared to $\alpha_v\beta_3$ (-). ++p < 0.01 compared to β_1 (-). Scale bars = 50μm. **(D)** Cell adhesion normalization and comparison between hMSCa and hDF. Error bars mean ± SD (n=4 samples per experimental condition). Statistical significance: paired, two-tailed t-test: *p < 0.05. DAO: dot array occupancy. hMSCa: adipose-derived human mesenchymal stromal cells. hDF: human dermal fibroblasts. Ctrl: control.

Finally, to validate our platform, $\alpha_v\beta_3$ and β_1 expression was determined by flow cytometry, which showed that hDF cells presented a lower $\alpha_v\beta_3$ and a higher β_1 expression than hMSCa, supporting the three main conclusions provided by our method. (1) Considering that α_v-class integrins initiate cell attachment by binding FN quicker than β_1-presenting integrins such as $\alpha_5\beta_1$ [37], the faster kinetics suggested a higher density of α_v-class integrins in C_2C_{12} and hMSCa than in BHK and hDF. (2) hMSCa and hDF presented a different integrin expression profile, being major the role of β_1-presenting integrins in hDF, and important but not unique in hMSCa. (3) $\alpha_v\beta_3$ and β_1 contributed similarly to hMSCa adhesion strengthening, despite the role of β_1 integrins was greater in hDF.

Integrin expression may be assessed by a complex technique such as flow cytometry, however, it does not provide information regarding their actual contribution to the whole adhesion process. More importantly, in such technique, cells are studied in suspension, a scenario in which intracellular pathways dependent on cell adhesion cannot be recreated. Our biosensor overcomes these issues by offering the possibility to study attached cells in an environment that mimics their natural niche and predicts both, integrin expression profile and their role in functional adhesion formation via integrin crosstalk and clustering. Thus, the platform provides crucial information directly applicable to the field of biomimetic material science.

In this vein, it is important to note that the particular contribution of integrins in a specific cell will dictate the adequate biofunctionalization strategy [41]. An example that illustrates this fact is the tri-peptide RGD, recognized as the essential cell adhesion site in FN and other natural components of the ECM [11,42]. Although multiple integrins bind RGD, the affinity for this motif varies among the different integrin classes. For instance, while both $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins bind RGD, the latter also requires the PHSRN synergy site, present in natural FN in close proximity to RGD, to establish cell adhesion [37,43,44]. Similarly, $\alpha_4\beta_1$ integrins also bind to the PRARI synergistic sequence [44]. Thus, our data suggests that despite the inclusion of synergy motifs such as PHSRN or PRARI may be beneficial in hMSCa, the presence of the sole RGD may promote an important cell viability. Contrarily, the accompaniment of RGD with the synergistic peptides in hDF is essential, considering the smaller contribution of $\alpha_v\beta_3$ and the potent effects of β_1.

With regard to the multifunctional hydrogel system showed in Fig. 2, the data obtained with our biosensor indicates that biofunctionalizing MSC-containing APA microcapsules with RGD would be an efficient approach. However, the outer hydrogel biofunctionalization may be different. For instance, in the event that the system was subcutaneously implanted and such outer hydrogel was degradable to enhance the invasion and restoration of the tissue by host cells like fibroblasts, the inclusion of whole proteins such as fibronectin or collagen would be a more adequate option.

Overall, the sensor enables to study in depth the affinity of cells for different substrates, it is not limited to FN. Moreover, by selecting the most suitable anti-integrin antibodies the platform allows the study of any receptor class in multiple cell types in parallel. The potential of this biosensor lies on its high sensitivity, accuracy, simplicity and low cost and will be of interest for the whole community of researchers interested in the design of biomimetic materials.

Having explored how to control MSC behavior in APA microcapsules and studied cell integrin balance to design adequate biofunctionalization strategies, we next focused on the third element: the strategy to enhance and prolong the immunomodulatory properties of MSCs. In particular, we explored the effectivity of a multifunctional system combining the effects of 3D biomimetic cell culture and sustained inflammatory licensing. To assess MSC-mediated immunomodulation, we studied the expression of a broad panel of immunomodulatory genes that encode for pivotal regulatory factors acting at different levels of the immunosuppressive response by real-time RT-qPCR.

Preliminarily, we evaluated the effect of inflammatory licensing on primary human MSCs (hMSCs) by exposing them to IFN-γ and TNF-α overnight and subsequently maintaining them in 2D culture on tissue culture plates (TCP). Gene expression of indoleamine 2,3-dioxygenase 1 (*IDO1*), prostaglandin-endoperoxide synthase 2 / Cyclooxygenase 2 (*PTGS2*) and galectin-9 (*GAL9*) was found to be importantly upregulated upon inflammatory licensing. These results are in agreement with previous studies that showed the combination of these two cytokines polarized hMSCs to an immunosuppressive phenotype [19], inducing hMSCs to secrete regulatory enzymes and soluble factors such as IDO or cyclooxygenase 2 (COX2) [45]. However, here, the effect was demonstrated to be transient, since after 3 days of 2D culture,

their expression significantly decreased. Considering the expression of the other genes in our panel was not affected by cytokine simulation, we divided them into two groups: genes responsive to inflammatory licensing (*IDO1*, *GAL9*, *PTGS2*) and not responsive (hepatocyte growth factor (*HGF*), interleukin-10 (*IL10*) and interleukin-1 receptor antagonist (*IL1RN*).

As above-mentioned, we hypothesized that a 3D biomimetic hydrogel would maintain expression of these immunomodulatory genes. To test this hypothesis, hMSCs were encapsulated in a range of artificial extracellular matrix (aECM) hydrogels after overnight licensing (Fig. 8). The matrix of aECM hydrogels consists of an interpenetrating network of alginate and fibrillar collagen type I, which is intended to mimic the architecture of the native extracellular matrix (ECM) [46]. Four types of aECM hydrogels were tested: soft viscoelastic, stiff viscoelastic, soft elastic and stiff elastic. Elasticity and stiffness were independently tuned by varying the mode and magnitude of alginate crosslinking. Viscoelastic alginate hydrogels exhibit rapid stress-relaxation behavior from the reversible ionic crosslinks at blocks of guluronic acid-rich regions (G-blocks). The addition of permanent covalent crosslinking makes the network more elastic, and this is accomplished with the incorporation of norborene (Nb) and tetrazine (Tz) that undergo bio-orthogonal inverse electron demand Diels-Alder reactions to “click” together the existing G-block ionic crosslinks [47,48]. After 3 days, the hydrogel matrix significantly promoted hMSC immunomodulatory potential, compared to 2D. Upregulation was observed in the whole panel of immunomodulatory genes, regardless of whether a specific gene was influenced or not by exposure to inflammatory cytokines (Fig. 9). This indicated an important

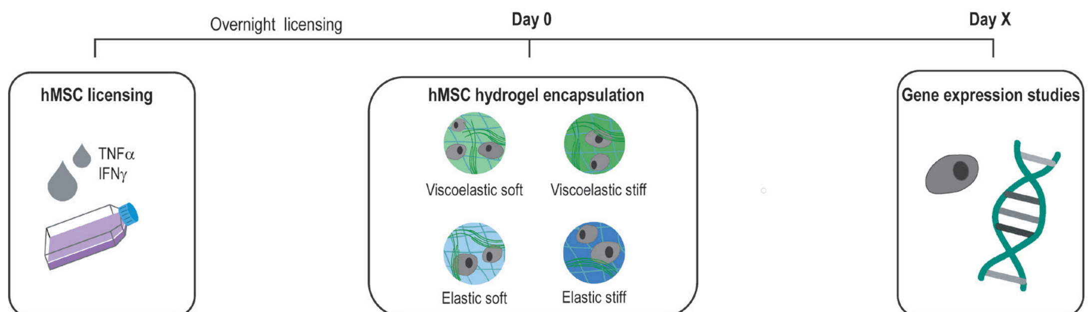


Fig. 8. Experimental procedure followed in the development of a strategy to boost the immunomodulatory potential of MSCs. After hMSC overnight preconditioning with interferon γ (IFN-γ) and tumor necrosis factor α (TNF-α), cells were detached and 3D hydrogel encapsulated. At different time-points, RNA was isolated from the cells for the subsequent RT-qPCR analysis. hMSCs: human mesenchymal stromal cells.

effect of the 3D biomimetic culture by itself, independent of licensing. However, by day 7 the expression of licensing responsive genes significantly decreased, whereas it was maintained for genes not regulated by IFN-γ / TNFα. These results suggested that the effect of the matrix persisted, while the licensing effect decreased by day 7 because of its transient nature.

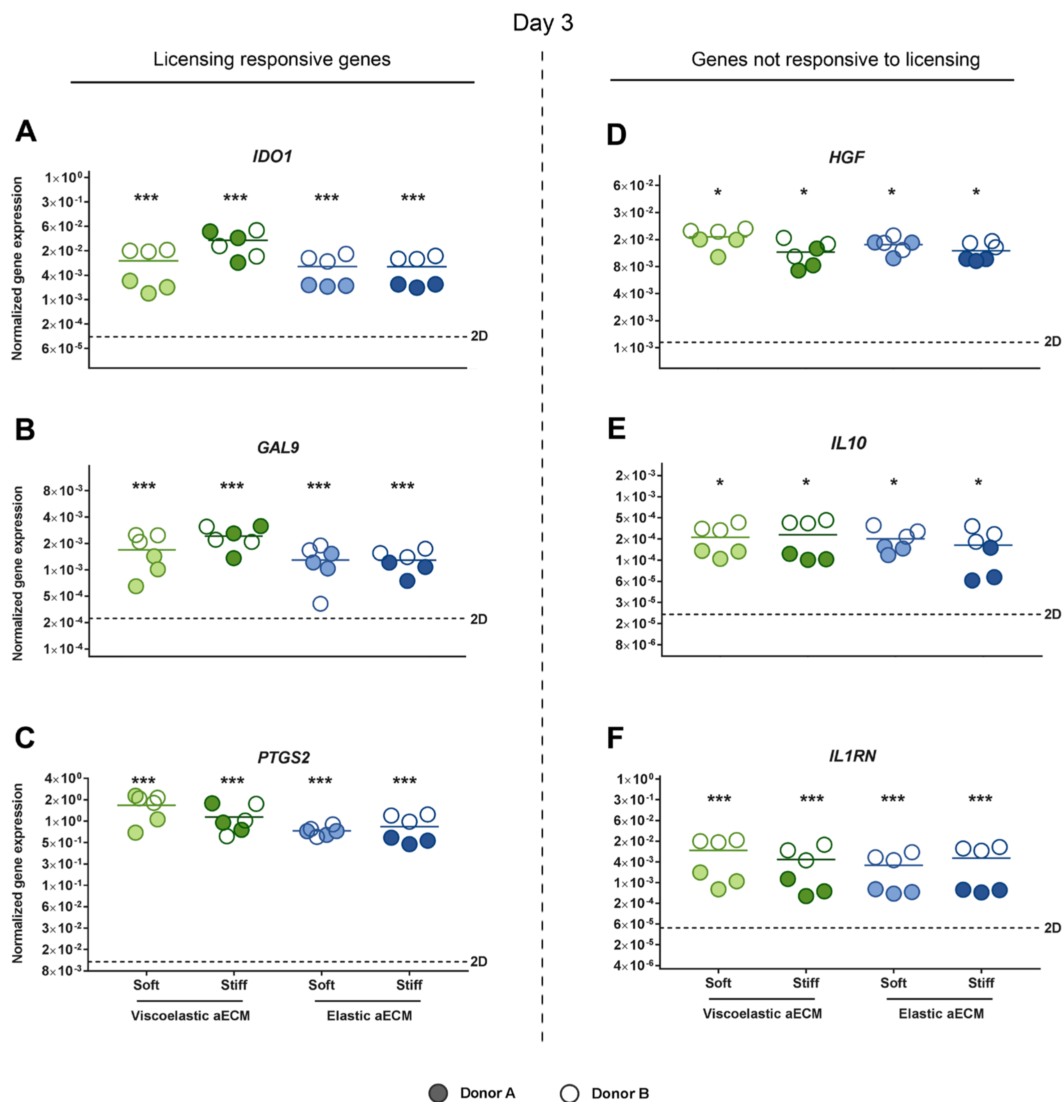


Fig. 9. Immunomodulatory gene expression by hMSCs 3D cultured in aECM hydrogels on day 3. Normalized gene expression of **(A)** *IDO1*, **(B)** *GAL9*, **(C)** *PTGS2*, **(D)** *HGF*, **(E)** *IL10* and **(F)** *IL1RN* by hMSCs encapsulated within aECM hydrogels 3 days after licensing with IFN-γ and TNF-α and subsequent encapsulation. Normalized to *GAPDH*. Statistical significance: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to cells cultured 2D. aECM: artificial extracellular matrix. hMSCs: human mesenchymal stromal cells. IFN-γ: interferon γ. TNF-α: tumor necrosis factor α.

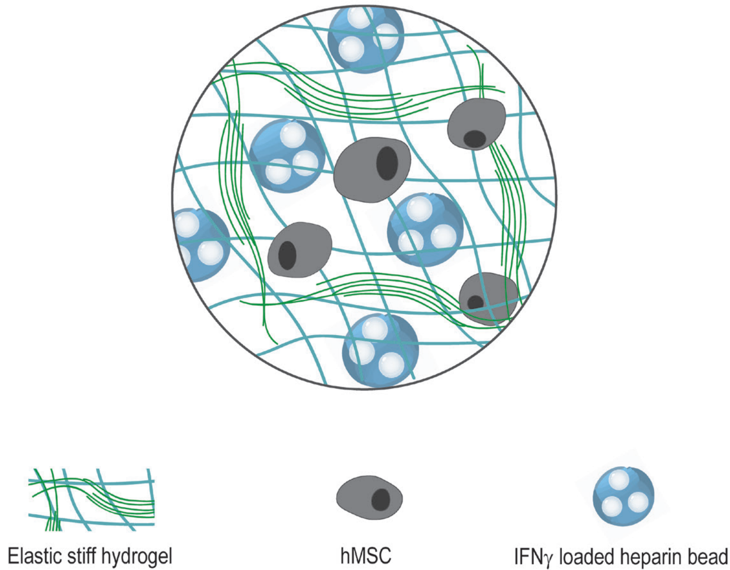


Fig. 10. Schematic representation of IFN-γ loaded iECM hydrogels. iECM: immunomodulatory extracellular matrix. IFN-γ: interferon γ. hMSC: human mesenchymal stromal cells.

Interestingly, no significant differences in hMSC immunomodulatory gene expression were detected among the aECM hydrogel types explored here. This may be due to the range of mechanical properties tested in this study, or an overwhelming impact of the inflammatory cytokines together with the 3D microenvironment. Further work is required to determine the mechanism by which 3D encapsulation in aECM enhances immunomodulatory potential.

In the attempt to prolong the expression of licensing-responsive immunomodulatory genes, we next combined the 3D biomimetic cell culture with sustained inflammatory licensing. With the aim of providing a continuous inflammatory microenvironment for hMSCs, heparin coated beads were loaded with IFN-γ and incorporated in elastic stiff aECM matrices. Since we did not detect differences among the mechanical variations of aECM, such hydrogel base was selected for being the most mechanically robust and thus, likely the most relevant for future implantation studies. As a result, multifunctional immunomodulatory extracellular matrix (iECM) hydrogels were obtained (Fig. 10). It is noteworthy that the binding of IFN-γ to heparin has been demonstrated to limit the extent of proteolytic degradations to one of its domains, which in turn, enhances the cytokine potency [49,50].

iECM hydrogels were demonstrated to prolong the licensing effect and therefore, the expression of major immunomodulatory genes such as *IDO1* and *GAL9* (Fig. 11). Further, the multifunctional system enabled the correct regulatory function of hMSCs: T cell proliferation was suppressed when lymphocytes were co-cultured with hMSCs encapsulated in iECM hydrogels. Importantly, such result indicates that the system permitted diffusion and release of hMSC-derived immunomodulatory factors, which is a key feature, because biomaterial formulations have been reported to hamper the biomolecule diffusion by their relatively large volume [51]. Additionally, the heparin beads did not sequester all the factors secreted by the cells, nor did they physically impede their diffusion. Moreover, it is important to note that the presence of IFN-γ showed no negative effects, suggesting that loaded IFN-γ likely did not escape the hydrogel system, or at least, not in significant quantities.

Together, the combination of 3D biomimetic cell culture and sustained inflammatory licensing was demonstrated to enhance the immunomodulatory potential of MSCs, while enabling a correct diffusion of the secreted bioactive factors. These findings have significant implications in hMSC therapies, since new strategies to boost their therapeutic potential are being extensively explored.

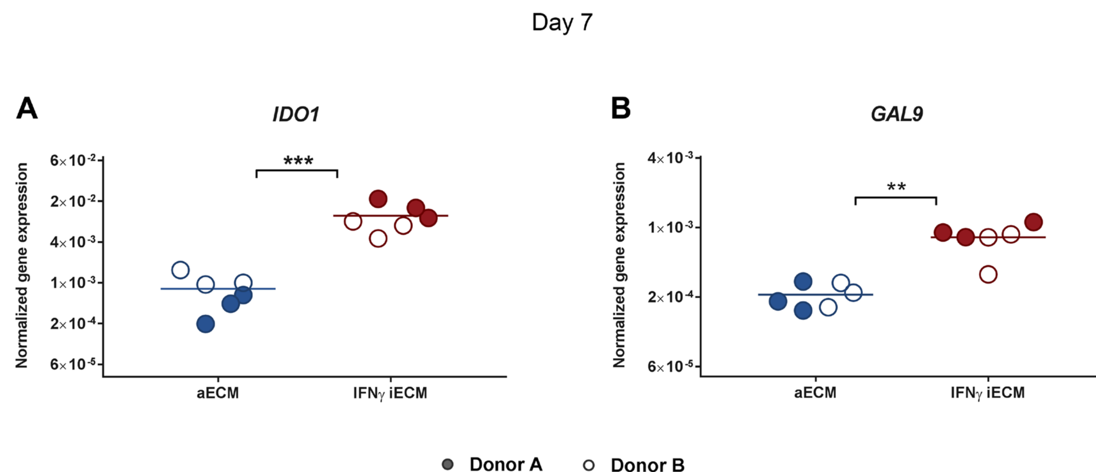


Fig. 11. Immunomodulatory gene expression by hMSCs encapsulated in iECM hydrogels. Normalized gene expression of **(A)** *IDO1* and **(B)** *GAL9* by hMSCs 7 days after encapsulation in iECM hydrogels. Normalized to *GAPDH*. Statistical significance: $^{**}p < 0.01$ and $^{***}p < 0.001$. aECM: artificial extracellular matrix. iECM: immunomodulatory extracellular matrix. hMSC: human mesenchymal stromal cells. IFN-γ: interferon γ. TNF-α: tumor necrosis factor α.

In such work, we observed the importance of gene expression assessment to determine the immunomodulatory potential of hMSCs. Real-time RT-qPCR is most predominantly the method of choice to do it, due to its high sensitivity and relative simplicity. However, the need to validate the expression of so-called housekeeping genes that are used to normalize the data under specific experimental conditions is often ignored and can lead to inaccurate results and flawed conclusions. Therefore, in the final step of this doctoral thesis, we systematically investigated how 3D encapsulation, the mechanical properties of the matrix and inflammatory licensing impact the expression of widely used reference genes in hMSCs. To the best of our knowledge, this gap needed to be filled considering the vast number of studies aiming to precondition hMSCs to enhance their regulatory properties.

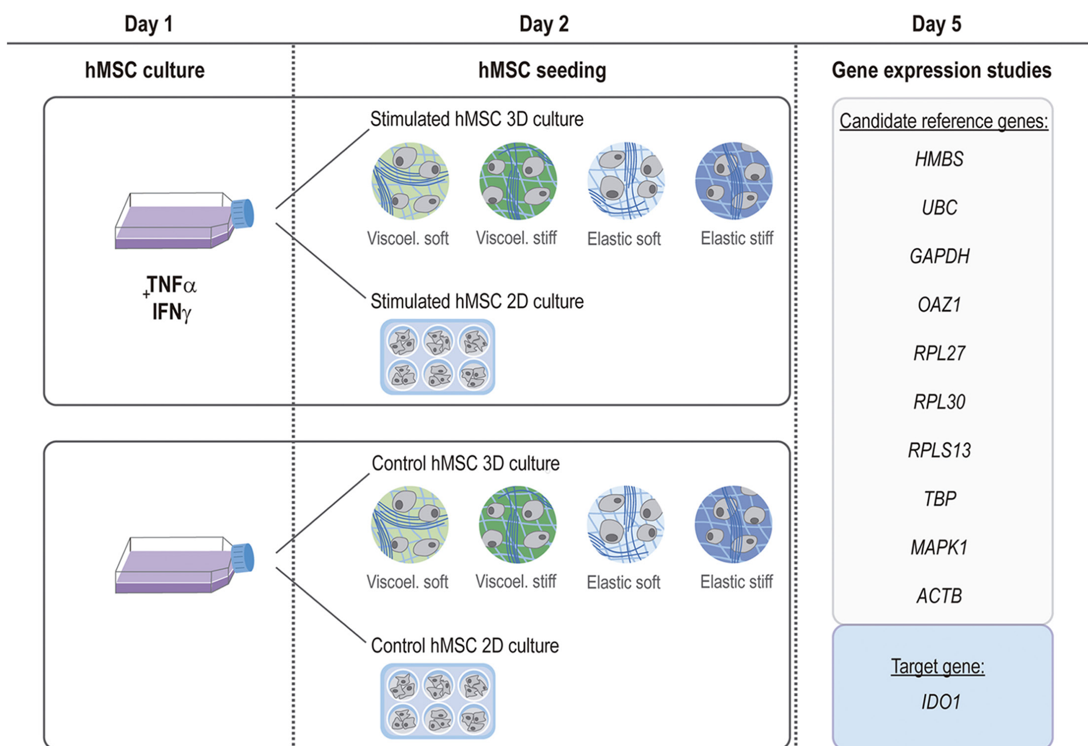


Fig. 12. Experimental procedure followed to determine the stability of reference genes upon 3D encapsulation and inflammatory licensing in hMSCs. Human primary mesenchymal stromal cells (hMSCs) were stimulated overnight (≈ 16 h) with interferon γ (IFN-γ) and tumor necrosis factor α (TNF-α) and subsequently detached and encapsulated in hydrogels with different mechanical properties (3D) or seeded in tissue culture plates (2D). The same procedure was followed with unstimulated control hMSCs. After 3 days of culture, RNA was extracted from the cells and real time RT-qPCR analysis of 10 different reference genes and the target gene *IDO1* was performed.

First, we evaluated reference gene stability in hMSCs across a pool of cells licensed with IFN-γ and TNF-α or non-licensed control cells either in 2D or 3D culture in the four different aECM hydrogel types previously described (Fig. 12). This stability assessment was performed by means of BestKeeper (BK), NormFinder (NF) and geNorm (GN) algorithms, which revealed the ribosomal proteins *RPS13* and *RPL30* as two of the most stable reference genes. This matches the results obtained in a meta-analysis conducted by de Jonge *et al.* [52], where *RPS13* and *RPL30* ranked the first and fourth reference genes, respectively, in terms of stability among multiple cell types and a multitude of experimental conditions. On the contrary, *ACTB* and *UBC* were scored as the least stable housekeeping genes by NF, presenting stability values of 0.35 and 0.36, respectively, far from the values of the rest of housekeeping genes. Supporting such result, BK and GN also ranked *ACTB* and *UBC* among the least stable candidates. Despite *ACTB* has been reported one of the 12 most widely used reference genes [52] in agreement with our results, its instability upon other experimental conditions has previously been demonstrated in multiple publications [53-55].

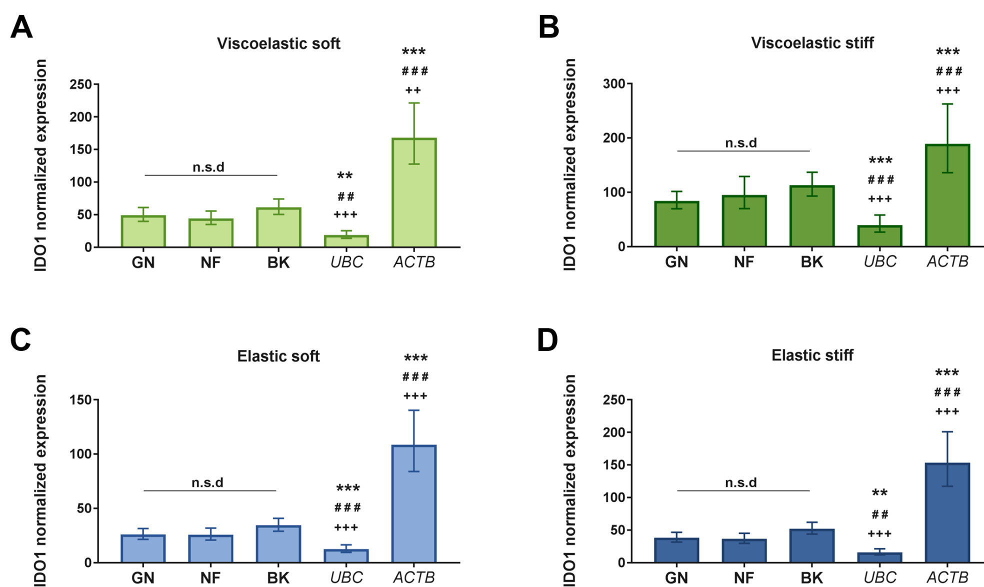


Fig. 13. Effect of 3D encapsulation on reference gene stability. *IDO1* expression in cells encapsulated in **(A)** soft viscoelastic **(B)** stiff viscoelastic **(C)** soft elastic and **(D)** stiff elastic gels was normalized to 2D cultured cells, using the reference gene combinations provided by GN and NF, the BK index or the reference genes *UBC* or *ACTB*. Values represent mean ± S.E. (n = 3 samples per experimental condition). Statistical significance: one-way ANOVA with Bonferroni multiple comparisons test: *p < 0.05, **p < 0.01 and ***p < 0.001 compared to GN ##p < 0.01 and ###p < 0.001 compared to NF and ++p < 0.01 and +++p < 0.001 compared to BK. NF: NormFinder. GN: geNorm. BK: BestKeeper. n.s.d.: no significant difference.

Subsequently, we determined that the poor stability of *ACTB* and *UBC* led to misleading results when analyzing the expression of the target gene *IDO1* in our experimental set-up. Real-time RT-qPCR data was analyzed following the $2^{-\Delta\Delta CT}$ method, also known as the Livak method [56]. We started exploring the effect of 3D hMSC encapsulation. The analysis determined downregulation of *ACTB* and upregulation of *UBC* in 3D cultured hMSCs in comparison to 2D. As a result, normalization to *ACTB* resulted in an overestimation of *IDO1*, whereas when using *UBC*, *IDO1* expression was underestimated (Fig. 13). These results are consistent with previous studies, where GN and NF algorithms identified *ACTB* among the three least stable reference genes in 3D cultivated bone marrow MSCs [53]. Moreover, Liu *et al*. ranked *ACTB* as the least stable candidate housekeeping gene in MSCs under dynamic hydrostatic pressure and concluded that *ACTB* is not a suitable internal control gene for mRNA assay in mechanobiology studies [57].

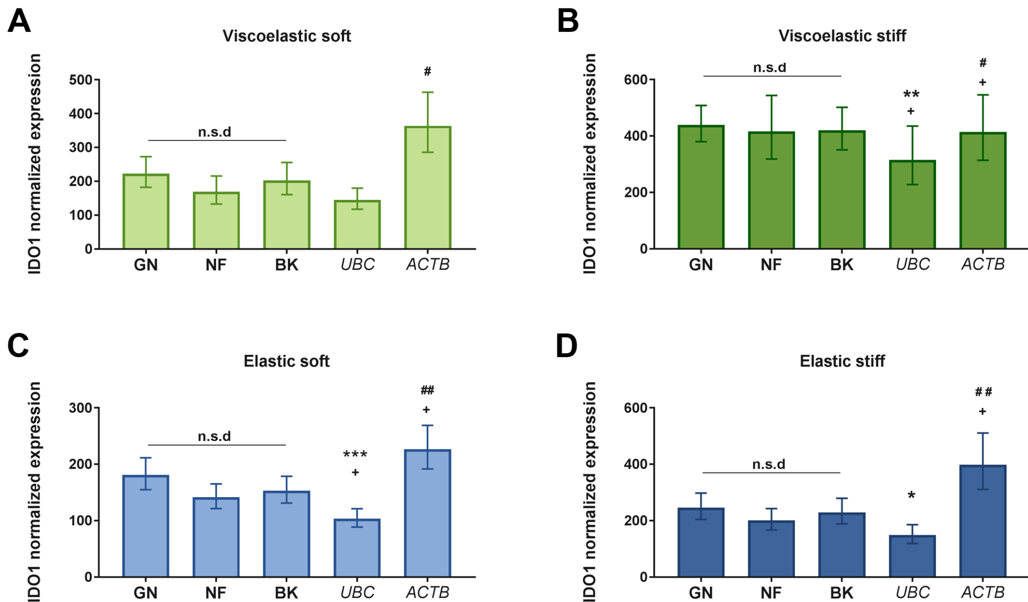


Fig. 14. Effect of inflammatory licensing on reference gene stability. *IDO1* expression in stimulated cells encapsulated in **(A)** soft viscoelastic, **(B)** stiff viscoelastic, **(C)** soft elastic and **(D)** stiff viscoelastic gels was normalized to the non-stimulated controls using the reference gene combinations provided by GN and NF, the BK index or the reference genes *UBC* or *ACTB*. Values represent mean ± S.E. (n = 3 samples per experimental condition). Statistical significance: one-way ANOVA with Bonferroni multiple comparisons test: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to GN $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to NF and $^{+}p < 0.05$ compared to BK. NF: NormFinder. GN: geNorm. BK: BestKeeper. n.s.d.: no significant difference.

Next, we evaluated the stability of the candidate reference genes upon our particular mechanical variations. While multiple studies have described the effects of the mechanical properties of the matrix on cell biology, the expression of *ACTB* and *UBC* remained stable in hMSCs encapsulated in aECM hydrogels with varying viscoelasticity and stiffness.

Finally, we assessed *ACTB* and *UBC* stability upon inflammatory licensing with IFN-γ and TNF-α. We observed the same trend detected in cell encapsulation, although these effects were not as drastic: *ACTB* expression was downregulated and *UBC* upregulated licensed cells, in comparison to control, non-stimulated cells, which led to *IDO1* over and underestimation, respectively (Fig. 14). In agreement with our results, a recent publication demonstrated the poor stability of some miRNA reference genes extensively employed to quantify the nucleic acid content of extracellular vesicles produced by MSCs, upon cell inflammatory licensing with IFN-γ [58].

Overall, these results indicate that the expression of some of the most widely employed reference genes, including *UBC* and *ACTB*, was importantly altered upon hMSC 3D encapsulation and upon inflammatory licensing. As a result, their use as housekeeping genes in such context, led to significant over and underestimation of target gene *IDO1* mRNA levels in real-time RT-qPCR studies. These findings highlight the pivotal importance of reference gene validation upon hMSC pre-conditioning intended to enhance their immunomodulatory potential.

Ultimately, in the present doctoral thesis we focused on the optimization of 3D hydrogel systems to provide new insights into the control of erratic cell responses, the design of adequate biofunctionalization strategies to promote cell viability and the enhancement of the immunomodulatory potential of MSCs. Together, our findings represent an important step forward in the development of cell encapsulation systems, having significant implications in MSC therapies.

References

[1] F. Gao, S.M. Chiu, D.A. Motan, Z. Zhang, L. Chen, H.L. Ji, H.F. Tse, Q.L. Fu, Q. Lian,Mesenchymal stem cells and immunomodulation: current status and future prospects, Cell. Death Dis. 7 (2016) e2062. https://doi.org/10.1038/cddis.2015.327.

[2] G.M. Spaggiari, A. Capobianco, H. Abdelrazik, F. Becchetti, M.C. Mingari, L. Moretta,Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2, Blood. 111 (2008) 1327-1333. https://doi.org/10.1182/blood-2007-02-074997.

[3] P. Terness, T.M. Bauer, L. Rose, C. Dufter, A. Watzlik, H. Simon, G. Opelz,Inhibition of allogeneic T cell proliferation by indoleamine 2,3-dioxygenase-expressing dendritic cells: mediation of suppression by tryptophan metabolites, J. Exp. Med. 196 (2002) 447-457. https://doi.org/10.1084/jem.20020052.

[4] A. Corcione, F. Benvenuto, E. Ferretti, D. Giunti, V. Cappiello, F. Cazzanti, M. Risso, F. Gualandi, G.L. Mancardi, V. Pistoia, A. Uccelli,Human mesenchymal stem cells modulate B-cell functions, Blood. 107 (2006) 367-372. https://doi.org/10.1182/blood-2005-07-2657.

[5] M. Benkhoucha, M.L. Santiago-Raber, G. Schneiter, M. Chofflon, H. Funakoshi, T. Nakamura, P.H. Lalive,Hepatocyte growth factor inhibits CNS autoimmunity by inducing tolerogenic dendritic cells and CD25+Foxp3+ regulatory T cells, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 6424-6429. https://doi.org/10.1073/pnas.0912437107.

[6] M. Francois, R. Romieu-Mourez, M. Li, J. Galipeau,Human MSC suppression correlates with cytokine induction of indoleamine 2,3-dioxygenase and bystander M2 macrophage differentiation, Mol. Ther. 20 (2012) 187-195. https://doi.org/10.1038/mt.2011.189.

[7] J.H. Ylostalo, T.J. Bartosh, K. Coble, D.J. Prockop,Human mesenchymal stem/stromal cells cultured as spheroids are self-activated to produce prostaglandin E2 that directs stimulated macrophages into an anti-inflammatory phenotype, Stem Cells. 30 (2012) 2283-2296. https://doi.org/10.1002/stem.1191.

[8] J.R. Ferreira, G.Q. Teixeira, S.G. Santos, M.A. Barbosa, G. Almeida-Porada, R.M. Goncalves,Mesenchymal Stromal Cell Secretome: Influencing Therapeutic Potential by Cellular Pre-conditioning, Front. Immunol. 9 (2018) 2837. https://doi.org/10.3389/fimmu.2018.02837.

[9] F.J. Vizoso, N. Eiro, S. Cid, J. Schneider, R. Perez-Fernandez,Mesenchymal Stem Cell Secretome: Toward Cell-Free Therapeutic Strategies in Regenerative Medicine, Int. J. Mol. Sci. 18 (2017) 10.3390/ijms18091852. https://doi.org/10.3390/ijms18091852.

[10] J. Galipeau, L. Sensebe,Mesenchymal Stromal Cells: Clinical Challenges and Therapeutic Opportunities, Cell. Stem Cell. 22 (2018) 824-833. https://doi.org/10.1016/j.stem.2018.05.004.

[11] A. Gonzalez-Pujana, E. Santos, G. Orive, J.L. Pedraz, R.M. Hernandez,Cell microencapsulation technology: Current vision of its therapeutic potential through the administration routes, Journal of Drug Delivery Science and Technology. (2017). https://doi.org/10.1016/j.jddst.2017.03.028.

[12] E. Santos, J.L. Pedraz, R.M. Hernandez, G. Orive,Therapeutic cell encapsulation: ten steps towards clinical translation, J. Control. Release. 170 (2013) 1-14. https://doi.org/10.1016/j.jconrel.2013.04.015.

[13] A. Gonzalez-Pujana, G. Orive, J.L. Pedraz, E. Santos-Vizcaino, R.M. Hernandez, Alginate Microcapsules for Drug Delivery, Alginates and their Biomedical Applications, Springer Singapore, 2018. https://doi.org/10.1007/978-981-10-6910-9.

[14] P. de Vos,Historical Perspectives and Current Challenges in Cell Microencapsulation, Methods Mol. Biol. 1479 (2017) 3-21. https://doi.org/10.1007/978-1-4939-6364-5_1.

[15] G.A. Paredes Juarez, M. Spasojevic, M.M. Faas, P. de Vos,Immunological and technical considerations in application of alginate-based microencapsulation systems, Front. Bioeng. Biotechnol. 2 (2014) 26. https://doi.org/10.3389/fbioe.2014.00026.

[16] S.V. Bhujbal, B. de Haan, S.P. Niclou, P. de Vos,A novel multilayer immunoisolating encapsulation system overcoming protrusion of cells, Sci. Rep. 4 (2014) 6856. https://doi.org/10.1038/srep06856.

[17] M. Krampera, L. Cosmi, R. Angeli, A. Pasini, F. Liotta, A. Andreini, V. Santarlasci, B. Mazzinghi, G. Pizzolo, F. Vinante, P. Romagnani, E. Maggi, S. Romagnani, F. Annunziato,Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells, Stem Cells. 24 (2006) 386-398. https://doi.org/10.1634/stemcells.2005-0008.

[18] N. Murphy, O. Treacy, K. Lynch, M. Morcos, P. Lohan, L. Howard, G. Fahy, M.D. Griffin, A.E. Ryan, T. Ritter,TNF-alpha/IL-1beta-licensed mesenchymal stromal cells promote corneal allograft survival via myeloid cell-mediated induction of Foxp3(+) regulatory T cells in the lung, FASEB J. (2019) fj201900047R. https://doi.org/10.1096/fj.201900047R.

[19] P. Jin, Y. Zhao, H. Liu, J. Chen, J. Ren, J. Jin, D. Bedognetti, S. Liu, E. Wang, F. Marincola, D. Stroncek,Interferon-gamma and Tumor Necrosis Factor-alpha Polarize Bone Marrow Stromal Cells Uniformly to a Th1 Phenotype, Sci. Rep. 6 (2016) 26345. https://doi.org/10.1038/srep26345.

[20] S.S. Ozturk, B.O. Palsson,Effect of medium osmolarity on hybridoma growth, metabolism, and antibody production, Biotechnol. Bioeng. 37 (1991) 989-993. https://doi.org/10.1002/bit.260371015.

[21] L. Chen, Y. Zhang, S. Li, X. Wang, N. Li, Y. Wang, X. Guo, S. Zhao, W. Yu, G. Sun, Y. Liu, X. Ma,Effect of plasma components on the stability and permeability of microcapsule, J. Biomed. Mater. Res. A. 102 (2014) 2408-2416. https://doi.org/10.1002/jbm.a.34907.

[22] B. Thu, P. Bruheim, T. Espevik, O. Smidsrod, P. Soon-Shiong, G. Skjak-Braek,Alginate polycation microcapsules. I. Interaction between alginate and polycation, Biomaterials. 17 (1996) 1031-1040. https://doi.org/10.1016/0142-9612(96)84680-1.

[23] J.W. Shin, D.J. Mooney,Extracellular matrix stiffness causes systematic variations in proliferation and chemosensitivity in myeloid leukemias, Proc. Natl. Acad. Sci. U. S. A. 113 (2016) 12126-12131. https://doi.org/10.1073/pnas.1611338113.

[24] H. Gurruchaga, J. Ciriza, L. Saenz Del Burgo, J.R. Rodriguez-Madoz, E. Santos, F. Prosper, R.M. Hernandez, G. Orive, J.L. Pedraz,Cryopreservation of microencapsulated murine mesenchymal stem cells genetically engineered to secrete erythropoietin, Int. J. Pharm. 485 (2015) 15-24. https://doi.org/10.1016/j.ijpharm.2015.02.047.

[25] A. Garate, J. Ciriza, J.G. Casado, R. Blazquez, J.L. Pedraz, G. Orive, R.M. Hernandez,Assessment of the Behavior of Mesenchymal Stem Cells Immobilized in Biomimetic Alginate Microcapsules, Mol. Pharm. 12 (2015) 3953-3962. https://doi.org/10.1021/acs.molpharmaceut.5b00419.

[26] A. Leal-Egana, A. Fritsch, F. Heidebrecht, A. Diaz-Cuenca, M. Nowicki, A. Bader, J. Kas,Tuning liver stiffness against tumours: an in vitro study using entrapped cells in tumour-like microcapsules, J. Mech. Behav. Biomed. Mater. 9 (2012) 113-121. https://doi.org/10.1016/j.jmbbm.2012.01.013.

[27] T. Richardson, S. Barner, J. Candiello, P.N. Kumta, I. Banerjee,Capsule stiffness regulates the efficiency of pancreatic differentiation of human embryonic stem cells, Acta Biomater. 35 (2016) 153-165. https://doi.org/10.1016/j.actbio.2016.02.025.

[28] C. Liu, Y. Liu, X.X. Xu, H. Wu, H.G. Xie, L. Chen, T. Lu, L. Yang, X. Guo, G.W. Sun, W. Wang, X.J. Ma, X. He,Potential effect of matrix stiffness on the enrichment of tumor initiating cells under three-dimensional culture conditions, Exp. Cell Res. 330 (2015) 123-134. https://doi.org/10.1016/j.yexcr.2014.07.036.

[29] R.M. Kleinberger, N.A. Burke, K. Dalnoki-Veress, H.D. Stover,Systematic study of alginate-based microcapsules by micropipette aspiration and confocal fluorescence microscopy, Mater. Sci. Eng. C. Mater. Biol. Appl. 33 (2013) 4295-4304. https://doi.org/10.1016/j.msec.2013.06.033.

[30] X. Ma, I. Vacek, A. Sun,Generation of alginate-poly-l-lysine-alginate (APA) biomicrocapsules: the relationship between the membrane strength and the reaction conditions, Artif. Cells Blood Substit. Immobil. Biotechnol. 22 (1994) 43-69. https://doi.org/10.3109/10731199409117399.

[31] Y.L. Wu, U.B. Maachani, M. Schweitzer, R. Singh, M. Wang, R. Chang, M.M. Souweidane,Dual Inhibition of PI3K/AKT and MEK/ERK Pathways Induces Synergistic Antitumor Effects in Diffuse Intrinsic Pontine Glioma Cells, Transl. Oncol. 10 (2017) 221-228. https://doi.org/10.1016/j.tranon.2016.12.008.

[32] X. Huang, R. Hang, X. Wang, N. Lin, X. Zhang, B. Tang,Matrix stiffness in three-dimensional systems effects on the behavior of C3A cells, Artif. Organs. 37 (2013) 166-174. https://doi.org/10.1111/j.1525-1594.2012.01546.x.

[33] Y. Takada, X. Ye, S. Simon,The integrins, Genome Biol. 8 (2007) 215-2007-8-5-215. https://doi.org/10.1186/gb-2007-8-5-215.

[34] V. Thulabandu, D. Chen, R.P. Atit,Dermal fibroblast in cutaneous development and healing, Wiley Interdiscip. Rev. Dev. Biol. 7 (2018) 10.1002/wdev.307. Epub 2017 Dec 15. https://doi.org/10.1002/wdev.307.

[35] Y. Wang, X. Chen, W. Cao, Y. Shi,Plasticity of mesenchymal stem cells in immunomodulation: pathological and therapeutic implications, Nat. Immunol. 15 (2014) 1009-1016. https://doi.org/10.1038/ni.3002.

[36] A. Mohr, R. Zwacka,The future of mesenchymal stem cell-based therapeutic approaches for cancer - From cells to ghosts, Cancer Lett. 414 (2018) 239-249. https://doi.org/10.1016/j.canlet.2017.11.025.

[37] K. Hozumi, K. Nakamura, H. Hori, M. Miyagi, R. Nagao, K. Takasaki, F. Katagiri, Y. Kikkawa, M. Nomizu,Mixed Fibronectin-Derived Peptides Conjugated to a Chitosan Matrix Effectively Promotes Biological Activities through Integrins, alpha4beta1, alpha5beta1, alphavbeta3, and Syndecan, Biores Open Access. 5 (2016) 356-366. https://doi.org/10.1089/biores.2016.0037.

[38] J. Lee, A.A. Abdeen, X. Tang, T.A. Saif, K.A. Kilian,Geometric guidance of integrin mediated traction stress during stem cell differentiation, Biomaterials. 69 (2015) 174-183. https://doi.org/10.1016/j.biomaterials.2015.08.005.

[39] D. Cox, M. Brennan, N. Moran,Integrins as therapeutic targets: lessons and opportunities, Nat. Rev. Drug Discov. 9 (2010) 804-820. https://doi.org/10.1038/nrd3266.

[40] M. Bharadwaj, N. Strohmeyer, G.P. Colo, J. Helenius, N. Beerenwinkel, H.B. Schiller, R. Fassler, D.J. Muller,alphaV-class integrins exert dual roles on alpha5beta1 integrins to strengthen adhesion to fibronectin, Nat. Commun. 8 (2017) 14348. https://doi.org/10.1038/ncomms14348.

[41] R. Cruz-Acuna, A.J. Garcia,Synthetic hydrogels mimicking basement membrane matrices to promote cell-matrix interactions, Matrix Biol. 57-58 (2017) 324-333. https://doi.org/10.1016/j.matbio.2016.06.002.

[42] J. Takagi,Structural basis for ligand recognition by RGD (Arg-Gly-Asp)-dependent integrins, Biochem. Soc. Trans. 32 (2004) 403-406. https://doi.org/10.1042/BST0320403.

[43] A. Mardilovich, J.A. Craig, M.Q. McCammon, A. Garg, E. Kokkoli,Design of a novel fibronectin-mimetic peptide-amphiphile for functionalized biomaterials, Langmuir. 22 (2006) 3259-3264. https://doi.org/10.1021/la052756n.

[44] A. Sharma, J.A. Askari, M.J. Humphries, E.Y. Jones, D.I. Stuart,Crystal structure of a heparin- and integrin-binding segment of human fibronectin, EMBO J. 18 (1999) 1468-1479. https://doi.org/10.1093/emboj/18.6.1468.

[45] F. Gao, S.M. Chiu, D.A. Motan, Z. Zhang, L. Chen, H.L. Ji, H.F. Tse, Q.L. Fu, Q. Lian,Mesenchymal stem cells and immunomodulation: current status and future prospects, Cell. Death Dis. 7 (2016) e2062. https://doi.org/10.1038/cddis.2015.327.

[46] C. Popov, T. Radic, F. Haasters, W.C. Prall, A. Aszodi, D. Gullberg, M. Schieker, D. Docheva,Integrins alpha2beta1 and alpha-11beta1 regulate the survival of mesenchymal stem cells on collagen I, Cell. Death Dis. 2 (2011) e186. https://doi.org/10.1038/cddis.2011.71.

[47] R.M. Desai, S.T. Koshy, S.A. Hilderbrand, D.J. Mooney, N.S. Joshi,Versatile click alginate hydrogels crosslinked via tetrazine-norbornene chemistry, Biomaterials. 50 (2015) 30-37. https://doi.org/10.1016/j.biomaterials.2015.01.048.

[48] K.H. Vining, A. Stafford, D.J. Mooney,Sequential modes of crosslinking tune viscoelasticity of cell-instructive hydrogels, Biomaterials. 188 (2019) 187-197. https://doi.org/10.1016/j.biomaterials.2018.10.013.

[49] H. Lortat-Jacob, F. Baltzer, J.A. Grimaud,Heparin decreases the blood clearance of interferon-gamma and increases its activity by limiting the processing of its carboxyl-terminal sequence, J. Biol. Chem. 271 (1996) 16139-16143. https://doi.org/10.1074/jbc.271.27.16139.

[50] E. Saesen, S. Sarrazin, C. Laguri, R. Sadir, D. Maurin, A. Thomas, A. Imberty, H. Lortat-Jacob,Insights into the mechanism by which interferon-gamma basic amino acid clusters mediate protein binding to heparan sulfate, J. Am. Chem. Soc. 135 (2013) 9384-9390. https://doi.org/10.1021/ja4000867.

[51] N. Huebsch, P.R. Arany, A.S. Mao, D. Shvartsman, O.A. Ali, S.A. Bencherif, J. Rivera-Feliciano, D.J. Mooney,Harnessing traction-mediated manipulation of the cell/matrix interface to control stem-cell fate, Nat. Mater. 9 (2010) 518-526. https://doi.org/10.1038/nmat2732.

[52] H.J. de Jonge, R.S. Fehrmann, E.S. de Bont, R.M. Hofstra, F. Gerbens, W.A. Kamps, E.G. de Vries, A.G. van der Zee, G.J. te Meerman, A. ter Elst,Evidence based selection of housekeeping genes, PLoS One. 2 (2007) e898. https://doi.org/10.1371/journal.pone.0000898.

[53] J. Rauh, A. Jacobi, M. Stiehler,Identification of stable reference genes for gene expression analysis of three-dimensional cultivated human bone marrow-derived mesenchymal stromal cells for bone tissue engineering, Tissue Eng. Part C. Methods. 21 (2015) 192-206. https://doi.org/10.1089/ten.TEC.2014.0230.

[54] A. Jacobi, J. Rauh, P. Bernstein, C. Liebers, X. Zou, M. Stiehler,Comparative analysis of reference gene stability in human mesenchymal stromal cells during osteogenic differentiation, Biotechnol. Prog. 29 (2013) 1034-1042. https://doi.org/10.1002/btpr.1747.

[55] I. de Lázaro, K. Kostarelos,Exposure to graphene oxide sheets alters the expression of reference genes used for real-time RT-qPCR normalization, bioRxiv. (2018) 469304. https://doi.org/10.1101/469304.

[56] K.J. Livak, T.D. Schmittgen,Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods. 25 (2001) 402-408. https://doi.org/10.1006/meth.2001.1262.

[57] J. Liu, L. Zou, J. Wang, Z. Zhao,Validation of beta-actin used as endogenous control for gene expression analysis in mechanobiology studies, Stem Cells. 27 (2009) 2371-2372. https://doi.org/10.1002/stem.160.

[58] E. Ragni, P. De Luca, C. Perucca Orfei, A. Colombini, M. Vigano, G. Lugano, V. Bollati, L. de Girolamo,Insights into Inflammatory Priming of Adipose-Derived Mesenchymal Stem Cells: Validation of Extracellular Vesicles-Embedded miRNA Reference Genes as A Crucial Step for Donor Selection, Cells. 8 (2019) 10.3390/cells8040369. https://doi.org/10.3390/cells8040369.

Conclusions

Based on the results obtained in the experimental studies, the main conclusions of this doctoral thesis are the following:

1. An adequate selection of osmolarity adjusting agents in the formulation of alginate-poli-L-lysine-alginate (APA) microcapsules successfully controlled mesenchymal stromal cell (MSC) behavior, with no need of altering the biomaterial or crosslinker type or proportion. The system was demonstrated to avoid erratic cell responses and as a result, provide a safer and more predictable therapeutic regimen *in vivo*.

2. The development of an extracellular matrix protein dot microarray biosensor enabled an in depth characterization of the biofunctionalization requirements of a particular cell type. The platform provided information regarding cell adhesion kinetics, integrin profile and integrin contribution to adhesion consolidation, resulting in a useful tool for the adequate design of biomimetic materials.

3. The development of a bioinspired multifunctional system combining three-dimensional (3D) biomimetic cell culture and sustained inflammatory licensing significantly enhanced and prolonged the expression of pivotal immunomodulatory genes by MSCs. Importantly, the system permitted a correct release and diffusion of the factors secreted by the cells, validating the functionality of the platform.

   Additionally, we determined that such experimental conditions significantly altered the expression of commonly used reference genes such as *ACTB* and *UBC*. Their use as housekeeping genes introduced significant errors in the assessment of the target gene *IDO1* mRNA levels, leading to over or underestimation of the immunomodulatory properties of MSCs. Together, these results highlight the importance of reference gene validation upon MSC pre-conditioning intended to enhance their immunomodulatory potential.

EUSKARAZKO BERTSIOA

Sarrera

Zelula estromal mesenkimaletan oinarritutako terapiak immunitate gaitzen tratamenduan: aurrerapen berriak eta etorkizuneko erronkak

Kapitulu hau *Expert Opinion On Drug Delivery* aldizkarira bidali da

Zelula estromal mesenkimaletan oinarritutako terapiak immunitate gaitzen tratamenduan: aurrerapen berriak eta etorkizuneko erronkak

Ainhoa Gonzalez-Pujana[a,b], Manoli Igartua[a,b], Edorta Santos-Vizcaino[a,b*], Rosa Maria Hernandez[a,b,*]

[a] NanoBioCel taldea, Teknologia Farmazeutikoko Saila, Farmazia Fakultatea, Euskal Herriko Unibertsitatea (UPV/EHU), Espainia.
[b] Sareko Ikerketa Biomedikorako Zentroa, Bioingenieritza, Biomaterial eta Nanomedikuntzan (CIBER-BBN), Espainia.
* Egile urgazleak: Edorta Santos-Vizcaino, Rosa Maria Hernandez.

LABURPENA

Sarrera: Zelula estromal mesenkimalek (*mesenchymal stromal cell* edo MSC) ezaugarri immunomodulatzaile garrantzitsuak dituzte. Hori dela eta, gaitz immuneak eta hanturazko gaixotasunak tratatzeko estrategia bikaina bihurtu dira. MSCen eragin immunomodulatzailean mekanismo desberdinek parte hartzen dute, eta haien artean, zelulek faktore bioaktiboak askatzeko duten gaitasuna funtsezkoa da. Molekula horiek, esaterako, prostaglandina E2 (PGE2) edo indoleamina 2,3-dioxigenasa (IDO) dira, berezko erantzun immunea zein erantzun immune adaptatiboa erregulatu ditzaketeenak. Ondorioz, MSCak asko erabili izan dira ikerketa preklinikoetan, giza entsegu klinikoei bidea zabalduz.

Artikuluaren edukia: Berrikuspen honetan MSCek askatzen dituzten faktore immunomodulatzaile garrantzitsuenetakoak aipatzen dira. Bestalde, ikerketa preklinikoetan egindako azken aurrerapenak laburbiltzen dira, zenbait gaixotasunei erreparatuz. Horien artean, ostalariaren aurkako mentu gaixotasuna (*graft-versus-host-disease* edo GvHD) edo hesteetako hanturazko gaixotasuna (*inflammatory bowel disease* edo IBD) daudelarik. Horrez gain, MSCetan oinarritutako terapia immunomodulatzaileen gaur egungo egoera deskribatzeko, azken faseko giza entsegu klinikoen emaitzak eta onarpen klinikoa lortu duten lehendabiziko MSCdun produktuak deskribatzen dira.

Adituaren iritzia: MSCetan oinarritutako terapien potentziala ikerketa anitzetan frogatu egin da. Halere, behinbetiko itzulpen klinikoa lortzeko, hainbat erronka gainditu behar dira oraindik, esaterako, protokoloen normalizazioa edota MSCen ezaugarri immunomodulatzaileak sustatzen dituzten estrategien garapena.

1. Sarrera

Zelula estromal mesenkimalak (*mesenchymal stromal cells* edo MSCak) aitzindari ez hematopoietikoak dira, eta ugaztunen ehun estromaletan aurkitzen dira. Horien artean, hezur-muina, zilbor hestea eta ehun adiposoa dira zelula hauen iturri erabilienak [1]. Duela hiru hamarkada, MSCen inguruan interes klinikoa nabarmen handitu zen [2], baina ikerlarien artean ez zegoen adostasunik haien ezaugarri definitzaileak erabakitzeko orduan. Hori dela eta, Terapia Zelularraren Nazioarteko Erakundeak (*The International Society for Cellular Therapy* edo ISCT) 2006. urtean giza MSCak definitzeko irizpide fenotipiko eta funtzionalak ezarri zituen, hiru alderdi nagusitan oinarrituta [2].

Lehenik eta behin, MSCek kultibo plastikoetan atxikitzeko ahalmena izan behar dute, proliferatzean fibroblasto moduko populazioak osatuz. Bigarrenik, MSCek CD73, CD90 eta CD105 gainazaleko antigenoak adierazi behar dituzte. Bestalde, ezin dituzte hurrengoak adierazi: CD45 leukozitoen markatzailea, CD34 aitzindari hematopoietikoen eta zelula endotelialen markatzailea, CD11b edo CD14 monozito/makrofagoen markatzaileak, CD19 edo CD79α B zelulen markatzaileak eta gizakien leukozitoen antigenoa (*human leukocyte antigen* edo HLA-DR) [2]. Azkenik, MSCek leinu mesodermikoen zeluletan desberdintzatzeko gaitasuna aurkeztu behar dute, hau da, adipozitoetan, kondrozitoetan eta osteoblastoetan [2].

Aipagarria da auto-berritzeko gaitasuna duten aitzindariak aurkitu direla giza hezur-muineko MSCen artean (*bone marrow MSC* edo BMMSC). Halere, ez dago argi ezaugarri hori jatorri desberdinetako MSCetan adierazten den ala ez. Hori dela eta, ISCT erakundeak aipatutako hiru irizpideak betetzen dituzten zelulak “zelula estromal mesenkimal multipotenteak” izendatzea gomendatzen du eta “zelula ama mesenkimal” terminoa auto-berritzeko gaitasuna duten zelulentzat erreserbatzea [3].

Hiru leinu horietan desberdintzatu ahal izateagatik, MSCak ehunen sendaketan eta birsorkuntzan alternatiba interesgarria dira. Hori dela eta, MSCen erabilera sakonki aztertu da ehunetako gaitzetan, batez ere hezur eta kartilagoaren berrikuntzan [4-6]. Berez, Ferreira eta kideek aipatu zutenez, 2015. urtera arte, MSCak erabiltzen zituzten ikerketa gehienak arazo muskuloskeletikoen tratamendurako bideratu ziren [7]. Hala ere, birsortze ahalmen horretaz gain, MSCek ezaugarri immunomodulatzaileak ere aurkezten dituzte. Hainbat ikerketek

jakinarazi dute MSCek berezko immunitatea eta immunitate adaptatiboa erregulatzen dituztela, zelula immune ugarien oinarrizko funtzioak modulatuz. Zehazki, MSCek hainbat zelulen aktibazioa, heltzea, proliferazioa edo jarduera zitotoxikoa moldatzen dutela egiaztatu da [8], haien artean, T eta B linfozitoak, zelula hiltzaile naturalak (*natural killer* edo NK) [9-11], zelula dendritikoak [12] eta monozito eta makrofagoak daudelarik [13,14].

Funtzio immunomodulatzaile hori gauzatzeko, mikroingurunearen eraginez MSCen fenotipoa hanturazkoa izatetik antiinflamatorio izatera aldatu behar da. Polarizazio aldaketa hori zelulen gainazalean adierazitako *toll-like*-delako hartzaileen (*toll-like receptors* edo TLR) bidez gertatzen da, ingurugiroan dauden zitokinak antzematen dituztenak [15]. Hanturazko egoera baten hasieran, hanturazko bitartekarien presentzia urria da, hala nola, α tumorearen nekrosi faktorearena (TNF-α) edota γ interferoiarena (IFN-γ). Hori dela eta, MSCen fenotipoa proinflamatorioa da eta zelulek hainbat faktore jariatzen dituzte erantzun immunologikoa aktibatzeko eta zelula immuneak lesioaren gunera migrarazteko [16]. Bestalde, hanturaren azken faseetan, zitokina inflamatorioen maila altuek MSCak fenotipo inmunoezabatzaile batera polarizatzen dituzte. Ondorioz, MSCak erantzun immunologikoa murrizten saiatzen dira, ehunak lesio oxidatiboetatik babestuz eta konponketa eta homeostasia sustatuz [16,17].

MSCek fenotipo hipoimmunogenikoa aurkezten dute. Alde batetik, ez dituzte HLA-DR molekulak (histokonpatibilitate konplexu nagusi II-ko (*major histocompatibility complex* edo MHC) azaleko hartzaileak) adierazten, IFN-γ bezalako molekulek estimulatzen dituztenean izan ezik. Bestalde, ez dituzte CD40, CD80, CD83, CD86 eta CD154 molekula ko-estimulatzaileak adierazten [18] (1A. Irudia). Horrela, hipoimmunogenikoak izanda eta ekintza immunoezabatzailea erakutsiz, MSCek potentzial handia daukate hanturazko gaitzak eta gaixotasun immuneak tratatzeko, hala nola, ostalariaren aurkako mentu-gaixotasuna (*graft-versus-host-disease* edo GvHD), hesteetako hanturazko gaixotasuna (*inflammatory bowel disease* edo IBD) edo esklerosi anizkoitza [19-21].

Berrikuspen honen bidez, MSCetan oinarritutako terapia antiinflamatorio eta immunomodulatzaileetan egindako azken aurrerapausoak aztertu nahi dira. Lehenik eta behin, MSCek sistema immunologikoan duten eragina berrikusiko da, askatzen dituzten faktore bioaktibo garrantzitsuenetarikoei erreparatuz. Ondoren, ikerketa preklinikoak eta azken faseko giza

entsegu klinikoak laburbilduko dira, arloaren gaur egungo ikuspegia eskainiz. Amaitzeko, adituaren iritziaren atalean, MSCetan oinarritutako terapiak praktika klinikora heltzeko oraindik gainditu behar diren erronkak eztabaidatuko dira.

2. MSCek askatzen dituzten faktore bioaktiboak

MSCen ekintza immunomodulatzailean mekanismo desberdinek parte hartzen dute. Alde batetik, zelula-zelula arteko elkarrekintzek efektu immunoezabatzaile garrantzitsuak dituzte, MSCek gainazalean adierazten dituzten ligando desberdinen bidez (1.B Irudia). Horien artean, PD-L1 ligandoak T zeluletan dauden PD-1 hartzaile inhibitzaileetara lotzen dira, eta, ondorioz, linfozitoen proliferazioa eta degranulazio zitotoxikoa ekiditen dute [22]. Era berean, MSCek FAS ligandoa (FASL) adierazten dute, T zeluletan dauden FAS hartzaileetara lotzen dena haien apoptosia eraginez [23]. Horrez gain, elkarreragin horrek MSCek monozito proteina kimiotaktikoa (*monocyte chemotactic protein* edo MCP-1) jariatzea eragiten du, T zelulak FASLren bitarteko apoptosira bideratzen dituena [24]. Gainera, T zelula apoptotikoen eraginez, makrofagoek β hazkuntza faktore transformatzailea (*transforming growth factor* β edo TGF-β) askatzen dute, $CD4^{+}$ $CD25^{+}$ $Foxp3^{+}$ T zelula erregulatzaileen presentzia sustatuz [24].

Hala ere, MSCek askatzen dituzten faktore bioaktiboen efektu parakrinoak haien jarduera terapeutikoaren arduradun nagusitzat jotzen dira [7,25] (1.C Irudia). Molekula horien artean interleukinak, entzima metabolikoak, hazkuntza faktoreak eta kimiokinak aurkitzen dira. MSCek molekula bioaktibo horiek transferitu ditzakete ere zelulaz kanpoko besikulak (extracellular vesicles edo EVak) askatuz [20]. Berez, frogatu da MSCetatik eratorritako EVek eragin zuzena dutela berezko edo hartutako erantzun immuneko zelula ugarien gainean, zeinen artean T eta B linfozitoak, monozitoak eta zelula dendritikoak dauden [20].

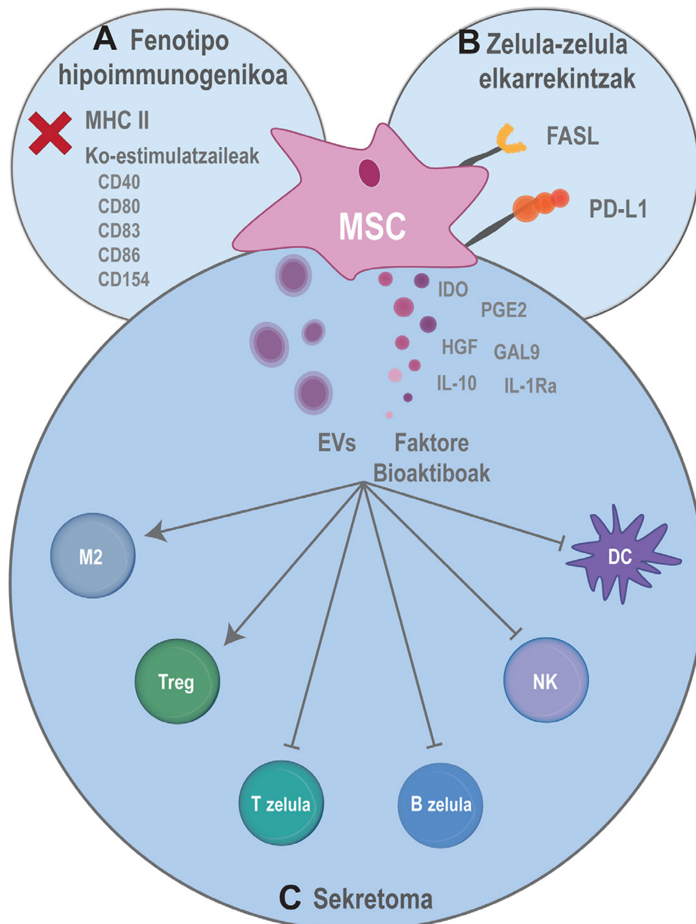
MSCek ekoizten duten efektore garrantzitsu bat indoleamina 2,3-dioxigenasa (IDO) da, hanturazko egoeretan askatzen den entzima metabolikoa [26]. IDOk triptofanoaren metabolismoaren lehendabiziko urratsa katalizatzen du, kinureninaren degradazio bidearen bitartez [27]. Ondorioz, IDO adierazten duten zelulek 2 efektu garrantzitsu eragiten dituzte haien mikroingurunean: (1) triptofanoa agortzea eta (2) kinurenina metabolito toxikoak sortzea [10,28]. Triptofanoaren eskasiak T linfozitoen ziklo zelularra etetea eta anergia eragiten du [29]. Horrez

gain, kinurenina metabolitoek T zelulen proliferazioa inhibitzen dute haiengan efektu zitotoxikoa izanez [10] eta zelula T erregulatzaileen bereizketa sustatzen dute ere [28]. Kinurenina metabolito toxikoek batez ere T zeluletan dute eragina, baina baita B eta NK zeluletan ere [10]. Azken horiei dagokionez, IDOren funtzio immunoezabatzaileak paper garrantzitsua du MSCek NK zeluletan eragiten duten inhibizioan [9]. Gainera, IDOk monozitoak interleukina-10 (IL-10) askatzen duten $CD206^{+}$ M2 makrofagoetan bereiztea suspertzen du, eta horrek, aldi berean, T zelulen supresioa bultzatzen du [13].

Ziklooxigenasa 1 (*cyclooxygenase 1* edo COX1), ziklooxigenasa 2 (*cyclooxygenase 2* edo COX2) eta prostaglandina sintetasa entzimek azido arakidonikotik ekoizten dute prostaglandina-E2 (PGE2) faktore antiinflamatorioa [30, 31]. MSCek era konstitutiboan askatzen dute PGE2, baina bere jariaketa nabarmen handitzen da IFN-γ, TNF-α edo interleukina-1β (IL-1β) bezalako hanturazko zitokinen presentzian [32, 33]. PGE2 faktoreak T zelulen proliferazioa inhibitzen du [33], linfozito T erregutzaileen sorkuntza sustatzen du [34] eta NK zelulen proliferazioa, zitotoxikotasuna eta zitokinen produkzioa inhibitzen ditu [9, 35]. Gainera, PGE2k M2 makrofagoen polarizazioa sustatzen du, haien IL-10 zitokinaren askapena handituz [36]. Oro har, hanturazko erantzunak inhibitzeagatik eta zelula T erregulatzaileak sustatzeagatik, PGE2 funtsezko faktorea da MSCen eragin immunomodulatzailean.

MSCek hazkuntza faktore hepatozitikoa (*hepatocyte growth factor* edo HGF) konstitutiboki adierazten dute ere. HGF faktoreak zelulen hazkuntza, migrazioa eta angiogenesia sustatzen ditu, eta efektu antifibrotiko garrantzitsuak aurkezten ditu [37,38]. Horrez gain, HGFk potentzial immunomodulatzaile indartsua ere badu. T zelulei dagokienez, HGFk bere proliferazioa eten egiten duela behatu da [39]. Gainera, gainazaleko aktibazio markatzaileen adierazpena erregulatzen du, IL-10 ekoizten duten linfozito T erregulatzaileen desberdintzapena sustatzen du eta interleukina-17 (IL-17) ekoizten duten T zelulak gutxitzen ditu ere [12]. Bestalde, HGF zelula dendritikoen funtzioak inhibitzeko gai da [12]. Interesgarria da zelula-zelula arteko elkarreragina garrantzitsua dela MSCek HGF ekoiztu dezaten. Berez, frogatu da HGFren ekoizpena desberdina dela MSCak eta zelula mononuklearrak kontaktuan edo kontakturik gabe ko-kultibatzean [40].

Galektinak zelulek jariatzen dituzten proteina disolbagarriak dira, eta ligando desberdinetara lotzeko ahalmenda dute. Hori dela eta, prozesu anitzetan parte hartzen dute, hala nola, hanturazko erreakzioetan edota erantzun immuneetan [41,42]. 15 galektina azpimoten artean, zenbait galektinek MSCen eragin immunomodulatzailean laguntzen dute. MSCek biziki adierazten dute galektina-1 (Gal-1), bere ekintza immunoerregulatzailea T zelulen proliferazioa ekiditzea delarik [43]. Gal-1 faktoreak zelula dendritiko tolerogenikoen sorrera sustatzen du ere [44]. Bestalde, MSCek galektina-9 (Gal-9) ekoizten dute hantura estimuluen eraginez.



1. Irudia. MSCen ezaugarri immunomodulatzaileak. (A) MSCak fenotipo hipoimmunogenikoa aurkezten dute. MSCen eragin immunoezabatzailea mekanismo desberdinen bidez gauzatzen da, haien artean **(B)** zelula-zelula arteko elkarrekintzak, MSCek gainazalean adierazten dituzten ligandoen bidez; eta bereziki, **(C)** faktore bioaktibo eta EVen ekoizpena. Laburdurak: MSCak: zelula estromal mesenkimalak (*mesenchymal stromal cells*). EVs: zelulaz kanpoko besikulak (*extracellular vesicles*). NK: zelula hiltzaile naturalak (*natural killer*). DC: zelula dendritikoak (*dendritic cells*). M2: M2 makrofagoak.

MSCek Gal-9 askatzean, galektina honek T [45] eta B [46] zelulen proliferazioaren aurkako efektuak dituela frogatu da. Gainera, Gal-9 faktoreak linfozitoek dituzten TIM-3 hartzaileetara lotzean, hauen apoptosia eragiten du [47].

IL-10 ezaugarri antiinflamatorio garrantzitsuak dituen zitokina da. T zelulen proliferazioa eten [48] eta linfozito T erregulatzaileen fenotipoa egonkortzen laguntzen duela jakin da [49]. Zelula dendritikoak IL-10 zitokinaren ekintzaren itu zelulak dira ere, haien arteko elkarrekintzak zelula dendritikoetan T zelulak aktibatzen dituzten zenbait gainazaleko molekulen eta hantura faktoreen adierazpena inhibitzen duelarik [50]. MSCen IL-10 zitokinaren ekoizpenaren inguruan eztabaida ugari sortu dira. Berez, zenbait argitalpenek MSCek zitokina hori ekoizteko ahalmena dutenik frogatu ez dela diote [51,52], eta beste batzuek, aldiz, MSCek IL-10 askatzeko duten gaitasuna aldarrikatzen dute [17,53]. Aurretik beste egile batzuek aipatu duten bezala, logikoa da pentsatzea MSCek IL-10 askatzen dutela haien ingurugiroaren arabera, adibidez TNF-α edo IFN-γ bezalako hanturazko zitokinen presentzian. Polemika hori alde batera utziz, MSCek beste zelula immune batzuk IL-10 ekoiztea eragiten dutela argi dago. Berez, MSCek jariatutako hainbat faktore bioaktiboen bidez T eta B linfozitoek edota makrofagoek IL-10 askatzea sustatzen da [12,36,55,56].

Interleukina-1 hartzailearen antagonista (*interleukin-1 receptor antagonist* edo IL-1Ra), naturalki sortzen den zitokina da, eta interleukina-1 faktorearen (IL-1) inhibitzaile lehiakorra da. IL1Ra, IL-1 hartzaileara lotzen da inolako erantzunik eragin gabe. Ondorioz, IL-1 hartzaileara lotzea ekiditen du, zitokina horren hanturazko efektua saihestuz [57]. Ondorioz, IL1Ra faktoreak neutrofiloak, makrofagoak eta linfozitoak erakartzen dituzten kimiokinen ekoizpena eta jariaketa murrizten ditu [58]. Gainera, MSCek askatutako IL1Ra biomolekulak makrofagoen polarizazioa M2 fenotiporantz bideratzen duela eta B zelulen desberdintzapena inhibitzen duela frogatu da [59]. Horrez gain, Th17 zelulen polarizazioa ere inhibitzen du [60]. Berriki, IL-1Ra ekoizten duten MSCek IL-1β eta TNF-α zitokinen ekoizpena eta jarduera blokeatzen dutela jakin da [61].

3. Ikerketa preklinikoak gaitz immune eta hanturazkoetan

MSCek izan ditzaketen efektu immunomodulatzaileak kontuan hartuz, gaitz anitzen tratamendurako promesa handia bilakatu dira. MSCekin egindako ikerketa preklinikoak ezinbestekoak dira giza entsegu klinikoen oinarriak ezartzeko. Ondorioz, MSCetan oinarritutako terapiak interesez aztertzen ari dira animalia eredu desberdinetan.

*3.1 Ostalariaren aurkako mentu-gaixotasuna (*graft-versus-host-disease *edo GvHD)*

Zelula ama hematopoietikoen transplante alogenikoa (*allogeneic hematopoietic stem cell transplantation* edo HSCT) arrisku handiko hainbat gaixotasun hematologikoen tratamenduan erabiltzen da. HSCTren konplikazio nagusia GvHD da, emailearen T zelulek hartzailearen zeluletan adierazten diren proteinen aurrean erreakzionatzean gertatzen dena. Zehazki, giza antigeno leukozitarioak (*human leucocyte antigen* edo HLA) dira proteina horien artean nabarmenak. Elkarrekintza horren ondorioz, hartzailearen bizitza arriskuan jartzen dituzten hanturazko erantzunak eta sistema immunologikoaren arazoak sortzen dira [62, 63]. GvHDren intzidentzia tasa altuek eta estrategia terapeutiko estandarren gabeziak alternatiba terapeutiko berriak garatzea eskatzen dute. Testuinguru horretan, MSCetan oinarritutako terapiak aukera itxaropentsua dira. Sagu humanizatuen ereduak oso erabiliak izan dira MSCen eragina gaixotasun honetan aztertzeko, gizakiaren immunobiologia era eraginkorrean simulatzen dute eta [64].

Ikerketa preklinikoek MSCek GvHDren kudeaketan potentzial handia dutela erakutsi arren, terapiaren eraginkortasuna lortzeko baldintza zehatzak behar direla behatu da [65,66]. Ildo horretan, Tisato eta kideen arabera, profilaxi gisa administratutako zilbor hestetik eratorritako MSCen (*umbilical cord-derived MSCs* edo UCMSCs) dosi errepikatuak GvHDaren garapena ekiditeko baliagarriak izan ziren. Aldiz, tratamendu hori ez zen eraginkorra izan gaixotasuna jada ezarrita zegoenean [65]. Ondorengo ikerketek jakinarazi zuten MSCetan oinarritutako terapiaren eraginkortasuna zelulak administratzen ziren unearen araberakoa zela. Horrela, emaitza positiboak atzeratutako administrazioak egin ondoren bakarrik lortu ziren [66]. Terapiaren eragin positiboen artean $CD4^{+}$ T zelulen proliferazioaren inhibizioa eta TNF-α bezalako hanturazko zitokinen maila serikoaren txikitzea egon ziren. Ondorioz, gibeleko eta hesteetako patologia murriztu zen eta saguen biziraupena luzatu zen. [66]. Hala ere, beste

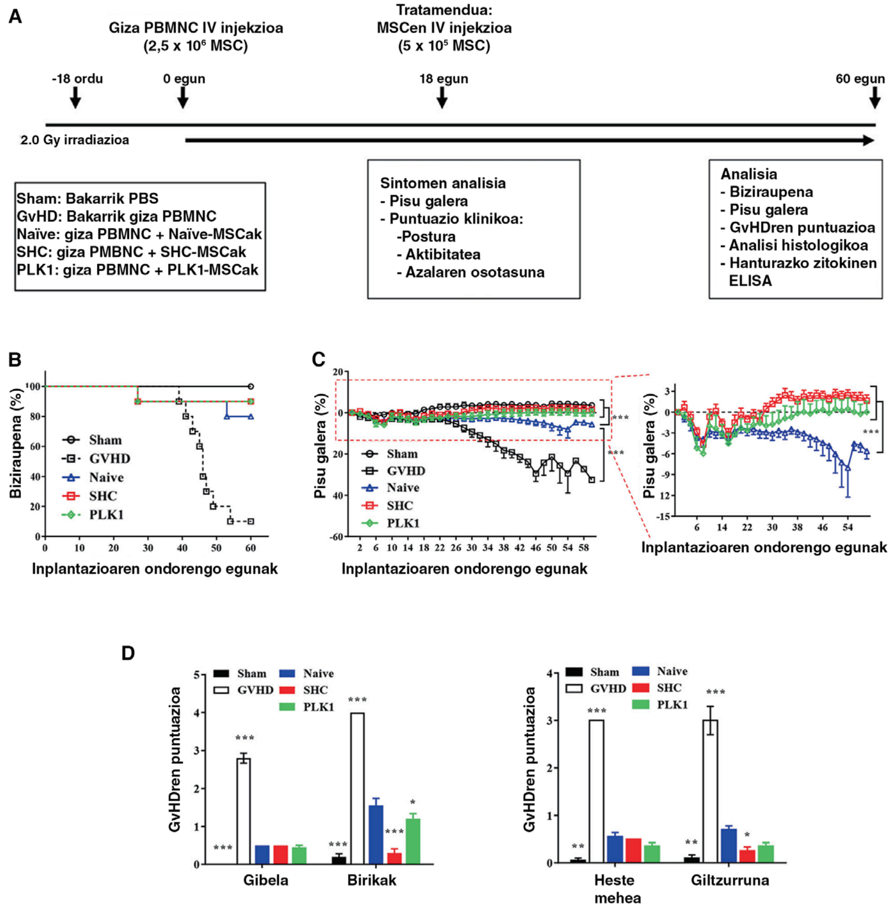
egile batzuek datu horiekin bat ez doazen emaitzak argitaratu dituzte. Besteak beste, ikerketa horietan ez zen GvHD saihestea lortu, ezta animalien hilkortasuna atzeratzea [67-69]. Ikerketen arteko ezberdintasunak, seguruenik, protokoloen arteko heterogeneotasunaren ondorioa dira, desberdintasun handiak daudelako gaixotasunaren etapan, zelulen jatorrian, dosian eta administrazioan edota ostalariek tratamenduarekiko duten sentiberatasunean. Arazo horri aurre egiteko, Zanotti eta kideek MSCak alginato mikrokapsuletan kapsularatzea proposatu zuten. Prozedura horrek protokolo zehatzak diseinatzea ahalbidetzen du eta baita zelulen dosia erraz estandarizatzea ere. Horrez gain, terapiaren biosegurtasuna hobetzen da. Alginato mikrokapsula horiek GvHD pairatzen zuten saguen larruazalpean inplantatzean, animalien biziraupena luzatu zen eta puntuazio klinikoa hobetu zen 8. egunean [70].

MSCen jarduera immunodulatzailea GvHDren tratamenduan hobetzeko ahaleginean, Kim eta kideek berriki proposatu zuten zelulak hipoxia eta kaltzio ioiekin baldintzatzea. Baldintzaturiko MSC horien administrazioak GvHD zuten sagu humanizatuen eredu batean sintomatologia nabarmen hobetzea lortu zuen. Ondorioz, animalien biziraupena luzatu egin zen eta pisu galera eta GvHDren itu organoetako lesio histopatologikoak esanguratsuki murriztu ziren, aurrez baldintzatu gabeko MSCen aldean (2. Irudia) [21]. Estrategia ezberdin bat jarraituz, HSCT sagu eredu batean UCMSCetik eratorritako EVek *in vivo* sintomatologia eta aldaketa histologikoak arindu zituzten, eta baita hilkortasuna murriztu ere. Efektu horiek, T zelulen kopuru absolutuaren eta interleukina-2 (IL-2), TNF-α eta IFN-γ zitokinen murrizketarekin lotuta egon ziren eta baita IL-10 zitokinaren maila handitzearekin ere [71].

*3.2 Hesteetako hanturazko gaixotasuna (*inflammatory bowel disease *edo IBD)*

IBD *Crohn* gaixotasuna eta ultzeradun kolitisa barne hartzen dituen gaitz kronikoa da. Arazo immunologikoen ondorioz, hanturazko prozesuak sortzen ditu digestio hodi osoan zehar [72]. Berez, desoreka immunologikoak ondo deskribatu izan dira IBD jasaten duten gaixoetan, besteak beste, T zelulen aktibazio mailaren igoera edota T zelula erregulatzaileen kopuru absolutuen murrizketa gertatzen direlarik [73]. MSCen ezaugarri antiinflamatorioak eta erregulatzaileak direla eta, zelula horietan oinarritutako terapiak sakonki aztertu dira IBDren ikerketa preklinikoetan.

MSCen administrazioak dextrano sodio sulfatoarekin (DSS) edo trinitrobenzeno azido sulfonikoarekin (TNBS) induzitutako IBD karraskari ereduetan emaitza onuragarriak lortu ditu. Zehazki, kolitisaren larritasun klinikoa hobetzen duela frogatu da, pisu galera, beherakoa eta hantura murriztuz eta baita biziraupena luzatuz ere [74-76]. Efektu terapeutiko hauek T linfozito



2. Irudia. Hipoxia eta kaltzio ioien bidez baldintzaturiko MSCak (SHC-MSCs) eta baita PLK1 adierazten zuten MSCak ere, kontrol *Naive*-MSCak baino eraginkorragoak izan ziren GvHDren tratamenduan. (A) Prozedura esperimentalaren azalpena. MSCak GvHD animali eredu humanizatu batean administratu ziren. Saguetan, 2.5 × 10^6 giza PBMNC injektatu ondoren, 5 × 10^5 MSC *naive* (kontrol taldea), SHC-MSCs (SHC taldea) edo PLK1 adierazten zuten MSCak (PLK1 taldea) administratu ziren. Sham delako taldean PBMNCen ordez, PBS administratu zen, GvHDrik adierazten ez zuen taldea izanda. **(B)** Biziraupen tasa (n = 10) eta **(C)** pisua (n = 10). **(D)** Puntuazio histopatologikoa GvHD itu organoetan (n = 10). Emaitzak batezbestekoa ± s.e.m. adierazten dute. Estatistika: *p < 0.05, **p < 0.01, ***p < 0.001, ANOVA test estatistikoa eginda, Bonferroni *post-hoc*-delakoa aplikatuz. Laburdurak: MSCak: zelula estromal mesenkimalak (*mesenchymal stromal cells*). IV: zain barneko administrazioa (*intravenous*). PLK-1: polo bezalako kinasa-1. GvHD: ostalariaren aurkako mentu-gaixotasuna (*graft-versus-host disease*). PBMNCs: odol periferikoaren zelula mononuklearrak (*peripheral blood mononuclear cells*). [21]. Erreferentziatik moldatua, ® 2018, Springer Nature.

erregulatzaileen indukzioarekin lotuta egon ziren, zeintzuk Th1 eta Th17 zelulen erantzunak gutxitu eta Th2 linfozitoen desberdintzapena sustatu zituzten *in vivo* [74-76]. Horrelako emaitza arrakastatsuak jatorri desberdineko MSCen erabilerarekin lortu dira [74,76,77]. Bestalde, zelulen administrazio uneak terapiaren eraginkortasunean eragina duela frogatu da. Berez, injekzio goiztiarrak nabarmen hobetu zuen DSSrekin induzitutako kolitisa gaixotasunaren jarduera indizeari zein puntuazio histologikoari dagokionez, atzeratutako injekzioaren aldean. Emaitza positibo hauek lortzearen mekanismoaren oinarriak M2 makrofago eta T linfozito erregulatzaileen polarizazioa izan ziren [75]. Bestalde, baldintzaturiko MSCen infusioa gaixotasuna modu eraginkorragoan murrizten duela frogatu egin da, kontrol MSCekin alderatuz. *In vitro* ko-kultibo ikerketek agerian utzi zuten baldintzaturiko MSCek PGE2 gehiago askatzen zutela, TNF-α eta IL-2 bezalako zitokina inflamatorioen maila murriztuz eta IL-10 ekoizten duten T zelulen funtzioa sustatuz [78].

Beste aldetik, posiblea da MSCen onura terapeutikoen probetxu hartzea zelulak zuzenean administratu gabe. Estrategia horietako batean, hezur muinetik eratorritako makrofagoak ehun adiposotik eratorritako MSCekin (ASC) edo ASCen baldintzatutako medioarekin ko-kultibatu ziren. Ko-kultiboak makrofagoak M2 fenotiporantz polarizatu zituen, haien IL-10 zitokinaren ekoizketa handituz eta T zelulak inhibitzeko gaitasuna areagotuz. Makrofago hauen infusio sistemikoak kolitisa inhibitu zuen saguetan, pisu galera eta hilkortasun tasa murriztuz [79]. Beste alternatiba batean, MSCetatik eratorritako EVen zain barneko administrazioa egin zen TNBSarekin induzitutako kolitisa zuten arratoietan. EVek koloneko kaltea murriztu zuten, zitokina proinflamatorioen ekoizketa mugatuz eta IL-10 maila handituz [80].

3.3 Esklerosi anizkoitza

MSCen eragin immunomodulatzailea gaixotasun auto-immuneen tratamenduan aztertu da, hala nola esklerosi anizkoitzan, lupus eritematoso sistemikoan edota alboko esklerosi amiotrofikoan [19]. Esklerosi anizkoitzak nerbio-sistema zentralaren hantura kronikoa, desmielinizazioa eta neurodegenerazioa eragiten ditu, eta bere garapenean T zelulek parte hartzen dute [81]. Ikerketa prekliniko anitzek frogatu dute MSCek gaixotasunaren emaitzak hobetzeko duten gaitasuna [82-84]. Zehazki, hanturazko infiltratuak, desmielinizazioa eta galera axonala gutxituz, MSCek gaitza arindu eta bir-gaixotzea murriztu ditzaketela behatu da [82].

Hala ere, esklerosi anizkoitzaren terapian eztabaida handia sortu da MSC autologoak erabiltzearen inguruan. Gaixotasunaren fase goiztiarra pairatzen zuten saguetatik eratorritako MSCak emaile osasuntsuengandik eratorritako zelulekin alderatzean ez zen inolako desberdintasunik antzeman [85]. Ostera, gaitzaren fase aurreratuetan zeuden saguetatik (sintomatologia larriagoa denean, buztanaren paralisia edo atzeko gorputz-adarren ahultasuna barne) eratorritako MSCak ez zuten eraginkortasun terapeutikorik erakutsi emaile osasuntsuen MSCekin alderatuta [86]. Ondorioz, MSC autologoen eraginkortasuna gaitzaren fasearen eta larritasunaren araberakoa zela frogatu zen [86]. Gaixotasunaren etapa aurreratuetan antzemandako potentzialaren galera, MSCak faktore bioaktiboak askatzeko duten gaitasunaren murrizketarekin lotuta dago [87]. Era berean, MSCen ahalmen antioxidatzailea negatiboki korrelazionatu zen gaitzaren fase aurreratuekin [88].

MSCen eta metilpdrednisolona edo rapamizina bezalako sendagaien arteko konbinazioak entzefalomielitis auto-immune esperimentaleko (*experimental autoimmune encephalomyelitis* edo EAE) ereduetan sintoma klinikoak hobetzen dituela behatu da. Zehazki, hanturazko infiltrazioa eta desmielinizazioa gutxitzen direla frogatu da [83,84]. Efektu horiek $CD8^+$ T zelulen jarduera zitotoxikoaren murrizketarekin eta T zelula erregulatzaileen kopuruaren igoerarekin erlazionatu dira [83,84]. Horrez gain, hanturazko zitokinen (IFN-γ, TNF-α edo IL-17) maila gutxitzeak eta zitokina anti-inflamatorioen (IL-4, IL-10) maila igotzeak funtsezkoa izan zen gaixotasuna arintzeko [83, 84]. MSCen potentzial immunomodulatzailea hobetzeko burutu zen ikerketa batean, MSCak IFN-γ zitokinarekin estimulatu ziren. Estimulatutako zelula hauek esklerosiaren sintomatologia era nabarmenean hobetu zuten, estimulatu gabeko zelulekin alderatuz. Efektu hori, T zelula erregulatzaileen kopuruaren igoerarekin eta hanturazko zitokinen jariaketaren murrizketarekin lotuta egon zen [89].

Bestalde, MSCen baldintzatutako medioa erabiltzea estrategia baliagarria bilakatu da esklerosi anizkoitzaren sintomatologia hobetzeko, zeluletan oinarritutako terapiak alde batera utziz. MSCek askatzen dituzten faktore bioaktiboak dituen medioa administratzean, oligodendrozitoen eta neuronen garapena hobetu zen, EAE saguen defizit funtzionalak murriztuz [90].

4. Gaitz immuneak eta hanturazkoak tratatzeko giza entsegu klinikoak

Azken urteetan, ikerketa preklinikoetan egindako aurrerapenek MSCetan oinarritutako giza entsegu klinikoei bidea eman diete. Berez, 2012. urtean Shi eta kideek aipatu zutenez, MSCak erabiltzen zituzten 100 entsegu kliniko baino gehiago erregistratuta zeuden clintrials.gov nazioarteko datu basean [51]. 2016an Gao eta kolaboratzaileek 400 ikerketen erregistroa aldarrikatu zuten [8] eta 2019ko abuztuan kopurua jada 900tik igo da.

Horien artean, III faseko entsegu klinikoen azterketak arloaren gaur egungo egoera oso ondo deskribatzen du (1. Taula). MSCekin egiten ari diren saiakuntza kliniko gehienak fase goiztiarretan egon arren [18], fase aurreratuetako entseguek MSCetan oinarritutako terapia klinikoen funtsak ezartzea espero da. 2009. urtean, *Osiris Therapeutics* enpresak MSCekin egindako III faseko lehenengo entsegu kliniko nagusia burutu zuen (NCT00366145). Haien produktua, Prochymal®, BMMSC alogenikoetan oinarrituta zegoen eta esteroideekiko errefraktarioa den GvHDaren tratamendura zuzenduta zegoen. Ikerketaren amaierako helburua ez zen bete, ez baitziren desberdintasun nabarmenik antzeman plazebo taldearekin konparatzean [91]. Halere, ikerketa hori abiapuntu garrantzitsua izan zen etorkizuneko entseguetarako. 2013. urtean, Prochymal® produktuaren aktiboak Mesoblast Inc. enpresak eskuratu zituen. Segituan, produktua Remestemcel-L® izendatuz, lehenengoan oinarritutako entsegu kliniko egokitzailea hasi zuen, GvHD errefraktarioa tratatzeko paziente pediatrikoetan (NCT02336230). 2018an, prentsa ohar batek jakinarazi zuen entseguak helburu nagusia bete zuela eta erantzun orokorra hobetzea lortu zuela (subjektuen %69ak tratamenduari erantzun zion, kontrol protokoloaren tasa %45a izanda). Emaitza arrakastatsu horiek kontuan hartuz, jada aipatu da produktu hori izan daitekeela Amerikako Estatu Batuetan Elikagai eta Sendagaien Administrazioak (*Food and Drug Administration* edo FDA) onartzen duen lehendabiziko MSCetan oinarritutako terapia [92]. Horrez gain, 2015eko irailean, Mesoblasten lizentziaduna den JCR Pharmaceutical Co. Ltd. enpresak Medikamentu eta Osasun Produktuen Agentzia (*Pharmaceuticals and Medical Devices Agency* edo PMDA) japoniarraren onarpena jaso zuen TemCell® produktuarentzat, GvHD larria tratatzeko [93].

2015ean, TiGenix S.A.U. enpresak III faseko saiakuntza klinikoa burutu zuen ASC alogenikoak erabiliz (Cx601) *Crohn* gaixotasuna jasaten zuten pazienteetan fistula perianala tratatzeko (NCT01541579). Ikerketa hau Cellerix S.A enpresak aurretik egindako entsegu baten

1. Taula. MSCen erabilera III. faseko giza entsegu klinikoetan gaitz immuneen eta hanturazko gaixotasunen tratamenduan. GvHD: ostalariaren aurkako mentu gaixotasuna (*graft-versus-host-disease*). ALS: alboko esklerosi amiotrofikoa (*amyotrophic lateral sclerosis*).

Aplikazioa	Babeslea / produktuaren izena	Egoera	Jatorria	Emaitzak	Identifikatzailea
Industriak babestua					
GvHD	Osiris therapeutics (*Prochymal*)	Amaitua (2009)	BMMSC alogenikoak	Helburu nagusia ez zen bete. Ez ziren desberdintasun nabarmenik antzeman plazebo taldearekin alderatuz [91]	NCT00366145
GvHD pediatrikoa	Mesoblast Ltd. (*Remestemcel-L*)	Amaitua (2018)	BMMSC alogenikoak	Helburu nagusia bete zen. 28. eguneko erantzuna nabarmen hobetu zen	NCT02336230
Crohn gaixotasunaren fistula	TiGenix S.A.U (*Cx601*)	Amaitua (2015)	ASC alogenikoak	Helburuak lortu ziren, eraginkortasuna eta segurtasuna frogatuz. 2. Mailako helburuak ere bete ziren, emaitz onak urtebete ondoren mantenduz. 2018ko martxoan, Europar Batasunaren onarpena lortu zen (*Alofisel*©) [95-97]	NCT01541579
Crohn gaixotasuna	Mesoblast Ltd. (*Prochymal*)	Aktiboa (2009)	BMMSC alogenikoak	Entseguaren amaiera data estimatua 2019ko otsaila zen. Oraindik ez dira emaitzarik argitaratu.	NCT01233960
ALS	Brainstorm cell therapeutics (*NurOwn*)	Errekrutatzen (2017)	BMMSC autologoak	Martxan	NCT03280056
Osteoartritisa	R-Bio (*JointStem*)	Errekrutatzen (2019)	ASC autologoak	Martxan	NCT03990805
Beste instituzioek babestua					
Artritis erreumatoidea	Royan Institutoa	Amaitua (2013)	Ez zehaztua	Eraginkortasuna eta segurtasuna frogatu ziren [104]	NCT01873625
Osteoartritisa	Stanford Unibertsitatea	Errekrutatzen (2018)	ASCak	Martxan	NCT03467919
Osteoartritisa	Emory Unibertsitatea	Errekrutatzen (2019)	ASC autologoak	Martxan	NCT03818737
GvHD	Guangdong Ospitalea	Errekrutatzen (2014)	Ez zehaztua	Martxan	NCT02291770
GvHD	Fujian Unibertsitatea	Errekrutatzen (2017)	Ez zehaztua	Martxan	NCT03631589

adaptazioa izan zen. Aurreko entsegu hori (NCT00475410), IBD pairatzen ez zuten pazienteetan fistula konplexuen tratamendura zuzenduta zegoen, eta amaitzean, 2009ko abuztuan, ez zuen desberdintasun esanguratsurik aurkitu ASC autologoak edo kontrol tratamendua erabiltzearen artean [94]. 2011n, Tigenix S.A.U. enpresak Cellerix eskuratu zuenean, aldaketa garrantzitsuak ezarri zituen NCT01541579 protokolo berriarentzat: ASC autologoak ASC alogenikoekin ordezkatu ziren, dosia 60 milioitik 120 milioi zeluletara handitu zen eta parte hartzaileek *Crohn* gaixotasuna jasan behar zuten (paziente horiek aurreko entseguan baztertu zituzten). Egokitutako entsegu honek emaitza arrakastatsuak lortu zituen, produktuaren eraginkortasuna eta segurtasuna frogatuz [95]. Emaitzak urtebetez mantendu ziren gutxienez. Berez, jarraipen-azterketak jakinarazi zuen Cx601 produktuarekin tratatutako gaixoen %56,3ak fistulen itxiera totala izan zutela, plazebo taldearen %38,6arekin alderatuta [96]. Galipeau eta kideek ikerketa hau MSCekin egindako lehenengo saiakuntza kliniko aurreratu arrakastatsua bezala deskribatu zuten [92]. Ondorioz, 2018ko martxoan, Europako Sendagaien Agentziak (*European Medicines Agency* edo EMA) Cx601 produktuaren onespena iragarri zuen Alofisel® izen komertzialarekin, Europan baimena lortu duen lehendabiziko MSC alogenikoetan oinarritutako produktua bilakatuz [97].

Fase aurreratuetako entsegu klinikoek MSCetan oinarritutako produktu terapeutikoak merkaturatzeko bidea ireki dute. Hala ere, oraindik esfortzu handia egin behar da gaur egun tratamendu eraginkorrik ez duten immunitate-gaitzetara zuzendutako produktu berriak garatzeko. MSCen efektu terapeutikoak arautzen dituzten mekanismoak eta zelulek haien mikro-ingurunearekin duten elkarreragina sakonki ulertzea nahitaezkoa da etorkizuneko entsegu klinikoak gidatzeko eta produktu farmazeutiko arrakastatsuak garatzeko.

5. Ondorioak

Faktore bioaktibo parakrino anitz ekoizteko eta askatzeko gaitasunak primerazko ezaugarri immunomodulatzaileak eskaintzen dizkie MSCei. Horrenbestez, MSCek potentzial handia erakusten dute gaitz immune eta hanturazko gaixotasun askoren tratamenduan, azterketa preklinikoetan frogatu den bezala. Animalia ereduekin egindako aurrerapenek entsegu klinikoen hazkunde garrantzitsua ekarri dute. Giza entsegu horietako askok haien helburuak lortu ez zituzten arren, etorkizuneko azterketa klinikoetarako erreferentzia garrantzitsua dira

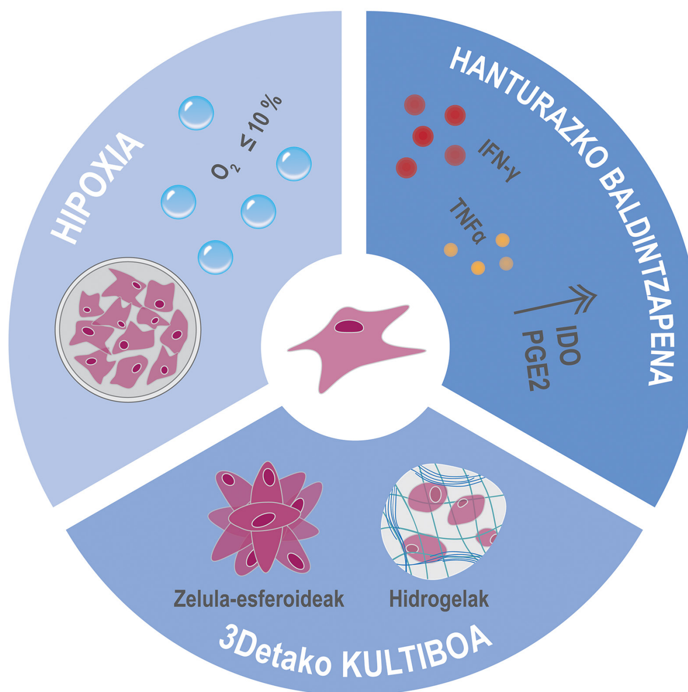
[91,94]. Berez, huts egin zuten entseguetan oinarritutako hainbat ikerketek emaitza positiboak lortu dituzte, Europan eta Japonian MSCetan oinarritutako lehenengo terapien onarpena lortuz [93,97].

6. Adituaren iritzia

Azken hamarkadetan lorpen handiak egin dira MSCetan oinarritutako terapietan. Hala ere, ikerketa askok emaitza kontraesankorrak argitaratu dituzte, estrategia terapeutiko honen aurrerapena motelduz. Desberdintasunak, seguruenik, funtsezko faktoreen arteko heterogenotasuna dela eta sortzen dira, esate baterako, zelulen jatorria (ehuna, emailea), dosia edo administratzeko protokoloak. Parametro horien artean dauden inkoherentziek era nabarmenean mugatzen dute MSCen onura terapeutikoa. Hortaz, itzulpen klinikoa lortzeko, MSC terapien estandarizazioa nahitaezkoa da. Ildo horretan, zelulen isolamendu, kultibo eta hedapen tekniken estandarizazioa eta kalitatea kontrolatzeko sistema sendoak behar dira oraindik. Era berean, *in vivo* administrazio protokoloen optimizazioa funtsezkoa da. Zain barneko administrazioaren ondoren, MSCak detektaezinak dira [98] eta ehunetako makrofago peribaskularrek ezabatzen dituzte [9], zelulen iraunkortasuna erabat mugatuz. Horrez gain, hainbat gaixotasunen tratamenduan, beharrezkoa da efektu terapeutikoak itu ehunean gauzatzea. Hori dela eta, administrazio protokolo berriak behar dira konpartimentu extrabaskularretan zelulak administratzeko. Bereziki, barrunbe peritonealean egindako administrazioa edo larruazalpeko administrazioa bide interesgarriak dira [92].

MSCen terapien emaitzetan eragin handia duen beste faktore bat zelulen potentzial immunomodulatzailea da. Potentzial hori faktore ugarien menpe dago, hala nola, MSCen jatorriko ehuna, emailearen osasun egoera eta adina edota inplantea egin aurretiko zelulen kultibo baldintzak [7]. Horretaz gain, tratatu nahi den gaixotasun zehatzean, adibidez, baliteke zelulen mikroingurunea egokia ez izatea MSCak fenotipo immunoezabatzaile baterantz polarizatzeko. Hori dela eta, etorkizunean funtsezkoa izango da MSCen potentzial immunomodulatzailea sustatzen duten estrategien garapena.

Gaur egun, ikertzaileek esfortzu handia egiten ari dira horrelako estrategiak garatzeko (3. Irudia). Alternatiben artean, MSCak hanturazko zitokinekin estimulatzea da nagusi. Zelulak



3. irudia. MSCak baldintzatzeko estrategia desberdinak. MSCen eragin immunomodulatzailea sustatzeko hainbat estrategia ikertzen ari dira gaur egun. Horien artean, zelulen kultiboa baldintza hipoxikoetan, zelulen baldintzapena hanturazko zitokinen bidez (hala nola IFN-γ edota TNF-α) eta hiru dimentsioetako (3D) kultiboa. Laburdurak: MSCak: zelula estromal mesenkimalak (mesenchymal stromal cells). IFN-γ: γ interferoia. TNF-α: α tumorearen nekrosi faktorea.

administratu aurretik IFN-γ edo TNF-α bezalako zitokinekin estimulatzean, hainbat funtsezko faktore immunomodulatzaileen askapena era nabarmenean hobetzen dela frogatu egin da, IDO eta PGE2 barne [8]. Hala ere, estrategia honen efektuak iragankorrak dira eta ikerketa gehiago behar dira bere efektuak mantentzen dituzten estrategiak garatzeko [99].

Beste alternatiba bat zelulak giro hipoxiko batean kultibatzea da, oxigeno eskaintza % 0-10 bitartekoa duena [100]. *In vivo* inguruneak hipoxikoak izan ohi direnez (%1-12 bitartekoak), MSCen *in vitro* kultiboa baldintza hauetan egiteak haien bideragarritasuna hobetzen du behin kaltetutako ehunean inplantatuta daudenean. Horrez gain, giro hipoxikoak molekula zitobabesleen askapena sustatzen duela frogatu da eta baita zelulen proliferazioa suspertzen duela ere, baina MSCen desberdintzapenik eragin gabe [7]. Halere, MSCek sentikortasun handia dute oxigeno tentsioarekiko eta aldaketa txikiek zelulen desberdintzapenean eta efektu

parakrinoetan eragin handia izan ditzakete. Hori dela eta, gaur egun existitzen diren protokoloen arteko aldakortasuna dela eta, kontraesanean dauden emaitzak argitaratu dira [7], eta oraindik teknikaren estandarizazioa beharrezkoa da.

Zelulen potentzial terapeutikoa hobetzeko MSCak hiru dimentsiotako (3D) ingurunean kultibatzea ere proposatu da, horrela zelulen mikroingurune naturala birsortzen baita. MSCak 3Dtan kultibatzeko gehien erabiltzen den estrategia zelula-esferoideen eraketa da, immunomodulazio gaitasuna handitzen duela frogatu baitu [101]. Berriki argitaratutako alternatiba ezberdin batean, MSCak hidrogel estaldura mehe batean kapsularatu ziren, ezaugarri immunomodulatzaileak hobetzeko hezur muineko transplante alogenikoaren sagu eredu batean [102]. Beste hidrogel sistema batzuek ere, hala nola, alginato-poli-L-lisina-alginato (APA) mikrokapsulek, MSCen ekintza hobetzen dutela erakutsi dute, jarduera metabolikoa eta hazkuntza faktore endotelial baskularraren (*vascular endothelial growth factor edo VEGF*) askapena handituz [103]. Hainbat ikerketek MSCak aurre-baldintzatzeko estrategia horien baliagarritasuna frogatzen duten arren, oraindik bere mekanismo zehatzak sakonki ulertzea beharrezkoa da, haien itzulpen klinikoa gauzatu ahal izateko.

Ikerketa preklinikoetan lortu diren aurrerapauso garrantzitsuek eta MSCetan oinarritutako lehendabiziko tratamenduen onarpen klinikoek, terapia zelularrean aro berri baten hasiera bultzatu dute. Gaur egun, MSCen ezaugarri terapeutikoak indartzen dituzten estrategiak garatzeko eta protokoloak estandarizatzeko burutzen ari diren ahaleginek, behin betiko itzulpen klinikora jauzia egiteko oinarriak finkatuko dituzte.

Eskerrak

A. Gonzalez Pujanak Eusko Jaurlaritzari doktoratu aurreko laguntza eskertzen dio (PRE_2018_2_0133). Proiektu hau neurri batean Eusko Jaurlaritzak finantziatu du (Talde kontsolidatuak, IT-907-16) eta baita MINECO SAF2017-82292-R proiektuak ere.

Erreferentzien zerrenda 48-53 orrialdeetan aurkitzen da.

Sarrera

Zelula-mikrokapsularatze teknologia: potentzial terapeutikoaren egungo ikuspegia inplantazio bidearen arabera

Journal of Drug Delivey Science and Technology, 42 (2017), 49-62.

DOI: https://doi.org/10.1016/j.jddst.2017.03.028

Zelula-mikrokapsularatze teknologia: potentzial terapeutikoaren egungo ikuspegia inplantazio bidearen arabera

Ainhoa Gonzalez-Pujana[a,b], Edorta Santos-Vizcaino[a,b], Gorka Orive[a,b,*], Jose Luis Pedraz[a,b], Rosa Maria Hernandez[a,b,*]

[a] NanoBioCel taldea, Teknologia Farmazeutikoko Saila, Farmazia Fakultatea, Euskal Herriko Unibertsitatea (UPV/EHU).
[b] Sareko Ikerketa Biomedikorako Zentroa, Bioingenieritza, Biomaterial eta Nanomedikuntzan (CIBER-BBN).
* Egile urgazlea: Rosa Maria Hernandez.

LABURPENA

Gaur egun, gaixotasun ugari kudeatzeko estrategia terapeutiko berrien premia dago. Estrategia horien artean, zelula bioaktiboen mikrokapsularatzea aukera interesgarria da. Tradizioz, teknologia horren helburu nagusia faktore terapeutikoen askapen iraunkorra izan da. Horretarako, zelulak biobateragarria den matrize esferiko batean biltzen dira, maiz mintz erdiiragazkor batekin estaltzen dena. Mintz honek, elikagaien, oxigenoaren eta zelulek jariatzen dituzten faktore terapeutikoen difusioa ahalbidetzen du eta aldi berean, zelulak ostalariaren erantzun immunetik babestuta egotea ere bermatzen du. Horren ondorioz, zelulek *de novo* ekoiztutako molekula aktiboen askapen iraunkorra lortzen da. Aplikazio honen potentzial handia dela eta, entsegu kliniko asko bideratu dira arlo honetan, mikrokapsularatze teknologia klinikara hurbiltzeko asmoz. Bestalde, azkenaldian, mikrokapsula klasikoen ezaugarriak moldatzen dituzten ikerketa anitz egin dira ehunen birsorkuntzan erabili ahal izateko. Mikrokapsulak inplantatzeko gorputzaren kokapen ugari ikertu direnez, artikulu honetan teknologia honen potentziala aztertuko da kapsulen administrazio bidearen arabera.

1. Sarrera

Azken hamarkadetan, teknologia farmazeutikoan aurrerapauso nabarmenak egin dira. Garapen honek, hainbat lorpen ekarri ditu arlo anitzetan, farmakozinetika edo farmako askoren administrazioan, esaterako. Hala ere, gaixotasun ugarien tratamenduan farmakoen askapenaren erregulazio zehatza funtsezkoa da, maiz sistema famarmazeutiko tradizionalekin lortu ezin daitekeena. Ondorioz, gaur egun, ikerketa talde askok erronka honi aurre egiteko lan egiten dute.

Zentzu honetan, potentzial izugarria erakutsi duen estrategia bat terapia zelularra izan da. Zelula primarioak, zelula amak, edota genetikoki moldatutako zelulak alternatiba terapeutiko gisa proposatu dira hainbat afekzioren tratamenduan, diabetesa edo nerbio-sistema zentraleko gaixotasunak, besteak beste [1]. Hala ere, terapia zelularrean egindako aurrerapenak mugatuak izan dira, estrategia honen zailtasunak direla eta. Horien artean, zelulen bideragarritasuna mantentzea eta ostalariaren erantzun immunologikoaren errefusa nabarmentzen dira [2]. Horregatik, ahalegin handiak egiten ari dira zelulak ostalariaren immunitate-erantzunetik babesten dituzten sistemak garatzeko. Helburu hori lortuta, farmako immunoezabatzaileen erabilera murriztuko litzateke, abantaila garrantzitsua, bai ikuspuntu terapeutiko batetik, bai ekonomikoki ere [3].

Ildo horretan, zelula-mikrokapsularatze teknologia arazo horiei aurre egiteko sistema bezala proposatu da. Teknologia honetan, zelulak material biobateragarri batez eratutako esfera polimerikoetan biltzen dira. Esfera hauek, mintz erdiiragazkor batekin estaltzen dira. Horrela, lortutako mikrokapsulak ostalarian inplantatzean, mintz honek, zelulek bizitzeko behar dituzten elikagaien eta oxigenoaren difusioa ahalbidetuko du, eta baita haiek ekoiztutako faktore terapeutikoena ere. Aldi berean, ostalariaren immunitate-erantzuneko zelulen eta antigorputzen aurka babestuta daude kapsularatutako zelulak, azken horien difusioa saihestuta dago eta. Kontzeptu horrek, 1933. urtean Biscegliek egindako ikerketa batean du oinarria. Zientzialariak, zelula tumoralak kapsularatu eta txerrien sabelaldeko barrunbean transplantatu ostean, haien biziraupena mantentzen zela frogatu zuen [4]. Hiru hamarkada ondoren, 1964an, Chang eta kideek mintz erdiiragazkorren erabilera hesi immunoisolatzaile bezala proposatu zuten [5]. Aurrerago, 1980an, Lim eta Sun ikertzaileek zelulen kapsularatze teknologia helburu terapeutikoekin erabil daitekeela frogatu zuten. Zehazki, arratoi diabetikoetan

kapsularatutako Langerhans irlak inplantatu ostean, gluzemia maila normalak berreskuratu zituzten. Hortik aurrera, kapsularatutako zelulen transplantearen inguruan asko ikertu da farmakoen askapen iraunkorra lortzeko estrategia bezala.

Farmakoen askapen iraunkorra mikrokapsularatze teknologiaren helburu terapeutiko nagusia izan da. Halere, mikrokapsulen diseinuan egindako moldaketak direla eta, sistema horiek beste aplikazioetan erabiltzea ahalbidetu da. Esaterako, mintz erdiiragazkorrik ez duten polimero biodegradagarriz eratuta dauden mikrosferak ehunen birsorkuntzan erabili izan dira.

Mikrokapsularatutako zelulen inguruan egindako ikerketa preklinikoen emaitza itxaropentsuak direla eta, entsegu kliniko asko burutzen ari dira gaur egun. Berrikuspen honen helburua zelulen mikrokapsularatze teknologiari buruzko ikuspegi orokor bat eskaintzea da, estrategia horren onura terapeutikoak azpimarratuz.

2. Zelula-kapsularatze teknologiaren potentzial terapeutikoa

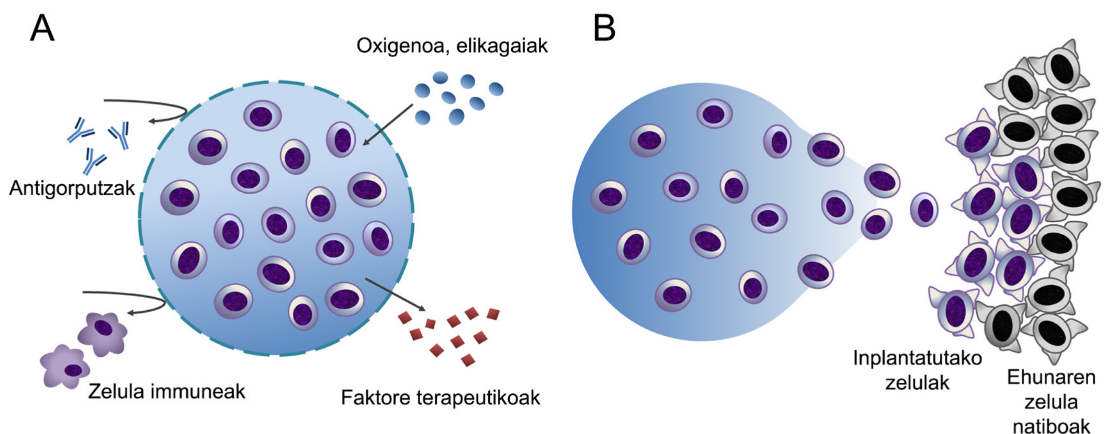
2.1. Molekula bioaktiboen askapen iraunkorra

Zelula bioaktiboen kapsularatzea estrategia interesgarria da farmakoen askapen egonkorra lortzeko. Alternatiba horretan, zelulek molekula aktiboak *de novo* ekoizten eta jariatzen dituzte. Horrela, faktore terapeutiko ezengonkorren administrazioa ahalbidetzen da, oso interesgarria dena haien efektua gauzatzeko ezaugarri biologiko eta fisiko-kimikoak mantendu behar dituztenen kasuan. Bestalde, sistemaren funtzionalitatea denboran zehar mantendu daiteke. Ikerketa askotan frogatu den bezala, kapsularatutako zelulen bideragarritasuna epe luzeetan mantentzen da eta ondorioz, ekoizten dituzten biomolekulen jariatze luzatua lortzen da [7]. Hori dela eta, admistrazio bakarra nahikoa izango litzateke denbora luzean farmakoaren askapen egonkorra lortzeko. Jardunbide klinikoan, abantaila horrek gaixoaren bizi-kalitatea era nabarmenean hobetuko luke, tratamenduarekiko atxikidura suspertuz.

Aipagarria da sistema-polimerikoak eskaintzen duen immunobabesa ezinbestekoa dela zelulen bideragarritasuna denboran zehar mantendu ahal izateko. Hori lortzeko, erabilitako biomaterialak degradaezinak eta egonkorrak izan behar dira. Kapsuletan bilduta, zelulak haien ingurugirotik isolatuta daude, iragazkortasun selektiboa duen mintzari esker. Horrela,

zelulek behar dituzten funtsezko elikagai eta oxigenoaren sarrera ahalbidetzen da kapsularen nukleora. Ostalariaren immunitate-erantzuneko zelulen eta pisu molekular handiko molekula efektoreen sarrera, aldiz, galarazita dago, hala nola, antigorputzena edo osagarri sistemaren faktoreena (1. Irudia A). Nolanahi ere, inplantatutako zelulen jatorriaren arabera, ostalariaren immunitate-erantzuna desberdina izango da. Hori dela eta, sistemaren formulazioa ezberdina izango da transplante alogenikoa edo xenogenikoa egin ahal izateko.

Alotransplanteetan, zelula emailea eta hartzailea espezie berekoak dira. Kasu honetan, transplantatutako zelulen eta ostalariaren immunitatearen arteko kontaktua ekiditzearekin nahikoa izaten da inplantearen errefusa saihesteko [8]. Ondorioz, zelulak biltzen dituen biomaterial polimerikoa soilik erabiltzea estrategia ona izan daiteke. Bestalde, xenotransplanteetan, zelula emailea eta hartzailea espezie desberdinekoak dira, erantzun immunologiko larriagoak eragiten dituena. Zehazki, zelulek epitopo xenogenikoak askatzen dituzte, antigorputz espezifikoen [9] eta hanturazko zitokinen [10] ekoizpena bultzatuz. Horrek, transplantearen biziraupena asko murrizten du. Aipatutako epitopo immunoerreaktiboen artean, galaktosil hondarrak (Gal) nabarmentzen dira. Epitopo horiek ostalariak berez ekoizten dituen anti-Gal eta ez-Gal M immunoglobulina (*immunoglobulin M* edo IgM) antigorputzekin elkarreragiten dute, berezko erantzun immune sendoak eraginez [11]. Hortaz, zelula xenogenikoak



1. Irudia. Zelula kapsularatze teknologiaren potentzial terapeutikoa. (A) Zelula bioaktiboak matrize ez degradagarrietan kapsularatzean faktore terapeutikoen askapen iraunkorra lortzen da. **(B)** Degradagarriak diren kapsulak zelulak askatzeko plataforma gisa erabil daitezke helburu berritzaileekin.

kapsularatzeko, sistemak eskakizun gehiago bete behar ditu, izan ere, egitura polimerikoak porotsuegiak dira barneko osagai biologikoei beharrezkoa den immunobabesa eskaintzeko. Hori dela eta, kapsulek estalki osagarriak behar dituzte iragazkortasuna murrizteko. Horrela, molekula txikien igarotzea soilik ahalbidetuko da (70 kDa baino txikiagoak), zelulek ekoiztutako hanturazko epitopoen irteera ekidituz [8]. Estalki horiek osatzeko erabiltzen diren biomaterialen artean, poli-L-lisina (PLL) [12,13] eta poli-L-ornitina (PLO) [14,15] polikatioiak nabarmentzen dira. Halere, gorputzean inplantatzean, positiboki kargatutako ioi horiek erantzun immuneak eragin ditzakete [16-19]. Arazo horri aurre egiteko, karga positiboak maskaratzen dituen bigarren estalki bat gehitzen da. Kasu gehienetan, azken estalki hori diluitutako alginatoarekin egiten da. Halere, oraindik ez dago argi bi estalki horien arteko interakzioa nahikoa den karga positibo guztiak neutralizatzeko.

Ondorioz, alginato geruzaren garrantzia zalantzan jarri da [20-21] eta estalkien konpozizioa teknologia honetan dauden faktore mugatzaileen artean garrantzitsuenetarikoa da oraindik. Horrez gain, kontuan hartu behar da polikatioiek eskaintzen duten biobateragarritasuna, erresistentzia mekanikoa eta egonkortasuna ez direla guztiz egokiak [22]. Hori dela eta, beste materialez egindako estalkiak erabiltzea proposatu da. Gaur egun gehien ikertzen ari direnen artean, kitosanoa [23,24], poli(metileno-ko-guanidina) [19,25] edo blokezko kopolimeroak [26,27] aurkitu ditzakegu.

Eragozpen hori egonda ere, xenotransplanteen kapsularatzearen inguruan gero eta gehiago ikertzen da, estrategia interesgarria baita alotransplanteekin konparatuz. Izatez, azken horien erabilera mugatua da emaile eskasia dela eta. Horregatik, kapsulen formulazioren moldaketa ezberdinak proposatu dira biobateragarritasunaren arazoa konpontzeko [28,29]. Horrez gain, aipagarria da azken urteetan, alo- edo xeno-transplante jatorria ez duten zelulekin lan egitea proposatu dela. Estrategia berri horien artean, zelula ama pluripotente induzituak (*induced pluripotent stem cells* edo iPSC) daude, jada zelulen mikrokapsularatze teknologian aztertu direnak [30]. Zelula horiek duten desberdintzapen eta proliferazio ahalmen nabarmengarria dela eta, zelula mota askoren iturri berriztagarria dira. Gainera, terapia zelularrean dauden arazo larrienetako asko ekiditzen dira iPSCak erabiltzean, ez baitute inolako erantzun immunologikorik edota eztabaida etikorik sortzen [30].

Immunobabesa eskaintzen duten kapsula-sistema horien optimizazioa lortuta, farmako immunoezabatzaileen erabilera asko murriztu daiteke. Helburu hori betetzea oso onuragarria izango litzateke pazienteentzat, produktu horien eragin desiragaitzak murriztuko lirateke eta, besteak beste, infekzioen arrisku altua edo minbizia garatzeko joera. Izan ere, aipatu denez, farmako immunoezabatzaileen erabilera ez da onargarria aplikazio batzuetan, pankrea-zelulen kapsularatzean, esaterako [9]. Ondorioz, erronka hori gainditzea nahitaezko eskakizuna da zelulen mikrokapsularatze teknologia klinikara eraman aurretik.

2.2 Medikuntza birsortzailea

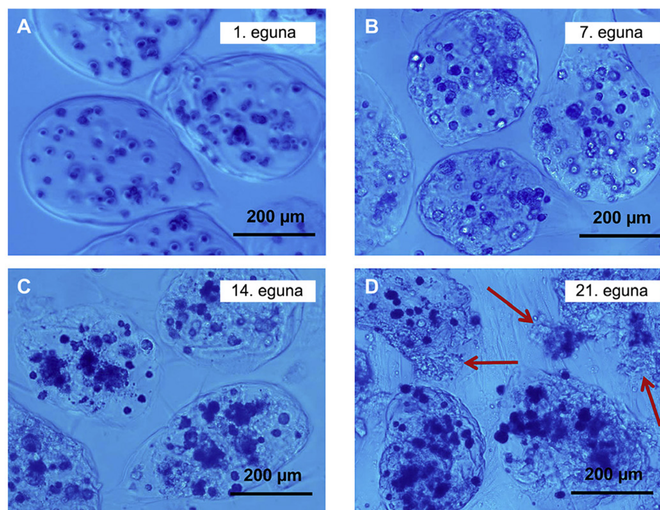
Zelulen mikrokapsularatzeak potentzial handia erakutsi du faktore bioaktiboen askapen egonkorrean, baina baita ehunen birsorkuntzaren arloan ere. Aplikazio honen helburu nagusia kaltetutako ehunean zelulak administratzea da. Horrela, alde batetik, zelulen txertaketa gertatzen da ehun espezifiko horretan, eta beste aldetik, zelulek lesioaren sendatzean parte hartzen duten hainbat faktore askatzen dituzte, hazkuntza-faktoreak eta zitokinak, besteak beste (1. Irudia B). Bi efektu desberdin horien bitartez, ehunaren birsorkuntza lortzen da.

Ehunen ingeniaritzan, zelula estromal mesenkimalen (*mesenchymal stromal cell* edo MSC) erabilera estrategia interesgarri gisa proposatu da. Zelula multipotenteak izateagatik, hezur, gantz edo kartilago zeluletan desberdintza daitezke. Orain dela gutxi argitaratutako lan batean, D1 MSCen analisi fenotipikoa egin zen eta haien desberdintzapen ahalmena frogatu zen [31]. Bestalde, hipoimmunogenizitatea erakusteaz gain, MSCek ezaugarri immunomodulatzaileak ere dituzte. Horrela, ehunaren birsortzea sustatzen duten mekanismo endogenoak estimulatzen dituzte, homeostasi immunea mantentzen den heinean [32, 33]. Berez, ikerketa askotan frogatu da giza MSCen (*human MSC* edo hMSC) aktibazioa eta migrazioa lesioa dagoen gunera. Han, ehunaren konponketa edo birsortzea sustatzen dute [33]. Bestalde, MSCek ehunaren baskularizazioa bultzatzen dute, ehunaren sendatze prozesua sustatuz eta baita inplantearen mentaketa hobetuz ere, oxigenoaren eta elikagaien eskaintza hobetzen baita.

Terapia honen helburua kontuan hartuta, kapsularatutako zelulen askapena beharrezkoa da. Hori lortzeko, matrizeak ezaugarri biodegradagarriak izan behar ditu. Ondorioz, kasu honetan, sistema ez da hesi immunoisolatzaile bezala erabiltzen, baizik eta zelulen mentaketa egokia

sustatzen duen euskarri *scaffold* gisa [22]. Degradazio prozesua parametro erabakigarria da terapiaren arrakasta lortzeko. Berez, degradazio abiadura zelulek *scaffold*-a ordezkatzeko behar duten denborarekin bat joan behar da. Hortaz, abiadura hori kontrolagarria izan beharko litzateke zelulen euspenaren eta askapenaren arteko oreka egokia lortzeko eta zelulek haien mikroingurunea eraberritu ahal izateko matrize extrazelularra (*extracellular matrix* edo ECM) sortzen duten heinean.

Ondorioz, biomaterialen aukera funtsezkoa da sistema era egokian funtzionatzeko. Orokorrean, bi motatako biomaterialak erabiltzen dira: berez biodegradarriak diren materialak edo ez-biodegradarriak diren konposatuak, degradagarri bihurtzen dituzten moldaketak jasan ondoren. Azken horien artean, adibide bat alginatoa da, zelula mikrokapsularatze teknologian polimerorik erabiliena. Alginatoa, biomaterial naturala da, berez degradaezina ugaztunetan ez baitute polimero kateen arteko loturak apurtzeko beharrezkoa den entzima. Ioien bidez elkargurutzatutako alginato hidrogelak ioi monobalenteen presentzian disolbatu daitezkeeen arren, komertzialki eskuragarri ditugun alginatoen batez besteko pisu molekularra zelulak kapsularatzeko aproposa da, baina altuegia giltzurrun bidezko iraizketa egokia lortzeko [34].



2. Irudia. Mikroesfera biodegradagarriak. hUCMSCak kapsularatzen dituzten oxidatutako alginato-mikropartikulen irudiak **(A)** egun 1, **(B)** 7 egun, **(C)** 14 egun eta **(D)** 21 egun ondoren. Mikroesferak 14 ordutan degradatzen hasi ziren. 21. egunerako, hainbat mikropartikula apurtzen hasi ziren (D irudian dauden geziak adierazita). [39] Erreferentziatik moldatua Elsevier-en baimenarekin (© 2011). Laburdurak: hUCMSC: giza zilbor hesteko zelula estromal mesenkimalak (*human umbilical cord mesenchymal stromal cells*).

Hori dela eta, biomaterialaren moldaketa derrigorrezkoa da medikuntza berritzailean erabili ahal izateko. Moldaketa-prozesu ugarien artean [35-38], aukera interesgarri bat kateen oxidazio partziala da, erreakzio honen bidez hidrolisia jasan dezaketen talde funtzionalak lortzen direlarik [34]. Formula honen abantaila nagusia oxidazio maila aldatuz degradazio abiadura kontrolatu daitekeela da. Esate baterako, oxidatutako alginatoa erabili zen ikerketa batean, polimeroak degradazio partziala erakutsi zuen 14. eta 21. egunetan, mikroesfera askoren egitura desegiten hasia zelarik (2. irudia) [39].

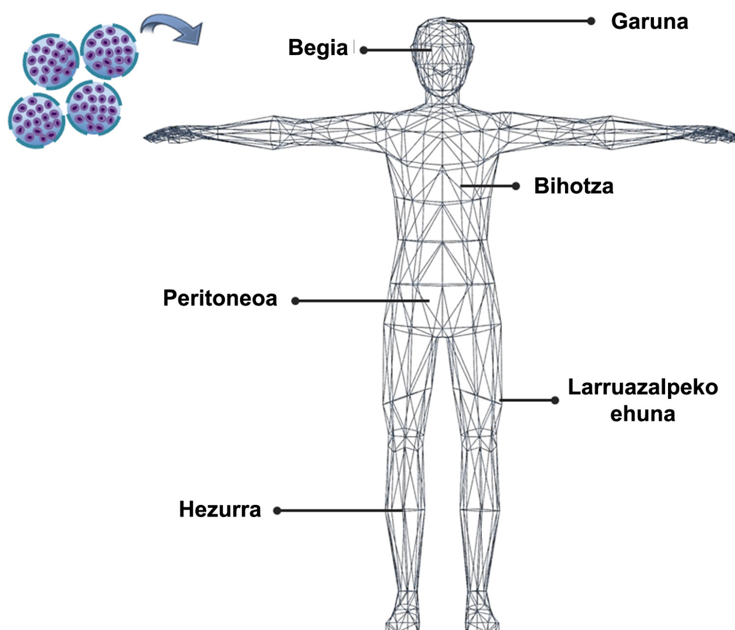
Beste aukera bat, kapsularatutako zelulek eurek matrizea degradatzea da. Estrategia honetan, alginatoen egiturara eransten dira zelulek askatzen dituzten metaloproteinasek (*metalloproteinases* edo MMP) haustu ditzaketen sekuentziak. Beraz, zelulak matrizearekin eta baita ostalariaren ehuna osatzen duten zelulekin ere elkarreragiten dute, sendatze prozesu naturala imitatuz [40, 41]. Ikerketa berri batean, beste aukera bat aztertu zen. Zehazki, kapsulen konposaketa moldatuz degradazio abiadura egokitu daitekeela erakutsi zen. Alginatoaren eta ioi dibalenteen kontzentrazioak doituz eta beste polimero batzuekin nahastuz, kapsulen degradazioa lortu zen. Hain zuzen ere, poloxamerraren gehiketak materialaren degradazioa arindu zuen era nabarmenean, bi astetan kapsulak guztiz haustea lortuz eta kapsularatutako zelulen askapena ziurtatuz ehunaren birsorkuntza gerta zedin [42].

3. Inplantazio kokalekuak

Mikroskapsularatutako zelulak gorputzaren hainbat kokapenetan administratu daitezke (3. irudia). Hori, abantaila kliniko garrantzitsua da, lokalizazio konplexuak tratatzea ahalbidetzen duelako, hala nola, garuna edo begiak. Onura horren benetako potentziala erakusteko, hurrengo atala inplantazio-kokalekuaren arabera banatu da.

3.1. Administrazio intraperitoneala

Barrunbe peritoneala kokapen egokia da mikrokapsularatutako zelulak administratzeko, bolumen handien injekzioa ahalbidetzen duelako [10]. Horrez gain, gune eskuragarria eta nahiko segurua da. Izan ere, frogatu egin da alginato purifikatuaz osatutako mikrokapsula hutsak (zelularik eramaten ez dutenak) intraperitonealki administratu ostean ez zutela gorputz



3. Irudia. Zelula kapsularatze teknologiaren administrazio bide nagusiak.

arrotzaren erreakzio esanguratsurik sortu [43]. Ondorioz, zelula-mikrokapsularatze teknologiaren hainbat aplikazio terapeutikoek abantaila honen probetxu hartu dute.

Zalantzarik gabe, intraperitonealki administratutako mikrokapsulen aplikazio nagusia diabetesaren tratamendua da. 1. motatako diabetesa (*type 1 diabetes* edo T1D) kontrolatzeko, intsulina era exogenoan eta pauta zorrotzetan administratzea beharrezkoa da. Zentzu honetan, zelulen kapsularatzea alternatiba egokia izan daiteke proteina horren askapen iraunkorra lortzeko [44,45]. Horren harira, Langerhansen irlen kapsularatzea aukera oso interesgarria da [45]. β zelula pankreatikoak jatorri ezberdinetatik lortu daitezke. Giza espezieari dagokionez, hildako emaileetatik zelula alogenikoak eskuratu ahal dira [46,47]. Immunoeskasia konbinatu larria (*severe combined immunodeficiency disorder* edo SCID) zuten sagu diabetiko ez-obesoekin (*non-obese diabetic* edo NOD) egindako ikerketa batean, giza-jatorriko Langerhansen irlak inplantatzeak hipergluzemiaren berehalako zuzenketa lortu zuen, eta prozesu esperimentalak iraun zuen bost asteen zehar mantendu zen efektua [48]. Hala ere, giza pankrea-emaileen eskasia dela eta, zelula horiek lortzeko iturri alternatiboak aztertu dira.

Eragozpen hori ekiditen duen aukera interesgarri bat xenotransplanteen erabilera da, besteak beste, txerri jatorriko pankrea zelulak. [24,49].

Irla pankreatikoen ordezko iturrien bila, zelulak genetikoki moldatzea intsulina ekoiztu dezaten estrategia baliotsua da [50]. Beste aukera interesgarri bat zelula amen erabilera da. Aurrerago aipatu den bezala, zelula amak beste zelula mota batzuetan desberdintzatzeko gai dira. Hori dela eta, zelula amen desberdintzapena intsulina jariatzen duten β zeluletan, prozedura egokia izan daiteke mikrokapsularatze teknologiaren bidez T1D tratatzeko. Bereziki, giza zelula amen kapsularatzea epitopo xenogenikorik askatzen ez duen eta era errazean eskalagarria den alternatiba da [51]. Bestalde, kapsulen konfigurazioari esker, zelulak hiru dimentsiotan (3D) hazten dira. Horren harira, frogatuta dago 3Dtan kapsularatutako zeluletan heldutasun markadore primarioen espresioa altuagoa dela bi dimentsiotan hazitako zelulekin konparatuz, eta horren ondorioz, intsulinaren askapena ere handiagoa dela [52]. Abantaila horiek guztiak direla eta, gero eta arreta gehiago jartzen ari da zelula amen kapsularatzean T1D tratatzeko [53,54]. Ikerketa berri batean, enbrioi zelula ametatik lortutako β zelula helduek gluzemiaren kontrol iraunkorra ezarri zuten C57BL/6J sagu immunogai eta diabetikoetan [55]. Gluzemiaren zuzenketa hori, ikerketak iraun zuen 174 egunetan zehar luzatu zen. Denbora tarte hori pasa ostean, sistema animalietatik berreskuratu eta analizatu ondoren, zelulen bideragarritasuna mantentzen zela egiaztatu zen, planteamendu honen potentziala agerian jarriz.

3.2 Larruazalpeko administrazioa

Larruazalpeko administrazioa oso erabilia izan da mikrokapsularatutako zelulak inplantatzeko. Administrazio prozedura erraza izateagatik, klinikoki aplikagarria da. Horrez gain, metodo azkarra, merkea eta sinplea da administrazio parenterala gauzatzeko. Bestalde, beste bide parenteralekin alderatuta, xurgapen abiadura motelagoa da, eragin iraunkorra bermatuz. Abantaila hori hainbat terapia aplikazioetan onuragarria izan daiteke.

Larruazalpeko administrazioa asko erabili izan da eritropoietina (EPO) askatzen duten mikrokapsularatutako zelulak inplantatzeko. Hormona honen bidez anemia kronikoa tratatu daiteke, eritropoiesia eta globulu gorrien desberdintzapena sustatzen baititu [56]. Sistemak EPOren askapen etengabea lortzen du eta horrela, farmakoaren ezegonkortasuna ekiditeaz gain, administrazio errepikatuen beharra desagertzen da.

Izan ere, eredu preklinikoetan, EPOren 300 eguneko askapen jarraitua lortu zen, terapia immunoezabatzailerik erabili gabe [57]. Beste ikerketa batean, inplantatutako zelulen bideragarritasuna eta funtzionalitatea aztertu ziren. Bertan, saguen hematokritoaren igoera antzeman zen, terapiaren balioa baieztatu zuena [58, 59].

Larruazalpeko administrazioaren bidez, antigorputz terapeutikoak ekoizten dituzten mikrokapsularatutako zelulak inplantatu dira ere. Estrategia hau erabilgarria da minbiziaren kontrako terapietan, antigorputzek zelula immuneekin elkarreragitean efektu immunomodulatzailea gauzatzen dute eta. Modulazio hori era desberdinetan lortu daiteke. Alde batetik, antigorputzek zelula immuneen funtzioa sustatzen duten hartzaileak jarraian estimulatu ditzakete. Bestalde, immunitatea inhibitzen duten hartzaileak blokeatu ditzakete. Antigorputz hauek ekoizteko, maiz *hybridoma* zelulak erabiltzen dira [60]. Esaterako, *hybridoma* zelula horien bidez lortutako anti-OX40 and anti-CD137 antigorputzak immunitate zelularra sustatzeko gai izan ziren [61]. Beste ikerketa batean, mikrokapsularatutako zelulek antigeno kartzinoenbrionarioen kontrako antigorputzak ekoizteko gaitasuna erakutsi zuten [62].

3.3 Begi-barneko administrazioa

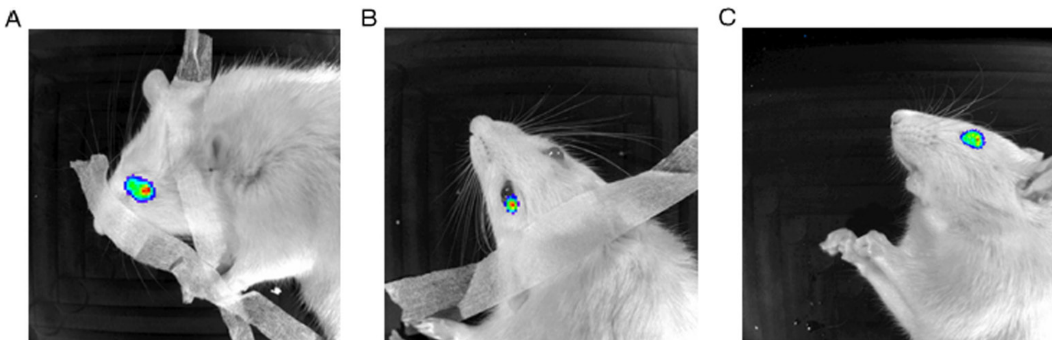
Gaur egun, hainbat begietako gaixotasun tratatzeko begi-barneko injekzioak erabiltzen dira, pazientearen eta osasun profesionalen erosotasunean eragin oso negatiboa izanez. Horrez gain, erretinaren mikroinguruneak, hesi hematoerretinalak eta begiaren anatomia bereziak administrazio egokia gauzatzea saihesten dute. Hori dela eta, arazo horiei aurre egiten dioten estrategia desberdinak ikertu dira.

Estrategia horietako bat zelulen kapsularatzea izan da. Mikrokapsulen begi-barneko administrazioak aipatutako zailtasunak murriztu ditzake, eta horrez gain, tresna baliagarria izan daiteke erretinaren gaitz ugari tratatzeko, esaterako, adinarekin lotutako makulako endekapena (*age macular degeneration* edo AMD) edo diabetesarekin lotutako edema makularra (*diabetic macular edema* edo DME). Gaixotasun horietan, hazkuntza faktore endotelial baskularrak (*vascular endothelial growth factor* edo VEGF) paper garrantzitsua jokatzen du. Izan ere, faktore horren eragin angiogenikoa gehiegizkoa bada, patologia desberdinak ager daitezke, haien artean, neobaskularizazio koroideoa [63]. Hori dela eta, VEGFren blokeoak potentzial handia du begiko gaitzak tratatzeko.

Adibidez, VEGF hartzailearen era disolbagarria (*kinase insert domain receptor* edo Flk-1/ KDR) ekoizten zuten C_2C_{12} mioblastoak mikrokapsularatu eta begi barnean administratzean, emaitza interesgarriak lortu ziren [64]. *In vitro* ikerketek zelulen bideragarritasuna eta sistemaren funtzionalitatea frogatu zuten, eta *in vivo*, kapsularatutako zelulen aktibitatea hiru aste baino gehiagoz monitorizatu ahal izan zen (4. Irudia). Horrez gain, lan honetan, *flow focusing* teknikaren bidez 100 µm-ko kapsulak garatu ziren, tamaina txikiko sistemak ekoiztearen erronkari aurre eginez. Aurrerapauso hori garrantzi handikoa izan zen zelulen mikrokapsularatze teknologian, gorputzaren kokapen zailenetara heltzea ahalbidetzen baitu, hala nola, begira edo nerbio sistema zentralera. Hori dela eta, aplikazio horietarako, kapsularatze sistema tradizionalak alde batera utzi dira, haien bidez kapsulen tamaina eta lodiera kontrolatzea ezinezkoa izateaz gain, zelulen distribuzio uniformea lortzea zaila delako. Bestalde, mikroteknologietan oinarritutako metodologiek kapsularatutako zelulen kopuruaren kontrol fina ahalbidetzen dute, eta baita sistemaren tamainaren eta itxuraren kontrola ere. Hori dela eta, mikrofluidika teknikak funtsezkoak bilakatu dira *conformal coating* - delakoak ekoizteko [65].

3.4 Hezur-barneko administrazioa

Terapia zelularra alternatiba baliagarria da hezur-ehunaren birsorkuntzan. Hala ere, zelulen administrazio metodo arrunten bidez zelulak ez dira hezur ehunean mantentzen eta haien bideragarritasuna eskasa da, terapiaren erabilera klinikoa asko murriztuz. Zentzu horretan, zelulen kapsularatzeak abantaila handiak ditu hezurren birsorkuntza sustatzeko. Alde batetik,



4. Irudia. Mikrokapsulen begi-barneko administrazioa. Kapsularatutako zelulen luminometria ikerketaren 3 asteetan zehar. Tratatutako begiek biziki distiratu zuten arratoi guztietan. Irudi bakoitzak entseguaren aste bakoitzeko taldeko arratoi bana erakusten du (n = 3) arratoi denen argazkia egon dadin **(A-C)**. [64] Erreferentziatik moldatua Elsevieren baimenarekin (© 2011).

mikrokapsulak administratzen diren kokapenean mantentzen dira, tamaina handiagoa izateagatik. Horrek, zelulen mentaketa eta ehunaren sendapena ahalbidetzen du. Bestalde, administrazio dosia prestatzean, sistema polimerikoak zelulak nahasketa eta injekzio prozesuen aurrean babesten ditu. Horrela, hezur akatsa era aproposean bete ahal da. Gainera, 3Dtako konfigurazioak zelulen kultiboa errazten du, hezurraren birsorkuntzan funtsezkoak diren prozesuak suspertuz, besteak beste, proliferazioa edota kaltzio eta hezur proteinen deposizioa [66].

Azken hamarkadetan, zelulen immobilizazioa oso erabilia izan da ehunen birsorkuntza naturala sustatzen duten zelulak administratzeko [38, 39]. Behin inplantatuta, sistemaren biodegradagarritasunak zelulen askapena ahalbidetzen du eta horren ondorioz, ehunaren sendapena hasten da. Hezurraren birsorkuntzaren lehendabiziko etapan, zelulak osteoblastetan desberdintzatu behar dira. Hori dela eta, MSCak alternatiba paregabekoa dira hezur-ingeniaritzan. Berez, aplikazio honetan hainbat jatorriko MSCak proposatu dira. Haien artean, erabilienak hezur-muineko MSCak (*bone marrow MSC* edo BMMSC) [67] eta zilbor hesteko MSCak (*umbilical cord MSC* edo UCMSC) [39] izan dira. Halere, hain erabiliak ez diren beste zelula batzuekin ere emaitza onak lortu dira. Hortzoietako MSCak (*gingival MSCs* edo GMSCs) horren adibide dira. Zelula horiek, hezur akatsean *in situ* mineralizatzeko gai izan ziren, aurretik inolako *in vitro* pre-mineralizazio tratamendurik izan gabe, eta desberdintzapen gaitasuna erakutsi zuten *in vitro* eta baita *in vivo* ere [68].

Ingurumenean dauden hazkuntza faktoreek MSCen desberdintzapen prozesua gidatzen dute. Hori dela eta, azken urteetan, ikerketa desberdinek hazkuntza faktoreen bidez zelula horien potentzial terapeutikoa nola sustatu daitekeen aztertu dute. Faktore horietako bat, hezur morfogenetikoaren proteina-2 (*bone morphogenetic protein* edo BMP-2) da [12]. Hezurraren proteina morfogenetiko hauek (*bone morphogenetic proteins* edo BPM) hazkuntza faktore beta eraldatzailearen (*transforming growth factor* β edo TGFβ) familiaren parte dira eta fun-tsezkoak dira hezurraren mantentze eta konponketa prozesuetan [69]. Lan interesgarri batean, iPSCetatik lortutako MSCak (*induced pluripotent stem cell-derived mesenchymal stromal cell* edo iPSMSC) BMP-2 faktorearekin bi astez estimulatu edo transduzitu ziren eta ondoren, biodegradarritasun azkarreko alginato matrizetan kapsularatu ziren. iPSMSCek, BMMSCek baino proliferazio ahalmen handiagoa erakutsi zuten. Horregatik, alternatiba

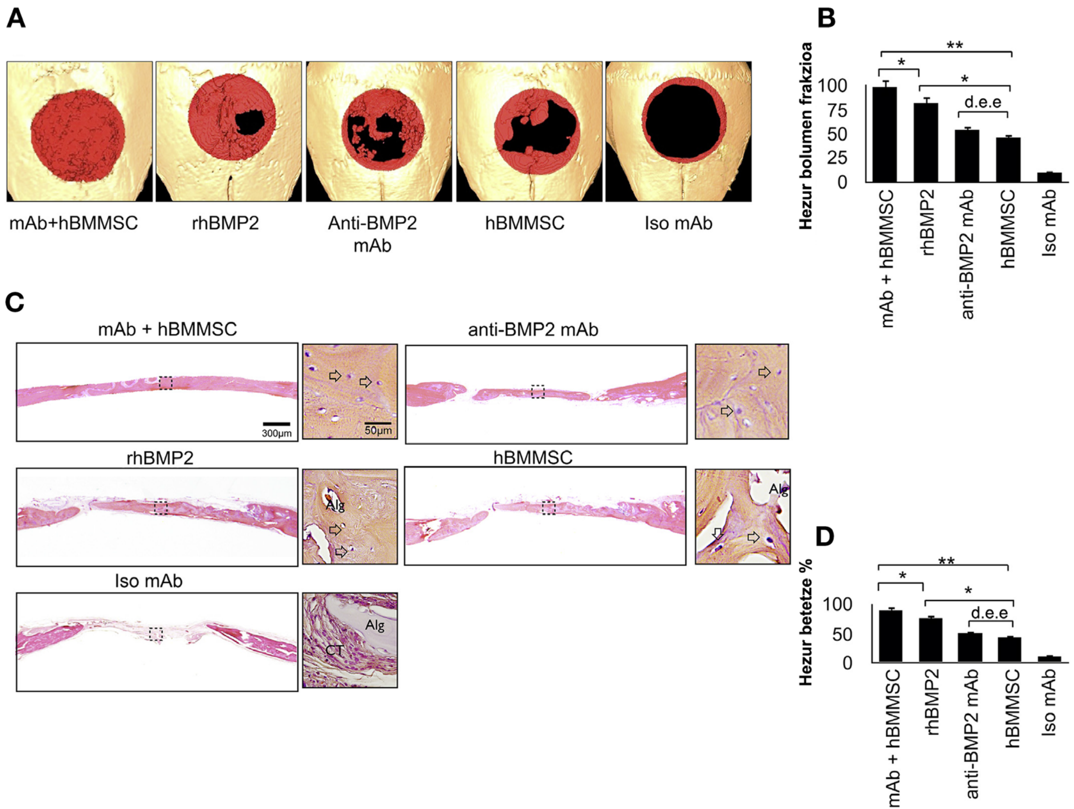
interesgarria dira, batez ere osasuna konprometitua duten pazienteentzat, haien BMMSC autologoek ehunaren birsorkuntza sustatzeko gaitasuna galdu baitute [70]. Kapsula horiek *in vivo*, arratoien garezur akatsetan administratu zirenean, zelulen bideragarritasuna hobetu zen eta ehunaren birsorkuntza nabarmenki sustatu zen. Berez, kontrolarekin alderatuta, hezur birsorkuntza 2-3 aldiz suspertu zen [70].

Beste estrategia interesgarri bat giza BMMSCak (*human BMMSCs* edo hBMMSC) eta anti-BMP-2 antigorputz monoklonalak batera kapsularatzea da. Horrela, antigorputzek BMP ligandoak biltzen dituzte, MSCei hezurrean desberdintzatzeko behar dituzten seinaleak eskainiz. Ikerketa batean, 5 mikrokapsula mota desberdin transplantatu ziren saguen garezur-gangaren akatsetan: (1) hBMMSCak eta anti-BMP2 antigorputzak (mAb + hBMMSC), (2) kapsularatutako giza BMP2 birkonbinatua (*recombinant human BMP-2* edo rhBMP2), (3) anti-BMP2 antigorputz monoklonalak (*anti-BMP2 monoclonal antibodies* edo anti-BMP2 mAb), (4) hBMMSCak soilik eta (5) kontrol negatibo gisa, BMP-2rako zehaztazunik ez duten isotipo antigorputz monoklonalak (*isotype monoclonal antibodies* edo Iso mAb). Emaitzek erakutsi zuten zelulek eragindako osteogenesia era nabarmenean hobetu zela hBMMSCak eta anti-BMP-2 antigorputzak ko-kapsularatzean (5. Irudia) [67]. Bestalde, beste ikerketa batean, BMSCek hezurrean desberdintzatzeko duten gaitasuna sustatzeko, BMP-2 eta VEGF geneekin transfektatu ostean, mikrokapsularatu eta inplantatu ziren [71]. Bi geneekin ko-transfektutako zelulek osteogenesia induzitzeko gaitasun handiagoa izan zuten, BMP-2 genearekin soilik transfektatutako zelulekin alderatuz. Emaitza horien azalpena VEGFaren efektu angiogenikoa izan daiteke, angiogenesia beharrezkoa baita hezur birsorkuntza gauzatzeko. Horrez gain, hazkuntza faktore horrek osteoblasto-bereizketa ere sustatzen du. Datu horiek agerian uzten dute MSCak hezurrean desberdintzatzeko premiazkoak direla hazkuntza faktoreen seinaleak. Ondorioz, hezur akatsen birsortze terapietan estrategia oso interesgarria da faktore hauen erabilera.

3.5. Garezur barneko administrazioa

Sistemikoki administratutako farmakoek zailtasun ugari dituzte garunera heltzeko. Hesi hematoentzefalikoak (*blood brain barrier* edo BBB) odola eta garun-parenkimaren arteko molekulen difusioa erregulatzen du. Horrela, hesi horrek garuna odolean dauden sustantzia

toxikoetatik babesten du. Burmuinaren babesa bermatzeko funtsezko egitura izan arren, BBBa oztopo handia da garun gaixoa tratatzeko [72]. BBBaren eragina ekiditeko, mikrokapsularatutako zelula terapeutikoak garunean bertan administratzea etorkizun handiko estrategia da. Alternatiba honetan, zelulek sustantzia terapeutikoak *de novo* ekoizten dituzte epe luzeko askapena lortuz eta administrazio sistemikoaren albo-ondorioak murrizten dira [73].



5. irudia. Hezurraren birsorkuntza. Anti-BMP2 antigorputzarekin (Anti-BMP2 mAb) kargatutako RGD-alginato mikrosferetan kapsularatutako hBMMSCek hezurraren birsorkuntza sustatzen dute tamaina handiko burezur akatsetan. **(A)** Tomografia mikro-konputazionalaren bidez lortutako irudiek saguen burezur akatsen konponketa erakusten dute. Birsortutako hezurra gorriz irudikatu da. **(B)** Tomografia mikro-konputazionalaren irudietan oinarrituta, hezur birsorkuntzaren analisi kuantitatiboa. **(C)** Saguen burezur akatsen konponketaren errepresentazio mikroanatomikoa, transplantearen 8 aste ondoren. Magnifikazio handiko (40x) eta txikiko (4x) hematoxilina-eosina irudiak. Geziak osteozitoak adierazten dituzte. **(D)** Burezur akatsen analisi histomorfometrikoak tamaina handiko burezur akatsetan hezur eraketa erlatiboa erakusten du. * p <0,05, ** p <0,01. n = 4 talde bakoitzean. Laburdurak: hBMMSC: giza hezur-muineko zelula estromal mesenkimalak (*human bone marrow mesenchymal stromal cells*). Alg: alginatoa. CT: ehun konektiboa (*connective tissue*). mAb + hBMMSCs: hBMMSC eta anti-BMP2 antigorputzen konbinazioa. rhBMP2: kapsularatutako giza BMP2 birkonbinatua (*recombinant human BMP-2*). Iso mAb: isotipo antigorputz monoklonalak (*isotype monoclonal antibodies*). d.e.e.: desberdintasun esanguratsurik eza. [67] Erreferentziatik moldatua Elsevier-en baimenarekin (© 2013).

Hala ere, administrazio eta erauzketa prozedurak oso inbasiboak dira, estrategia honen potentziala murriztuz.

Faktore neurotrofikoek neuronen biziraupen, desberdintzapen eta funtzio normala mantentzen laguntzen dute. Hori dela eta, faktore hauek askatzen dituzten mikrokapsularatutako zelulak administratzeak hainbat abantaila izan ditzake gaixotasun neurodegeneratiboen tratamenduan. Gaitz hauetako bat Alzheimerra (*Alzheimer's disease* edo AD) da, dementzia motarik ohikoena. Neuroendekapenezko gaixotasun honetan neuronak suntsitzen dira, funtzio kognitiboen mailaz mailako eta erabateko narriadura ekarri arte. Gaitzaren garapenarekin zerikusia duten bi faktore sakonki aztertu izan dira: tau proteina eta beta amieloide peptidoa. Zehazki, tau proteina hiperfosforilatua duten haril neurofibrilarrak eratzen dira neuronetan eta proteina amiloideak metatzen dira, plaka senil eta neuritiko modura. Gaitzaren tratamenduan, VEGF bezalako faktore angiogenikoen administrazioa proposatu da efektu neurobabeslea bilatuz. Saguekin egindako ikerketa preklinikoetan, VEGF askatzen zuten mikrokapsularatutako zelulek hiperfosforilatutako tau proteinaren maila murriztu zuten. Bestalde, ehunaren baskularizazioa areagotu zen, proteina amieloideen argitzea suspertuz. Orokorrean, efektu horiek zelulen bideragarritasuna eta saguen kognizioa nabarmenki hobetu zituzten [74]. Era berean, beste ikerketa batek frogatu zuen VEGFak hipokanpoaren giro dentatuan zelulen proliferazioa sustatu zuela. Hori estrategia oso interesgarria da garunean gauzatzen den proteina amieloideen metaketa tratatzeko [75].

Nerbioen hazkuntza faktorea (*nerve growth factor* edo NGF) eta zelula glialetatik eratorritako faktorea (*glial cell line-derived neurotrophic factor* edo GDNF) ekoizten dituzten zelulak Parkinson gaixotasunaren (*Parkinsons' disease* edo PD) tratamendu gisa proposatu dira. PD nerbio-sistema zentralaren gaixotasun neuroendekatzailea da. Bertan, dopamina sortzen duten substantzia nigrako neuronak etengabe galtzen dira. Horren ondorioz, endekapen motorea gertatzen da, sintoma nagusiak mugitzeko zailtasuna, moteltasuna, gogortasuna eta dardara direlarik. NFG eta GDNF bezalako faktoreen askapen egonkorraren bidez, neuronen funtzioa sustatzea eta ondorioz, gaixotasunaren sintomatologia zuzentzea bilatzen da. Gaur egungo tratamenduen helburua dopamina maila igotzea da, mugimendu arazoak murrizteko. Tratamendu eraginkorra izan arren, denborarekin efektua desagertzen da, ez baitu gaitzaren eboluzioa geldiarazten. Ostera, faktore neurobabesleek neuronen bideragarritasuna eta

funtzioa hobetzen dute, endekapen-prozesua motelduz, denborarekin eraginkor izateari uzten dion dopamina mailaren igoera eragin beharrean [76]. Bereziki, kapsularatutako zelulek arratoi gaixoen gorputz ildaskatuan GDNFa askatzeko ahalmena erakutsi zuten 3 hilabetez, animalien jokabidea zuzenduz [77]. Beste lan batean, NGFa askatzeko ahalmena zuten zelulak mikrokapsularatu eta kapsularatu gabeko zelula kromafinekin batera inplantatu ziren, azken hauen bideragarritasuna sustatzeko. Emaitza interesgarriak lortu ziren, berez, zelula kromafinen biziraupena luzatu zen eta ondorioz, animalien biraketa-portaera hobetu zen [78, 79]. Beste estrategia interesgarri bat glukagoia bezalako peptidoa (*glucagon-like peptide-1* edo GLP-1) askatzen duten kapsularatutako zelulak administratzea da. Garunean, GLP-1 errezeptoreen aktibazioa efektu neurobabesle eta neurotrofikoak ditu, gaitz anitzen tratamenduan alternatiba interesgarria izanez, esaterako alboko esklerosi amiotrofikoa (*amyotrophic lateral sclerosis* edo ALS) [80], AD [81], edo garuneko lesioak [82-83].

Faktore neurotrofikoak burmuinean bertan administratzean emaitza onak lortu direnez, oso interesgarria da era naturalean faktore hauek ekoizten dituzten zelulen mikrokapsularatzea. Horien artean, plexo koroideoetako zelulak (*choroid plexus cell* edo CPC), garun ehunaren sendaketa sustatzen dute, faktore terapeutikoen ekoizpenaren bidez [84,85]. Adibidez, zelula hauek ADren tratamenduan aztertu dira [86]. Arratoietan AD eragin zen eta ondoren, kapsularatutako CPCak inplantatu ziren garun-kortexean. Neurogenesia areagotzeaz gain, tratamenduak neuronen bideragarritasuna hobetu zuen, apoptosia murriztuz. Ondorioz, animalien errekuperazio orokorra gauzatu zen, epe luzeko oroimena hobetuz.

3.6. Bihotz barneko administrazioa

Miokardio-infartuaren ohiko kudeaketak sintomak arindu eta narriadura prozesua moteldu ditzake, baina ez du ehunaren funtzioa berreskuratzen. Arazo horri aurre egiteko, MSCetan oinarritutako terapiak alternatiba gisa proposatu dira ehun baskularraren bisorkuntzarako. Izan ere, zelula horiek birsortze prozesuan parte hartzen duten molekula aktibo ugari jariatzeko ahalmena dute, hala nola hazkuntza faktore hepatozitikoa (*hepatocyte growth factor* edo HGF) edo VEGF. Hala ere, inplantatutako zelulak administrazio lekuan mantentzea erronka handia da, faktoreen jariaketa iraunkorra lortzeko. Bihotzaren kasuan, bere etengabeko uzkurdura mugimenduek injektatutako zelulen galera eragiten dute [87]. Izan ere, ikerketa

desberdinek frogatu dute kapsularatu gabe administratutako zelulen % 0,1-15 bakarrik mantentzen dela miokardioan [88-90].

Arazo hori ekiditeko, MSCen kapsularatzea alternatiba egokia bihurtu da. Mikrokapsulen tamaina handiagoak zelulak bihotz ehunean mantentzea errazten du, eta beraz, haiek ekoiztutako faktore terapeutikoen etengabeko askapen lokala lortzen da [91]. Adibide interesgarri batean, gantz-ehunetik eratorritako MSCak (*adipose tissue-derived stem cells* edo ASC) genipinarekin gurutzatutako alginato eta kitosano mikrokapsuletan bildu eta *in vivo* xenotransplantatu ziren arratoien infartatutako bihotzetan. Estrategia honek, zelulen atxikipena hobetzeaz gain, fibrosia eta bihotz-disfuntzioa murriztu zuen [92].

Bestalde, *in vivo* administrazioetan, kapsularatutako zelulen biziraupena eta migrazioa jarraitzeko aukera izateak inplantearen segurtasuna era nabarmenean sustatzen du. Ikerketa interesgarri batean, sistema berri bat garatu zen helburu honekin. Zehazki, txerritik eratorritako ASCak alginatoan mikrokapsularatu ziren eta burdin oxido superparamagnetikoarekin (*superparamagnetic iron oxide* edo SPIO) egindako nanopartikulekin konbinatu ziren [93]. *In vivo* ikerketetan, zelulen bideragarritasun egokia lortu zen eta haien detekzioa entsegua amaitu arte gauzatu zen. Beste lan batean, kapsularatutako giza MSCen jarraipena egitea lortu zen biolumineszentziaren bidez. Horrez gain, kapsularatzeak zelulen atxikipena hobetu eta orbainen eraketa murriztu zuen [94]. Emaitza garrantzitsu hauek lortu arren, ikerketa gehiago behar dira zelula horiek bihotzaren birsorkuntzan duten eginkizuna erabat ulertzeko.

4. Entsegu klinikoak

Mikrokapsularatze teknologiaren balioa jada frogatu da entsegu preklinikoetan animalia eredu desberdinekin, klinikarako jauzia suspertuz. Estrategia honek duen potentziala dela eta, giza entsegu kliniko ugari burutu dira. Zentzu horretan, 1. taulak zenbait entsegu garrantzitsu biltzen ditu.

Ikerketa horietan, mikrokapsularatutako zelulak gaixotasun desberdinak tratatzeko erabili dira. Halere, ahalegin nagusia diabetesaren inguruan egin da. Kapsularatutako zelulekin burutu zen lehendabiziko entsegu klinikoa 1994. urtean izan zen [95] eta, gaur egun oraindik,

1. Taula. Mikrokapsularatutako zelulekin egindako entsegu klinikoak. APA: alginato-PLL/PLO-alginato. MSC: zelula estromal mesenkimalak (mesenchymal stromal cells), GLP1: glukagon bezalako peptidoa 1 (*glucagon-like peptide-1)*, PLO: poli-L-ornitina, CYP2B1: zitokromo P450 2B1.

Ikertzailea edo konpainia	Zelulen jatorria	Biomateriala	Inplantearen kokapena	Immunosupresioa	Erref.
Diabetesa					
Soon-Shiong *et al*.	Langerhans-en irla alogenikoak	APA mikrokapsulak	Barrunbe peritoneala	Bai	[95]
Calafiore *et al.*	Langerhans-en irla alogenikoak	APA mikrokapsulak	Barrunbe peritoneala	Ez	[96,97]
Tuch *et al.*	Langerhans-en irla alogenikoak	Ba^{+2} alginato mikroesferak	Barrunbe peritoneala	Ez	[98]
Jacobs-Tulleneers-Thevissen *et al.*	Langerhans-en irla alogenikoak	Ca^{+2}/Ba^{+2} alginato mikroesferak	Barrunbe peritoneala	Ez	[48]
Living Cell Technologies (LCT)	Langerhans-en irla xenogenikoak (txerria)	APA mikrokapsulak	Barrunbe peritoneala	Ez	[99]
Garezur-barneko hemorragia					
Brinker *et al.*	GLP-1 ekoizteko transfektatutako MSCs alogenikoak	Alginato mikrokapsulak	Garuna	Ez	[104]
Parkinson gaixotasuna					
Living Cell Technologies (LCT)	Plexo koroideoetako zelula xenogenikoak (txerria)	Alginato-PLO mikrokapsulak	Garuna	Ez	[105]
Pankreako minbizia					
Löhr *et al.*	CYP2B1 ekoizten duten 293 zelula alogenikoak	Zelulosa-sulfato mikrokapsulak	Tumorearen odol-hodiak	-	[109-112]

estrategia berriak aztertzen ari dira ikerketa anitzetan [10]. Soon-Shiong eta kideen lana aitzindaria izan zen arlo honetan. Bertan, paziente immunodeprimitu baten gluzemia indizea kontrolatu zen bederatzi hilabetez [95]. Aurrerapauso honen ondoren, hainbat ikerketek frogatu dute zelulen kapsularatze teknologiaren potentziala diabetesaren tratamenduan. Farmako immunoezabatzaile konkomitanteen erabilera alde batera utzi eta inplantearen administrazioaren ondoren, ez dira albo efekturik edo sistema immunearen sentsibilizaziorik antzeman [96, 97]. Horrez gain, denbora luzean intsulina maila mantentzea lortu da [98] eta baita zelulen bideragarritasun egokia eta glukosarekiko erantzuna ere hiru hilabeteko transplanteetan [48]. Gaur egun, *Living Cell Technologies* (LCT) enpresa lanean ari da DIABECELL® produktuaren entsegu klinikoetan. Ikerketak 1996. urtean hasi ziren eta hortik aurrera, emaitza itxaropentsuak aurkeztu dira [99 - 103]. Zehazki, kapsulen atxikipena frogatu dute administrazioaren bederatzi urte eta erdi ondoren. Berriki, webgunearen buletinak IIb / III faseko ikerketaren hasieraren berri eman du [10].

Garezur-barneko odoljarioaren kasuan, hematoma kentzeko kirurgiaren ondoren, ebakuntzaren emaitza hobetzeko, GLP1 faktore antiinflamatorio eta neurobabeslea askatzen zuten kapsularatutako zelulak inplantatu ziren. I / II faseko entsegu klinikoek tratamenduaren segurtasuna ebaluatu zuten eta ez ziren antzeman kirurgiarekin ezta inplantatearekin harremandutako alboko efekturik [104].

PDri dagokionez, LCT enpresaren ekarpena nabarmena izan da [105]. Haien produktua, NTCELL®, datu prekliniko itxaropentsuak lortu zituen PDren eredu batean [106]. 2015eko ekainean amaitutako I / IIa faseko entsegu kliniko batean, kapsulen segurtasuna eta efektu klinikoa aztertu ziren. Emaitzek frogatu zuten inplanteak seguruak eta ondo onartuak izan zirela. Horrez gain, paziente guztien sintoma klinikoak hobetu ziren eta efektua mantendu zen 26 aste igaro ondoren [107]. 2016ko martxoan, IIb faseko entsegua hasi zen dosi eraginkorrena bilatzeko [105]. 2019ko maiatzean ikerketa horren aurretiko emaitzak argitaratu ziren,produktuaren efektua frogatuz. Hala ere, III. faseko entseguak egitea beharrezkoa da oraindik tratamenduaren balioa egiaztatzeko [108].

Kapsularatutako zelulen bidez, pankreako minbiziaren tratamendua ere ikertu da giza pazienteetan burututako entsegu klinikoetan. Bereziki, P450 2B1 (CYP2B1) zitokromoa espresatzen

zuten zelulak erabili ziren. CYP2B1, *ifosfamide* agente zitotoxikoa metabolizatzean bere metabolito aktiboak ekoizten ditu. Egoera normal batean, konbertsio hori gibelean gauzatzen da eta metabolito zitotoxikoak tumorearen ingurura heltzen dira zirkulazio sistemikoaren bidez. Hala ere, albo efektu sistemikoek terapiaren erabilera era nabarmenean mugatzen dute. Zelulen kapsularatzeak arazo hori gainditu dezake. Mikrokapsulak tumorearen inguruan inplantatu daitezkeenez, profarmakoaren eraldaketa kaltetutako lekuan gertatzen da eta ondorioz, *ifosfamide*ren dosi baxuagoa administratu daiteke. I / II faseko entsegu kliniko batean, *ifosfamide* farmakoaren 1 g / m^2 / eguneko dosiarekin, gaixoen biziraupena bikoiztu zen. Terapiaren segurtasuna ere frogatu zen, paziente bakar batean antzeman baitziren tratamenduarekin harremandutako albo efektuak [109 - 111]. Ondorengo II. faseko entsegu batean, *ifosfamide* farmakoaren dosia 2 g / m^2 / eguneko igo zen. Bertan, terapiaren eraginkortasuna antzekoa izan zen, baina alboko efektu larriagoak antzeman ziren [112]. Emaitza itxaropentsu horiek lortuta, hurrengo faseetako entseguak burutzea espero da.

Laburtuz, gaur egun, hainbat estrategiek mikrokapsularatze teknologia klinikara gerturatzea dute helburu. Ikerketa horien arrakasta gaitz askoren kudeaketa hobetzeko abiapuntua izango litzateke.

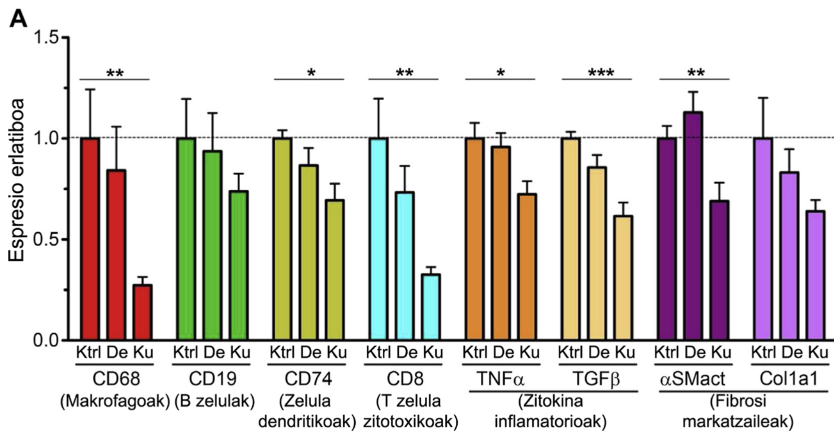
5. Etorkizuneko ikuspegia

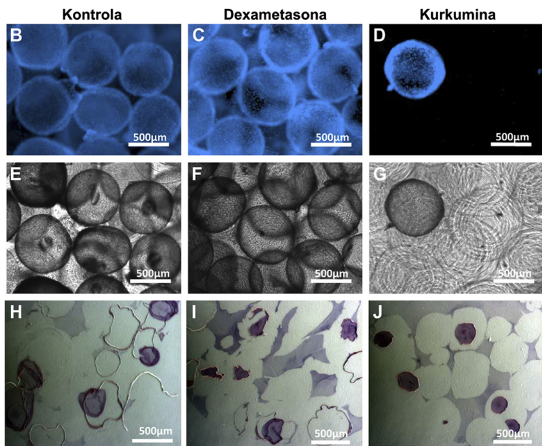
Zelulen kapsularatze teknologiaren inguruko lehendabiziko ikerketak duela lau hamarkada hasi ziren jada. Azken urteetan aurrerapen esanguratsuak egon arren, oraindik hainbat erronka gainditu behar dira estrategia hau klinikara hurbiltzeko.

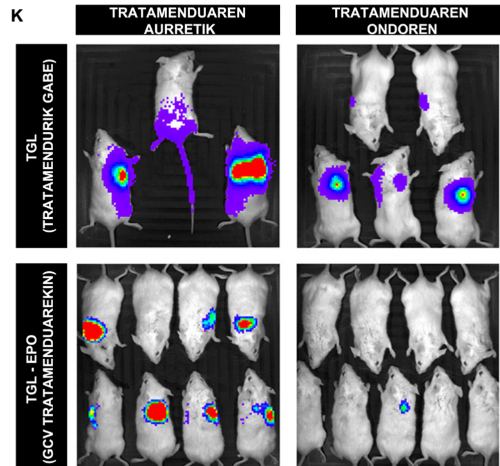
Erronka horien artean, biobateragarritasuna nabarmena da. Inplantearen funtzionalitatea ostalariaren sistema immunologikoaren menpe dago. Izan ere, kapsulak administratzen direnean, sistema immuneak prozesu ugari eragiten ditu, haien artean, hantura, fibrosia, gorputz arrotzen zelula erraldoien eraketa eta lesioak inplantazio gunean. Erantzun hori kaltegarria da kapsularatutako zelulentzat, eta baita pazienteentzat ere, ondoeza eta mina eragin ditzake eta. Horren harira, biobateragarritasunarekin lotutako faktoreak sakonki aztertu zituen ikerketa berri batek. Zehazki, estreptozozina (STZ) farmakoaren bidez C57B6/J saguetan diabetesa eragin zen eta mikrokapsulak inplantatu zitzaien. Kapsulak animalietatik esplantatu ondoren,

hauen azaleran markatzaile immunologiko eta fibrotikoen espresioa ikertu zen (6.A irudia) [113]. Arazo honi aurre egiteko, hainbat planteamendu aztertu izan dira, alternatiba batzuek emaitza onak aurkeztu dituztelarik. Adibidez, farmako antiinflamatorioen ko-administrazioa estrategia egokia izan daiteke. Hanturak basodilatazioa eragiten du eta ondorioz, iragazkortasuna areagotzen da, zelula immuneen migrazioa erraztuz inplantearen gunera. Hanturakontrako sendagaien artean, kurkuminak [113], dexametasonak [114,115] edo pentoxifilinak [116] emaitza onak erakutsi dituzte. Era berean, hantura bitartekarien antagonisten erabilera ere baliagarria izan da, hala nola, mugikortasun handiko talde kutxa 1 proteina (*high mobility group box protein 1* edo HMGB1) [117]. Dexametasona edo kurkuminarekin kargatutako mikrokapsulekin burututako ikerketa batean, inplanteak administratu ziren eta bi hilabete ondoren berreskuratu zirenean, biobateragarritasuna era nabarmenean hobetu zela behatu zen (6.B-J irudia) [113]. Beste aukera bat kapsularen egituran ezaugarri antiinflamatorioak dituzten motiboak eranstea da. Ildo horretan, sulfatatutako alginatoak [118] edo interleukin 1 hartzaileak (IL-1R) [29] erabili dira, zeintzuek zitokina anitzen efektua gutxitu zuten eta ondorioz, zelulen bideragarritasuna sustatzeko ahalmena erakutsi zuten. Azkenik, aipagarria da inplantearen biobateragarritasuna hobetzeko, zelula efektoreekin batera MSCak [32,119-122] edo Sertoli zelulak [123,124] ko-kapsularatzea alternatiba interesgarria dela. Zelula horiek efektu immunomodulatzaile baliotsuak eragin ditzakete, administratutako zelula efektoreen patua hobetuz eta horrela, inplantearen funtzionalitatea eta epe luzeko iraupena suspertuz.

Biobateragarritasuna ere kontuan hartuta, kapsulen tamainaren inguruan eztabaida handia egon da. Ikerketa batean, 1,5 mm-tik gorako diametroa zuten material esferikoek diametro txikiagoa zutenak baino bideragarritasun hobea erakutsi zuten. Lan horretan, formaren garrantzia ere aztertu zen. Ikertzaileen arabera, gorputz arrotzen erreakzioak ekiditeko ez da nahikoa tamaina handitzearekin, forma ere kontuan hartu behar da. Ondorioz, 1,5 mm-ko alginatozko esferak animalietan administratu zituzten eta emaitzek agerian utzi zuten ez zela metaketarik egon sei hilabetez [125]. Hala ere, orokorrean, ahalegin handia egin da mikrokapsulen tamaina txikitzeko [8], kapsula txikiak beharrezkoak baitira gorputzaren kokapen zehatzetara heltzeko, garunera edo begietara, besteak beste. Horrez gain, teknologia honetan, orobat onartuta dago kapsula txikietan (< 350 μm) nutrienteen eta oxigenoaren difusioa hobea dela tamaina ertaineko (350-700 μm) edo handiko kapsuletan (> 700 μm) baino [126]. Izan ere, kontuan hartu behar da faktore horien garraio egokia ezinbestekoa dela zelulen







6. irudia. Zelula kapsularatze teknologiaren erronkak: biobateragarritasuna eta biosegurtasuna. C57B6/J sagu diabetikoetan inplantatutako alginato mikrokapsuletan gertatutako fibrosiaren karakterizazioa. **(A)** Administrazioaren hilabete bat ondoren, mikrokapsulak berreskuratu ziren eta qPCR teknikaren bidez ostalariaren markatzaile immunologiko eta fibrotikoak aztertu ziren. Analizatutako markatzaileak hurrengoak izan ziren: CD68: makrofagoak, CD19: B zelulak, CD74: zelula dendritikoak, CD8: zelula T zitotoxikoak, TNF-α eta TGF-β: zitokina inflamatorioak eta aSMact eta Col1a1: fibrosiarekin lotutako markatzaileak. Emaitzak: batezbesteko ± s.e.m., (n = 7). * / ** / *** $p < 0,05$ / $p < 0,01$ / $p < 0,001$ adierazten dute hurrenez hurren. Laburdurak: TNF-α: α tumore nekrosi faktorea. TGF-β: hazkuntza faktore β eraldatzailea (*transforming growth factor* β). αSMact: muskulu leuneko α-actina (α-*smooth muscle actin*). Col1a1: 1A1 kolagenoa. s.e.m: batezbestekoaren errore estandarra (*standard error of the mean*). Administrazioaren 2 hilabete ondoren, mikrokapsulak berreskuratu eta ostalariaren DNA koloreztatu zen. Mikrokapsula kontrolen taldearen **(B)** eta dexametasona **(C)** edo kurkumina **(D)** zuten mikrokapsulen irudiak. **(E-G)** Mikrokapsula berdinen fase-kontraste irudiak. **(H-J)** Hematoxilina-eosina tindaketaren irudiak. **(K)** Argiaren igorpena GCV tratamenduaren aurretik eta ondoren aztertu zen TGL-EPO taldean (GCV tratamenduarekin) eta baita TGL taldean ere (tratamendurik gabekoa). Laburdurak: GCV: ganziklobir farmakoa. EPO: eritropoietina. S.D.: desbideratze estandarra (*standard deviation*). A-J [113] erreferentziatik moldatua (© 2013), K [135] erreferentziatik moldatua Elsevieren baimenarekin (© 2012).

bideragarritasuna mantentzeko. Ondorioz, tamaina egokia aukeratzeak erronka izaten jarraitzen du, oreka lortu behar baita elikagaien garraio zuzenaren eta erantzun immunologiko minimoaren artean.

Kapsula barneko mikroingurunea kontrolatzea beste desafio garrantzitsu bat da. Zelulak haien inguru naturaletik kanpo kultibatzeak biologia zelularrari buruzko ikuspegi zabal bat izatea eskatzen du. Zelulak ez dira indibidualki hazten, haien inguruan dauden seinale askoren beharra daukate. Horien artean, matrize extrazelularreko (*extracellular matrix* edo ECM) molekulak, morfogeno disolbagarriak, hazkuntza faktoreak eta inguruan dituzten beste zelulak nabarmen dira [127]. Ehunaren fisiologia elementu horien arteko elkarrekintzen menpe dago. Hori dela eta, zelulen funtzioa eta bideragarritasuna sustatzeko ehun naturala imitatzea estrategia interesgarria da. Zelulen mikroingurunea imitatzeko, kapsulak motibo desberdinekin eraldatu daitezke. Aukera bat ECMeko proteinak eranstea da [22]. Hala ere, alternatiba hori albo batera utzi eta ECMeko proteinen peptido-sekuentzia laburrak erabiltzen dira gehienbat. Peptido horiek proteinaren funtzionalitatea mantentzen dute, eta tamaina askoz txikiagoa dutenez, errazagoa dute zelulekin elkarri eragitea. Zalantzarik gabe, peptido motiborik erabilienetakoa arginina-glizina-aspartiko azidoa (RGD) da [31,128-130]. Peptido hori, ECMeko proteina ugariren parte da, esaterako fibronektinarena, kolagenoarena edo lamininarena, eta bere erabilerak emaitza interesgarriak lortu ditu [31,128-130]. Beste adibide batzuk YIGSR (Tyr-Ile-Gly-Ser-Arg) eta IKVAV (Ile-Lys-Val-Ala-Val) dira, lamininan aurkitu daitezkeenak [51,131]. Bestalde, kapsulen matrizeak funtzionalizatzeko beste hainbat estrategia aztertu dira. Esaterako, ECM liofilizatuaren hautsa mikrokapsuletara gehitzea [132], hazkuntza faktoreak lotzeko domeinuak eranstea [133] edo kapsula osatzen duen polimeroa galaktosilatutako kitosanoarekin eraldatzea [134].

Kapsularatze teknologia klinikara eramateko inplantearen segurtasuna bermatzea ezinbestekoa da. Izan ere, mikrokapsulen erabilera segurua izatea derrigorrezkoa da, eta zoritxarrez, oraindik ezin da esan helburu hori lortu denik. Hori dela eta, segurtasun arazoak ekiditeko, zenbait ikerketek aztertu dute kapsulen funtzioa nola aktibatu eta desaktibatu. Ikertutako bide bat kapsularatutako zelulen genoman gene suizidak sartzea da. Horrela, ekoizten dituzten faktore terapeutikoen askapena gelditu daiteke gene hori induzitzen duen estimulu bat emanda. Hain zuzen ere, azterketa arrakastatsu batean, ganziklobir (GCV) farmakoaren bidez

desaktibatu ziren genoma eraldatuta zeukaten zelulak. Zehazki, kapsularatutako zelulak hiru faktoreekin transfektatu ziren: (1) *TGL triple-fusion reporter* genearekin (*Herpes Simplex virus type 1 thymidine-kinase* (HSV1-TK) gene suizida kodifikatzen duena), (2) proteina berde fluoreszentearekin (*green fluorescent protein* edo GFP) eta (3) *Firefly Luciferase* ($SFG_{NES}TGL$) bektorearekin (Fig. 6K) [135]. Era berean, *TetOn / off* sistemak faktore terapeutikoen askapen kontrolatua gauzatzeko tresna baliagarria izan daitezke [12-136].

Azkenik, aipagarria da teknologia hau klinikara eramateko biosistemaren eskalagarritasuna ere lortu behar dela. Gaur egun, badaude kapsula kopuru handia ekoiztea ahalbidetzen duten *high throughput*-delakoen sistemak [137, 138]. Hala eta guztiz ere, laborategien arteko aldakortasuna oztopo bat da eskalagarritasun egokia lortzeko, emaitzak ez baitira errepikakorrak. Ikerketa batean, arazo hori gainditzeko ahalegin bikaina egin zen. Hain zuzen ere, kapsulak ekoizteko hainbat sistema konparatu ziren eta laborategien arteko aldakortasuna sortzen duten faktore teknikoak identifikatu ziren. Ikertzaileen arabera, eskalagarritasuna eta errepikakortasuna lortzeko, derrigorrezkoa da kapsulen ezaugarrietan eragina duten bost parametro konkretu deskribatzea. Faktore horiek hurrengoak dira: (1) erabilitako polimeroa, (2) iragazkortasuna, (3) gainzaleko ezaugarriak, (4) biobateragarritasuna eta (5) kontserbazio baldintzak [139]. Horrez gain, zelula mikrokapsulen ekoizpena oso garestia da, teknologiaren konplexutasuna dela eta. Berez, zelulak kultibatzeko denbora epe luzeak behar dira eta erabiltzen diren erreaktiboak garestiak dira. Hori, eskalagarritasuna lortzeko beste oztopo bat da, sendagaia merkea izatea funtsezkoa baita pazienteentzat eskuragarria izateko [22]. Bestalde, produktuak eskalatu baino lehen, erakundeen onarpena derrigorrezkoa da. Europar batasuneko (EB) erakundeek terapia aurreratuetako osasun produktuentzako araudia argitaratu zuten (1394/2007 (EC) araudia). Araudi hori, Europan zehar osasun produktuen mugimendu librea bermatzeko diseinatu zen, EBko merkatuan sarbidea errazteko eta Europako enpresen lehiakortasuna sustatzeko. Aldi berean, gaixoentzako osasun babes maila altuena bermatzea bilatzen du araudi honek. Interesgarria da, araudi horrek kontuan hartzen duela terapia aurreratuetako produktu askok material biologikoak (ehunak, zelulak) eta egitura kimikoak (polimerozko *scaffold*ak, esate baterako) konbinatzen dituztela. Hori dela eta, produktu hauentzat eskakizunak egokitu behar direla aipatzen da, ez baitira farmazia tradizionalean komertzializatzen diren produktuenak bezalakoak [140].

6. Ondorioak

Zelula bioaktiboen mikrokapsularatze teknologian aurrerapauso handiak egin dira azken urteotan, eta ondorioz, potentzial handiko hainbat tratamendu garatu dira. Teknologia honen abantaila nagusia sistemak duen aparteko moldagarritasuna da. Kapsula egituratzen duten biomaterialen ezaugarrien arabera, sistemaren funtzionalitatea aldatu egingo da eta aplikazioa desberdina izango da. Alde batetik, faktore terapeutikoen askapen iraunkorra lortzeko helburuarekin, biomaterial ez-biodegradagarriekin osatutako esfera polimerikoak mintz erdiiragazkor batekin inguratzen dira. Horrela, zelula bioaktiboak sistema hauetan kapsularatzean, *de novo* ekoizten dituzten faktore terapeutikoak etengabe askatzen dira. Kontuan hartu behar da zelula motaren arabera askatuko den biomolekula desberdina izango dela, eta horrela, aplikazio anitzetan erabil daitekeela estrategia hau. Bestalde, material biodegradagarriak erabiltzeak (berez degradagarriak direnak edo biodegradagarriak izateko moldatuak izan direnak) kapsularatutako zelulak ehun konkretu batean askatzea ahalbidetzen du, helburu berritzaileekin.

Kapsularatze teknologiaren garapen garrantzitsua burutu diren azterketa prekliniko ugariei esker gauzatu da. Hori dela eta, sistemaren inguruan ezagutza handia lortu da, estrategia hau aplikazio klinikora hurbilduz. Nahiz eta oraindik hobekuntza anitz behar diren inplanteen biosegurtasuna eta biobateragarritasuna lortzeko, alorrean burututako entsegu klinikoek teknologia itxaropentsu hau klinikarako jauziaren bidean jarri dute.

Eskerrak

A. Gonzalez Pujanak Eusko Jaurlaritzari doktoratu aurreko laguntza eskertzen dio. Proiektu hau neurri batean Eusko Jaurlaritzak finantziatu du (Talde kontsolidatuak, IT-907-16) eta baita Euskal Herriko Unibertsitateak ere (UPV/EHU) (UFI11/32).

Erreferentzien zerrenda 81-89 orrialdeetan aurkitzen da.

Hipotesia eta helburuak

Zelula estromal mesenkimalek (*mesenchymal stromal cells* edo MSC) hainbat faktore immunomodulatzaile askatzeko gaitasuna dute eta, beraz, potentzial handia erakusten dute hanturazko gaixotasun eta gaitz immune ugari tratatzeko. Hala ere, MSCetan oinarritutako terapien arrakasta lortzeko, zelula horien administrazio prozedurak optimizatu beharra dago. Zain barneko administrazioa erabiltzean, MSCak detektaezinak dira eta ostalariaren sistema immuneak zirkulaziotik ezabatzen ditu, zelulen iraunkortasuna erabat mugatuz. Horrez gain, hainbat gaixotasunen tratamenduan MSCen eragin terapeutikoa itu ehunean gauzatzea beharrezkoa da.

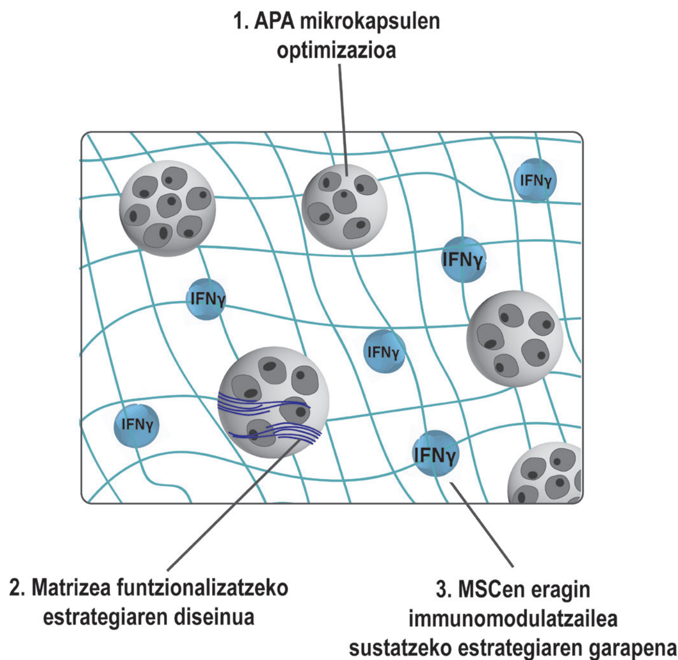
Zentzu horretan, zelulak kapsularatzen dituzten hiru dimentsiotako (3D) sistemek arazo horiek ezabatzen dituzte, zelulen iraunkortasuna hobetuz eta, ondorioz, haien efektuak luzatuz. Aukera interesgarri bat zelulak alginato-poli-L-lisina-alginato (APA) mikrokapsulen barnean administratzea da. Estrategia honetan, zelulak alginatoz osatutako mikroesfera polimeriko batean kapsularatzen dira, mintz erdiiragazkor batekin estaltzen dena. Sistema honek eskaintzen duen abantaila nagusia inplantatutako zelulen immunobabesa da. Zehazki, mintz erdiiragazkorrak ostalariaren erantzun immuneko zelulen eta antigorputzen difusioa saihesten du, eta aldi berean, zelulek bizitzeko behar dituzten elikagaien eta oxigenoaren difusioa ahalbidetzen du, eta baita haiek askatutako faktore terapeutikoena ere. Hori dela eta, zelulak ostalariaren erantzun immunetik babestuta daude, ekoizten dituzten faktore terapeutikoen askapena luzatuz. Hala ere, kontuan hartzekoa da sistema honen funtzionalitatea zelulen portaeraren menpe dagoela. Ondorioz, zelulen jokabidea kontrolatzeko ezintasunak terapiaren eraginkortasuna eta segurtasuna arriskuan jartzen ditu.

Bestalde, kapsularatutako zelulen bideragarritasuna mantentzea funtsezkoa da sistema epe luzez funtziona dezan. Ildo horretan, 3Dtako sistemak matrize extrazelularrean (*extracellular matrix* edo ECM) naturalki dauden egiturekin biofuntzionalizatzeari buruz asko ikertu da. Zelulak egitura horiek antzemateko gai dira integrina izendatutako mintz-hartzaileen bidez. Elkarrekintza horren ondorioz, zelulen funtzio zuzena suspertzen duten kaskada intrazelularrak gertatzen dira. Zelulen biziraupena hobetzeko, biofuntzionalizazio estrategia desberdinak erabil daitezke. Alde batetik posiblea da ECMean dauden proteinak osorik eranstea. Beste aldetik, zelulen atxikipena sustatzen duten ezinbesteko guneak soilik gehitu daitezke. Halere, integrina klase bakoitzaren afinitatea desberdina da ECMeko motibo bakoitzarentzako. Hori

dela eta, funtzionalizazio estrategia eraginkorrak diseinatzeko, lehendabizi erabiliko den zelula mota zehatzak aurkezten dituen integrina motak ikertzea funtsezkoa da.

Horren harira, oso garrantzitsua da aipatzea MSCen mikroinguruneak zelulen ekintza immunomodulatzailean eragin zuzena duela. Hori dela eta, gerta daiteke MSCak administratzen diren kokalekuaren ezaugarriak aproposak ez izatea MSCek fenotipo immunoezabatzailea erakutsi dezaten. Ondorioz, MSCen potentzial immunomodulatzailea sustatzen duten estrategien garapena erronka handi bat da gaur egun. Aztertzen ari diren aukeren artean, MSCen 3Dtako kultiboa eta zelulak γ interferoia (IFN-γ) bezalako hanturazko zitokinekin baldintzatzea nabarmen dira.

Aurreko guztia kontuan hartuz, hipotetizatu genuen MSCak kapsularatzen dituzten APA mikrokapsulak, biofuntzionalizazio estrategia egokia eta IFN-γ bidez lortutako hanturazko baldintzapena batzen dituen funtzio anitzeko sistema baten garapena oso baliagarria izango litzatekeela (1) MSCak ostalariaren sistema immunetik babesteko, (2) zelulen bideragarritasuna sustatzeko eta (3) haien eragin immunoezabatzailea sustatzeko.



1. Irudia. Tesi honen helburuen irudikapen eskematikoa.
APA: alginato-poly-L-lisina-alginato. MSC: zelula estromal mesenkimalak (*mesenchymal stromal cells*).

Hala ere, hain konplexua den sistema hori garatzeko, ezinbestekoa da aldez aurretik osagai bakoitzaren eraginkortasuna balioztatzea. Beraz, tesi honen helburu nagusia hiru osagaien inguruan ikuspegi sakon bat lortzea eta haien erabilera optimizatzea da, elementu horiek hurrengoak izanik: (1) APA mikrokapsulak, (2) matrizeen biofuntzionalizazioa eta (3) MSCen eragin immunomodulatzailea bultzatzen duen estrategia, IFN-γ hanturazko zitokinaren etengabeko baldintzapenean eta 3Dtako kultiboan oinarrituta (1. Irudia).

Beraz, tesi honen helburu zehatzak honakoak dira:

1. APA mikrokapsuletan kapsularatutako MSCen portaera kontrolatzea, terapia seguruagoa bilakatzeko, eta ondorioz, faktore terapeutikoen askapen aproposagoa lortzeko *in vivo*.

2. ECMeko proteinetan oinarritutako mikroarray biosentsore baten garapena, zelula konkretu baten atxikipen beharrak karakterizatzeko, biofuntzionalizazio estrategia arrakastatsuak diseinatzeko tresna gisa.

3. Bioinspiraturiko sistema funtzio anizdun bat garatzea 3Dtako kultiboa eta hanturazko baldintzapen iraunkorra konbinatuz, zelulen eragin immunoezabatzailea suspertzen duen konponbide integral gisa.

   Horrez gain, zelulen gaitasun immunomodulatzailea maiz denbora errealeko RT-qPCR teknikaren bidez aztertzen denez, MSCen ekintza immunomodulatzailea sustatzeko erabiltzen diren baldintza esperimental jakin hauek MSCen erreferentzia gene ohikoenen espresioan duten eragina aztertzea, emaitzen normalizazio aproposa egiteko eta ondorio okerrak ekiditeko.

Lan esperimentala

1. Kapitulua

Osmolaritate doitzaileen eragina mikrokapsularatutako zelulen portaeran: farmakoen askapen seguruagoen eta aurresangarriagoen bila

Drug delivey, 24 (2017), 1654-1666.

DOI: https://doi.org/10.1080/10717544.2017.1391894

Osmolaritate doitzaileen eragina mikrokapsularatutako zelulen portaeran: farmakoen askapen seguruagoen eta aurresangarriagoen bila

Ainhoa Gonzalez-Pujana[a,b], Aitor Rementeria[c], Francisco Javier Blanco[d], Manoli Igartua[a,b], Jose Luis Pedraz[a,b], Edorta Santos-Vizcaino[a,b,*], Rosa Maria Hernandez[a,b,*]

[a] NanoBioCel taldea, Farmazia Fakultatea, Euskal Herriko Unibertsitatea (UPV/EHU), Espainia.

[b] Sareko Ikerketa Biomedikorako Zentroa, Bioingenieritza, Biomaterial eta Nanomedikuntzan (CIBER-BBN), Espainia.

[c] Onddo eta Bakterien Biomika taldea, Euskal Herriko Unibertsitatea (UPV/EHU), Espainia.

[d] CIBER-BBN-*Bioscaff Cartílago*, INIBIC Ikerketa Biomedikoaren Institutua, Espainia.

* Egile urgazleak: Edorta Santos-Vizcaino, Rosa Maria Hernandez.

LABURPENA

Zelulen mikrokapsularatzea alginato matrizetan asko ikertu da sendagaien askapen iraunkorra lortzeko. Hala ere, zelula-jokabidearekiko kontrol ezak terapiaren eraginkortasuna eta segurtasuna arriskuan jartzen ditu. Artikulu honetan, mikrokapsulak formulatzean osmolaritate doitzaileen aukera egokiak zelulen portaera erregulatzea errazten duela frogatu da. Gure datuek agente hauek kapsulen eraketa prozesuan eragina dutela erakutsi zuten, alginatoaren erretikulazio maila eraldatuz. Ondorioz, eritropoietina (EPO) ekoizten zuten D1 zelula estromal mesenkimalak kapsularatzean, ezaugarri mekaniko desberdinak zituzten mikrokapsulak lortu ziren doitzaile geldoak edo elektrolitoetan oinarritutako doitzaileak erabiltzean. Ezaugarri mekanikoek zelulen jokabidean eragina dutenez, erantzun zelular desberdinak antzeman ziren bai *in vitro*, baita *in vivo* ere. Zehazki, manitol agente geldoa erabiltzean, kapsulek matrize permisiboa aurkeztu zuten, zelulen proliferazio tumorala ahalbidetuz. Horren ondorioz, nukleo nekrotikodun zelula-agregatu handiak eratu ziren, terapiaren eraginkortasuna eta segurtasuna arriskuan jarriz. Bestalde, elektrolito-osmolaritate doitzaileek, esaterako kaltzioa edo sodioa, kapsulen erretikulazio sendoagoa ahalbidetu zuten, zelulen proliferazioa kontrolatuz eta EPOren askapen egonkorra lortuz *in vivo*. Zelulen espresio genikoa aztertzean, osmolaritate doitzaileen eginkizuna erabakigarria izan zela egiaztatu zen. Berez, desberdintasun nabarmenak antzeman ziren bi taldeen artean biziraupenean, migrazioan eta desberdintzapenean parte hartzen duten geneak ikertzean. Orokorrean, osmolaritate doitzaileek potentzial handia erakutsi zuten matrizearen ezaugarri mekanikoak moldatzeko eta ondorioz, zelula-erantzunak erregulatzeko, terapia seguruagoak lortuz

2. Kapitulua

Matrize extrazelularraren proteinetan oinarritutako mikroarray-biosentsorea: integrinen profilaketa eta zelula-biomaterial interakzioen karakterizazioa

Sensors and Actuators B: Chemical, 299 (2019), 126954.

DOI: https://doi.org/10.1016/j.snb.2019.126954

Matrize extrazelularraren proteinetan oinarritutako mikroarray-biosentsorea: integrinen profilaketa eta zelula-biomaterial interakzioen karakterizazioa

Ainhoa Gonzalez-Pujana[a,b*], Edorta Santos-Vizcaino[a,b*], Maite García-Hernando[c,d], Beatriz Hernaez-Estrada[a,b], Marian M. de Pancorbo[d], Fernando Benito-Lopez[c], Manoli Igartua[a,b], Lourdes Basabe-Desmonts[e,f,#], Rosa Maria Hernandez[a,b,#]

[a] NanoBioCel taldea, Farmazia Fakultatea, Euskal Herriko Unibertsitatea (UPV/EHU), Espainia.

[b] Sareko Ikerketa Biomedikorako Zentroa, Bioingenieritza, Biomaterial eta Nanomedikuntzan (CIBER-BBN), Espainia.

[c] *Lab-on-a-Chip* teknologiarako Mikrosistema eta Material Analitikoen taldea, Euskal Herriko Unibertsitatea (UPV/EHU).

[d] BIOMICs ikerketa taldea, Lascaray Ikergunea, Euskal Herriko Unibertsitatea (UPV/EHU), Espainia.

[e] Ikerbasque, Zientziarako Euskal Fundazioa, Espainia.

[f] BIOMICs-mikrofluidika ikerketa taldea, Mikrofluidika Sorta, Euskal Herriko Unibertsitatea (UPV/EHU), Espainia.

* Egileek era berean jardun zuten lan honetan. [#]Egile urgazleak: Lourdes Basabe-Desmonts, Rosa Maria Hernandez.

LABURPENA

Zelulen atxikipena sustatzen duten biomaterialen ikerketan, funtsezkoa da zelula mota jakin batean ematen diren integrina-substratu elkarrekintzak aztertzea, horrela biofuntzionalizazio estrategia egokiak diseinatzeko. Lan honetan, mikroarray-biosentsore bat diseinatu zen substratu konkretu batean zelulen atxikipenaren karakterizazio zehatza lortzeko. Zehazki, 20 μm-ko fibronektina irla zirkularretan oinarritutako biosentsore bat garatu zen. Tresna analitiko horrek, zelulen atxikipen-zinetika ebaluatzeaz gain, integrinen profilaketa eta haien eragina atxikipenaren sendotzean ikertzea ahalbidetu zuen. Atxikipen zinetika lau zelula motetan ikertu zen: hamster kumearen giltzurrun fibroblastoetan (*baby hamster kidney* edo BHK), giza fibroblasto dermikoetan (*human dermal fibroblast* edo hDF), sagu C_2C_{12} mioblastoetan (*C_2C_{12} mouse myoblasts* edo C_2C_{12}) eta giza gantz zelula estromal mesenkimaletan (*human adipose mesenchymal stromal cells* edo hMSCa). Haien atxikipen zinetikak konparatzean, fibroblastoetan abiadura motelagoa antzeman zen. Datu hori azalduz, integrin profil desberdinak behatu ziren hDF eta hMSCa zelulak alderatzean. hDFren kasuan, β_1 integrinak izan ziren atxikipenaren arduradun nagusiak. Bestalde, hMSCa zeluletan, β_1 integrinek atxikipenean eragina izan arren, beste integrin klase batzuk ere nabarmendu ziren. Horretaz gain, gure analisiak zelulen atxikipenaren sendotzean zeuden desberdintasunak agerian utzi zituen. hMSCa zeluletan, $\alpha_v\beta_3$ eta β_1 integrinek antzeko funtzioa erakutsi zuten. Kontraz, β_1 integrinek garrantzi handiagoa izan zuten hDF zeluletan. Aurreko guztia kontuan hartuz, gure biosentsorearen bidez, zelulen atxikipena sustatzen duten biomaterial berriak garatzeko funtsezkoa den informazioa lortu zen, biomedikuntzaren arlo anitzetan premiazkoa dena, alegia, terapia zelularrean edota ehun-ingeniaritzan.

3. Kapitulua

Zelula estromal mesenkimalen ekintza immunomodulatzailea sustatzeko bioinspiraturiko hidrogel funtzio anizdunak

Kapitulu hau *Biomaterials* aldizkarira bidali da

Zelula estromal mesenkimalen ekintza immunomodulatzailea sustatzeko bioinspiraturiko hidrogel funtzio anizdunak

Ainhoa Gonzalez-Pujana[a,b*], Kyle H. Vining[c,d*], David K. Y. Zhang[c,d], Edorta Santos-Vizcaino[a,b], Manoli Igartua[a,b], Rosa Maria Hernandez[a,b #], David J. Mooney[c,d #]

[a] NanoBioCel taldea, Farmazia Fakultatea, Euskal Herriko Unibertsitatea (UPV/EHU), Espainia.
[b] Sareko Ikerketa Biomedikorako Zentroa, Bioingenieritza, Biomaterial eta Nanomedikuntzan (CIBER-BBN), Espainia.
[c] John A. Paulson Ingeniaritza eta Zientzia Aplikatuen Fakultatea, Harvard Unibertsitatea, Cambridge, AEB.
[d] Biologian oinarritutako Ingeniaritzaren Wyss Institutua, Harvard Unibertsitatea, Cambridge, Massachusetts, AEB.
* Egileek era berean jardun zuten lan honetan. [#]Egile urgazleak: Rosa Maria Hernandez, David J. Mooney.

LABURPENA

Zelula estromal mesenkimalen (*mesenchymal stromal cells* edo MSCak) eragin immunomodulatzailea dela eta, haien erabilera terapeutikoa oso baliagarria izan daiteke. Hala ere, haien aplikazioa nahiko mugatua dago, ezaugarri immunomodulatzaile horiek iragankorrak direlako eta zelulen mikroingurunean dauden hainbat faktoreen menpe daudelako. Azterlan honetan, MSCen ekintza immunomodulatzailea sustatzeko, funtzio anitzeko sistema bat garatu genuen, zelulen hiru dimentsiotako (3D) kultibo biomimetikoa eta hanturazko baldintzapen iraunkorra konbinatuz. Lortutako hidrogelak matrize extrazelular immunomodulatzaile (*immunomodulatory extracellular matrix* edo iECM) bezala izendatu ziren. iECM hidrogelen matrizea klik funtzionalizatutako alginatoz eta I. motako kolageno fibrilarrez osatutako sare interpenetratzailea izan zen, matrize extrazelular naturalaren arkitektura imitatzeko helburuarekin diseinatu zena. Bertan, γ interferoiarekin (IFN-γ) kargatutako heparina mikroesferak gehitu genituen. Zelulak kapsularatu ondoren, 3Dtako kultiboak giza MSCen (*human MSC* edo hMSC) ekintza immunomodulatzailea era nabarmenean bultzatu zuen, hainbat gene immunomodulatzaileen espresioa handituz 2Dtan kultibatutako zelulekin alderatuta. Horrez gain, IFN-γ-dun heparina mikroesferek etengabeko hanturazko mikroingurunea mantentzea lortu zuten, hanturazko baldintzapenaren menpe espresatzen diren gene garrantzitsuen adierazpena luzatuz, hala nola, indoleamina 2,3-dioxigenasa 1 (*IDO1*) edo galektina-9 (*GAL9*). Bestalde, iECM hidrogelek hMSCen eragin immunomodulatzaile egokia ahalbidetu zuten. Zehazki, iECMetan kapsularatutako hMSCak eta T zelulak ko-kultibatzean, bigarrenen proliferazioa inhibitu zen. Horrek hMSCek ekoiztutako faktoreen askapen eta hedapen egokia gertatzen zela egiaztatu zuen eta, beraz, plataformaren funtzionamendu zuzena agerian utzi zuen. Emaitza guzti horiek, hMSCen ekintza terapeutikoa sustatzen duen funtzio anitzeko sistema balioztatzen dute.

---

1. Sarrera

Zelula estromal mesenkimalek (*mesenchymal stromal cell* edo MSC) paregabeko ekintza immunomodulatzailea dutela jakin da eta ondorioz, haien erabilera oso interesgarria da gaitz immuneetan eta baita hanturazkoetan ere. MSCek berezko immunitatea eta immunitate adaptatiboa erregulatzen dituztela behatu da, zelula immune desberdinen oinarrizko funtzioak modulatuz. Zehazki, MSCek hainbat zelulen aktibazioa, heltzea, proliferazioa edo jarduera zitotoxikoa moldatzen dutela behatu da [1], haien artean, monozitoak eta makrofagoak [2,3], zelula dendritikoak [4], edota T, B eta zelula hiltzaile naturalak (*natural killer cells* edo NK) diren linfozitoak daudelarik [5-7]. MSCen eragin immunomodulatzailea mekanismo desberdinen konbinazio konplexuaren emaitza da, haien artean zelula-zelula kontaktuak eta faktore bioaktibo disolbagarrien askapena daudelarik, hala nola, interleukinen, entzima metabolikoen eta hazkuntza faktoreen askapena. Zehazki, zelulek ekoizten dituzten faktore bioaktiboen eragin parakrinoak haien jarduera terapeutikoaren arduradun nagusitzat jotzen dira [8,9].

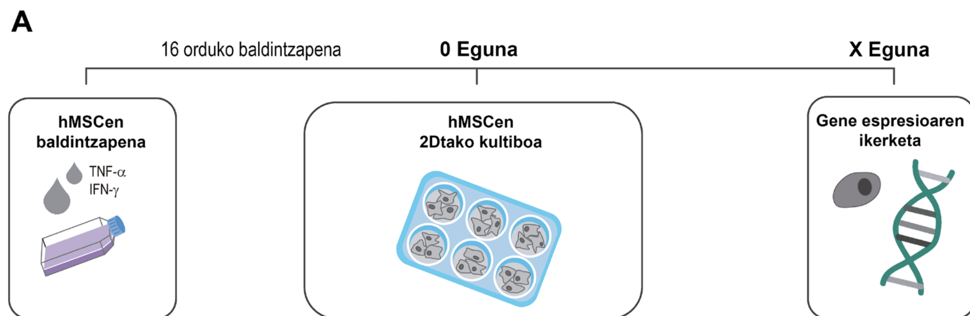
Halere, MSCen ezaugarri immunomodulatzaileek izaera iragankorra dute eta haien ingurunean dauden faktoreen menpe daude, zelula hauen erabilera erabat mugatuz. Muga horiek gainditzeko eta MSCen ahalmen immunomodulatzailea hobetzeko, ikertzaileek hainbat estrategia aztertu dituzte. Horietako bat zelulak mikroingurune naturalean dauden hanturazko zitokinekin baldintzatzea da, hala nola, γ interferoiarekin (IFN-γ) edo α tumorearen nekrosi faktorearekin (TNF-α). Estrategia honek MSCen fenotipo immunoezabatzailea sustatzen duela frogatu da, faktore bioaktiboen askapena hobetuz. Hala eta guztiz ere, MSCen hanturazko aurre-baldintzapenaren eragina iragankorra da [10-12]. MSCen potentzial terapeutikoa kultibo baldintzen menpe dago ere. Hiru dimentsiotako (3D) kultiboak MSCen ekintza immunomodulatzailea eta antiinflamatorioa suspertzen duela frogatu da [13]. Gaur egun, MSCen eragina bultzatzeko 3Dtako kultiborik erabiliena zelula-esferoideen eraketa da [13-15], zelula-zelula kontaktuak areagotzean MSCen ekintza suspertzen baitu. Horrez gain, MSCen mikroingurunearen ezaugarri mekanikoek ere eragina dute zelulen biologian. Berez, matrizearen gogortasunak eta elastizitateak faktore bioaktiboen askapenean eragina dutela frogatu da [16]. Halere, matrizearen ezaugarri mekaniko zehatzen eragina beste baldintzapen estrategiekin batera aztertzea ezinbestekoa da oraindik. Bestalde, garrantzitsua da aipatzea ikerketa askok gene edo faktore immunomodulatzaile bakar bat aztertzen dutela, MSCen potentzial erregulatzailearen ikuspuntu orokorra ematen ez duena.

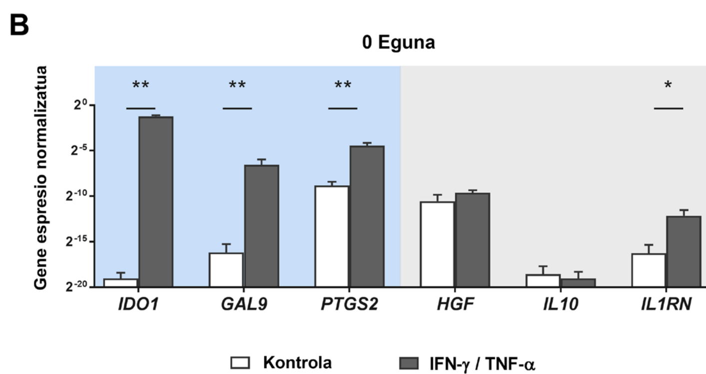
Azterlan honetan, MSCen ekintza immunomodulatzailea sustatzeko baldintzapen estrategia desberdinak konbinatzea proposatu genuen. Zehazki, bioinspiraturiko sistema funtzio anizdun bat diseinatu genuen, giza hezur muineko MSCak (*human MSC* edo hMSC) kapsularatzeko. Horretarako, hanturazko baldintzapen iraunkorra eta 3Dtako kultibo biomimetikoa ahalbidetzen duten ezaugarri mekaniko desberdineko hidrogelak konbinatu genituen.

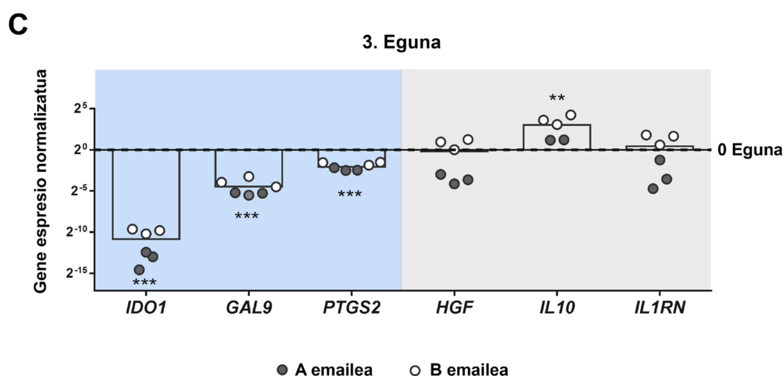
Gure sistemaren matrizea klik prozedura gauzatzeko funtzionalizatuta dagoen alginatoz eta I. motako kolageno fibrilarrez osatutako sare interpenetratzaile batean oinarrituta zegoen, matrize extrazelular (*extracellular matrix* edo ECM) naturalaren arkitektura imitatzeko, eta ondorioz, zelulen bideragarritasuna bultzatzeko helburuarekin diseinatu zena. Bertan, IFN-γ hanturazko zitokinarekin kargatutako heparina mikroesferak gehitu genituen. Funtzio anitzeko sistema honen eragina hainbat gene immunomodulatzaile garrantzitsuen espresioan aztertu zen denbora errealeko RT-qPCR teknikaren bidez, zehazki, indoleamina 2,3-dioxigenasa 1 (*IDO1*), prostaglandina sintetasa 2 (*PTGS2*), galaktina-9 (*GAL9*), hazkuntza faktore hepatozitikoa (*hepatocyte growth factor* edo *HGF*), interleukina-10 (*IL10*) eta interleukina-1 hartzailearen antagonista (*interleukin-1 receptor antagonist* edo *IL1RN*) geneetan [17-22]. Gene horiek emaitza adierazgarriak lortzeko asmoz aukeratu ziren, erantzun immunoezabatzailearen maila desberdinetan jarduten duten faktoreak kodifikatzen baitituzte. Sistemaren funtzionalitatea funtzio anitzeko sisteman kapsularatutako hMSCak eta T zelulak ko-kultibatuz aztertu zen.

2. Emaitzak eta eztabaida

Hasteko, hanturazko baldintzapenak gene immunomodulatzaile garrantzitsuen espresioan duen eragina aztertu genuen. Horretarako, 1. A irudian erakusten den prozedura jarraitu genuen: IFN-γ eta TNF-α hanturazko zitokinekin 16 ordu inguru inkubatu genituen zelulak, eta ondoren, bi dimentsiotan (2D) kultibatu genituen, zehazki, kultibo plaketan (*tissue culture plate* edo TCP). Hanturazko baldintzapenaren ostean (0 eguna) *IDO1*, *PTGS2* eta *GAL9* geneen espresioa esanguratsuki handitu egin zen, baldintzatu gabeko zelulekin konparatuz. Emaitza horiek literaturarekin bat datoz, bi zitokina hauen konbinazioak hMSCen fenotipo immunoezabatzailea bultzatzen duela dioenak [12], hMSCen faktore bioaktiboen ekoizketa sustatuz [24]. *HGF*, *IL10* eta *IL1RN* geneetan, aldiz, hanturazko baldintzapenak ez zuen eraginik izan (1.B irudia). Hori dela eta, gure geneak bi taldetan klasifikatu genituen: hanturazko

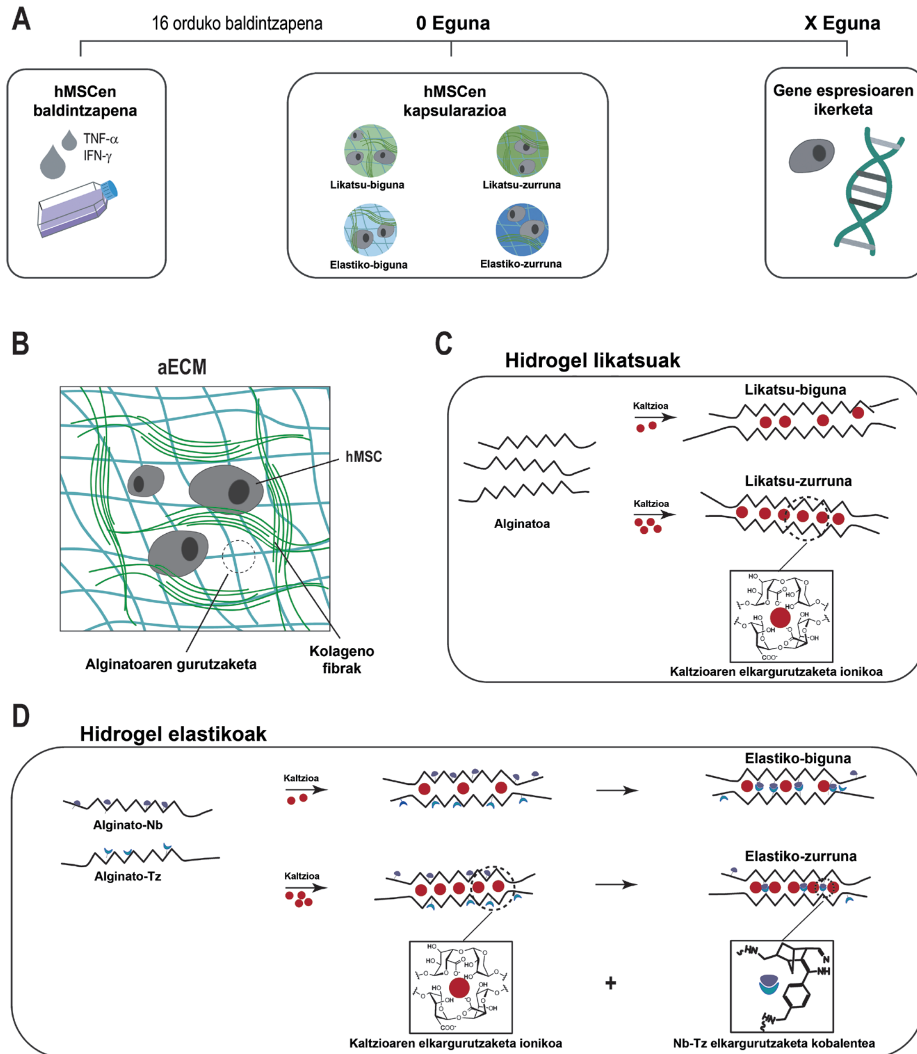






1. Irudia. 2Dtan kultibatutako hMSCen gene immunomodulatzaileen espresioa. (A) Prozedura esperimentalaren eskema. hMSCak IFN-γ eta TNF-α zitokinekin ≈16 orduz baldintzatu ondoren, kultiborako plaketan (TCP: *tissue culture plate*) kultibatu ziren (2D). Denbora puntu desberdinetan, zelulen gene espresioa ikertu zen denbora errealeko RT-qPCR teknikaren bidez. **(B)** *IDO1*, *GAL9*, *PTGS2*, *HGF*, *IL10* eta *IL1RN* geneen espresio normalizatua hanturazko baldintzapenaren ostean, baldintzatu gabeko kontrol zelulekin alderatuz. Normalizazioa egiteko *GAPDH* genea erabili zen. Estatistika: $^{*}p < 0.05$, $^{**}p < 0.01$. **(C)** *IDO1*, *GAL9*, *PTGS2*, *HGF*, *IL10* eta *IL1RN* geneen espresio normalizatua hanturazko baldintzapenaren ostean 2Dtan 3 egunez kultibatutako hMSCetan, 0 egunean behatutako espresioarekin alderatuz. Normalizazioa egiteko *GAPDH* genea erabili zen. Estatistika: $^{***}p < 0.001$, $^{**}p < 0.01$. Laburdurak: hMSC: giza zelula estromal mesenkimalak (*human mesenchymal stromal cells*). IFN-γ: γ interferoia. TNF-α: α tumorearen nekrosi faktorea.

baldintzapenaren aurrean erantzun zutenak: *IDO1*, *GAL9*, *PTGS2*; eta erantzun ez zutenak: *HGF*, *IL10* eta *IL1RN*. 2Dtako kultiboan 3 egun igaro ondoren, *IDO1*, *PTGS2* eta *GAL9* geneen espresioa era nabarmenean murriztu zen, hanturazko baldintzapenak eragin iragankorra duela frogatuz (1.C irudia).



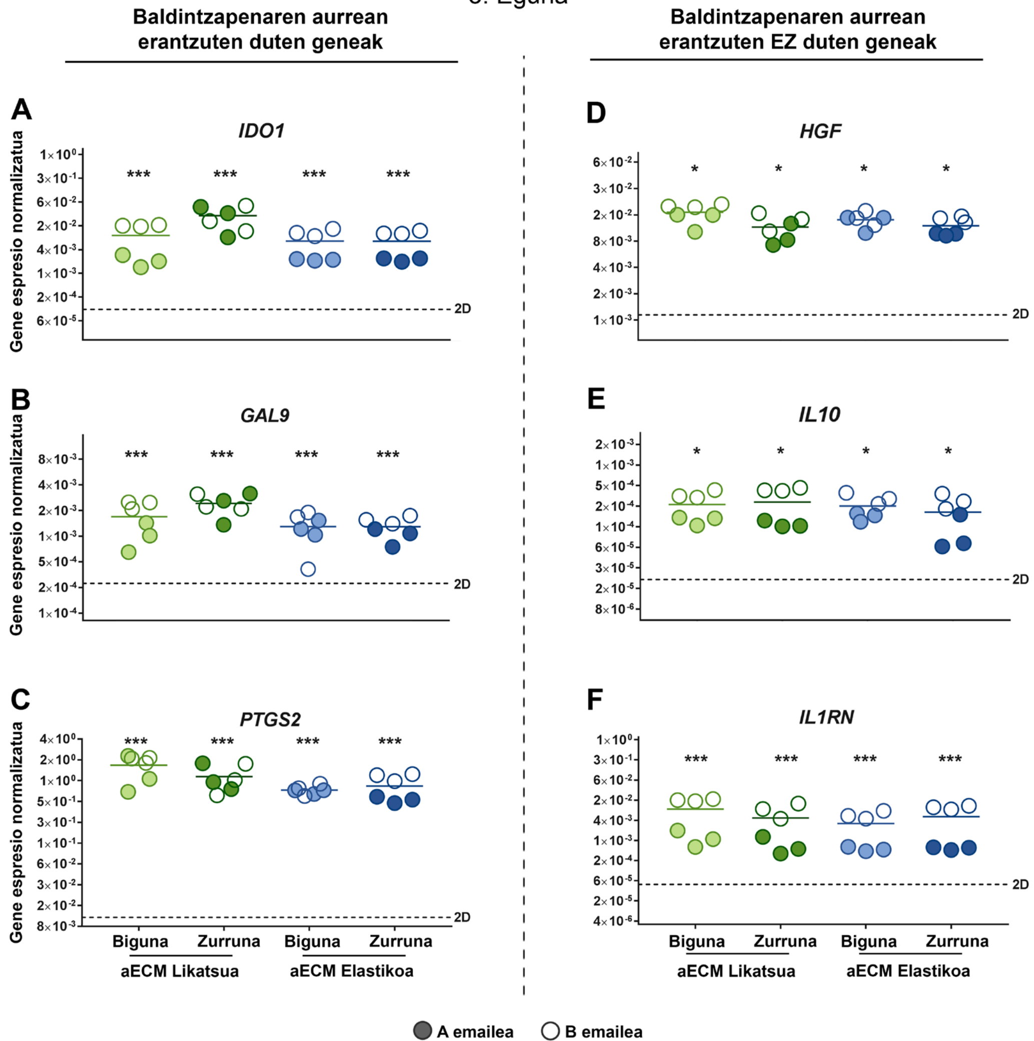
2. Irudia. aECM hidrogelak. (A) Prozedura esperimentalaren eskema. hMSCak IFN-γ eta TNF-α zitokinekin ≈16 orduz baldintzatu ondoren, hidrogel desberdinetan kapsularatu ziren (3D). Denbora puntu desberdinetan, zelulen gene espresioa ikertu zen denbora errealeko RT-qPCR teknikaren bidez. **(B)** aECM hidrogelen egitura. **(C)** Alginatoa kaltzioarekin elkargurutzatu zenean, hidrogel likatsuak lortu ziren. **(D)** Elkargurutzaketa ionikoa eta Nb / Tz taldeen arteko elkargurutzaketa kobalentea konbinatzean hidrogel elastikoak lortu ziren. Laburdurak: hMSC: giza zelula estromal mesenkimalak (*human mesenchymal stromal cells*). IFN-γ: γ interferoia. TNF-α: α tumorearen nekrosi faktorea. aECM: matrize extrazelular artifizial hidrogelak (*artificial extracellular matrix hydrogels*). Nb: norborenoa. Tz: tetrazina.

Ondoren, 3Dtako kultiboak gene immunomodulatzaile horien adierazpena sustatuko zuelaren hipotesiaren inguruan jardun genuen. Asmo horrekin, aldez aurretik 16 ordu inguru hanturazko zitokinekin baldintzaturiko hMSCak ECM artifizial (aECM) bezala izendatutako hidrogeletan kapsularatu genituen (2. Irudia A). aECM hidrogelen matrizea alginatoz eta I. motako kolageno fibrilarrez osatutako sare interpenetratzailea da, ECM naturalaren arkitektura imitatzeko eta baita zelulen biziraupena suspertzeko ere garatu genuena [25] (2. Irudia B). aECM hidrogelen lau mota ikertu ziren: likatsu-biguna, likatsu-zurruna, elastiko-biguna eta elastiko-zurruna (2. Irudia C-D), aurreko azterlan batean karakterizatu zirenak [16]. Alginatoaren gurutzaketa eta magnitudea aldatuz, hidrogelen elastikotasuna eta zurruntasuna era independentean moldatu ziren. Hidrogel likatsuek, estres-erlaxazio erantzun arina dute, gurutzaketa ioniko itzulgarria dutelako azido guluroniko ugariko blokeetan (G blokeetan). Norborenoa (Nb) eta tetrazina (Tz) taldeak gehitzean, gurutzaketa kobalente iraunkorra gauzatzen da, sarea elastikoa bihurtuz. Zehazki, Nb eta Tz taldeak matrizean gehitzean, Diels-Alderren alderantzizko erreakzio elektroi hartzaile bio-ortogonala ematen da, dagoeneko existitzen diren lotura ionikoak “klikatuz” [16, 23].

Zelulak aECM hidrogeletan 3 egunez kultibatu ostean, gure paneleko gene immunomodulatzaile guztien espresioa nabarmenki handitu egin zen, hanturazko baldintzapenaren aurrean erantzun (3. Irudia A-C) zein ez (3. Irudia D-F). Emaitza horiek agerian utzi zuten 3Dtako kultiboaren eragina hanturazko baldintzapenaren ekintzaren independentea izan zela. Ezaugarri mekaniko desberdinak zituzten lau aECM hidrogel moten artean ez ziren desberdintasunik antzeman.

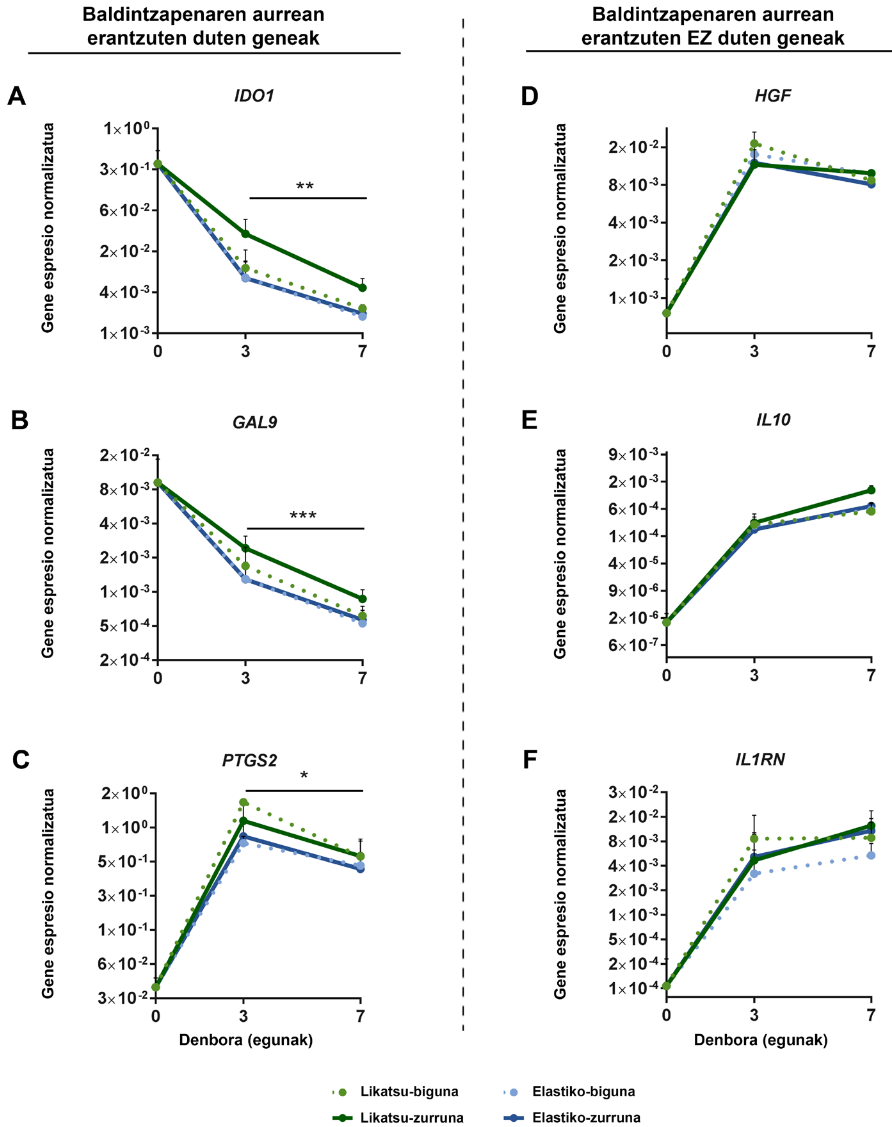
Hurrengo pausuan, 3Dtako kultiboaren eraginaren iraupena aztertu genuen. Horretarako, prozedura berdina jarraitu genuen eta denbora puntu desberdinetan gene immunomodulatzaileen espresioa ikertu genuen. 7. egunari erreparatuz, 2 joera desberdin behatu genituen gure gene panelean: alde batetik hanturazko baldintzapenaren aurrean erantzuten duten geneen espresioa nabarmenki murriztu zen 3. egunarekin konparatuz (4. Irudia A-C). Bestalde, baldintzapenari erantzuten ez dioten geneen espresioa, aldiz, mantendu egin zen (4. Irudia D-E). Emaitza horiek 3Dtako kulturaren eragina iraunkorra dela iradokitzen dute, eta kapsularatu aurreko hanturazko baldintzapenarena, ostera, iragankorra.

Literaturan hainbat azterlanek frogatu dute matrizearen ezaugarri mekanikoek MSCen biologian eragina dutela [26-28]. Hala ere, esperimentu honetan ez ziren desberdintasun nabarmenik behatu mekanikoki desberdinak ziren aECM hidrogel moten artean. Gure hipotesia hanturazko baldintzapenaren eta 3Dtako kultiboaren eragin handiak ezaugarri mekanikoen ekintza



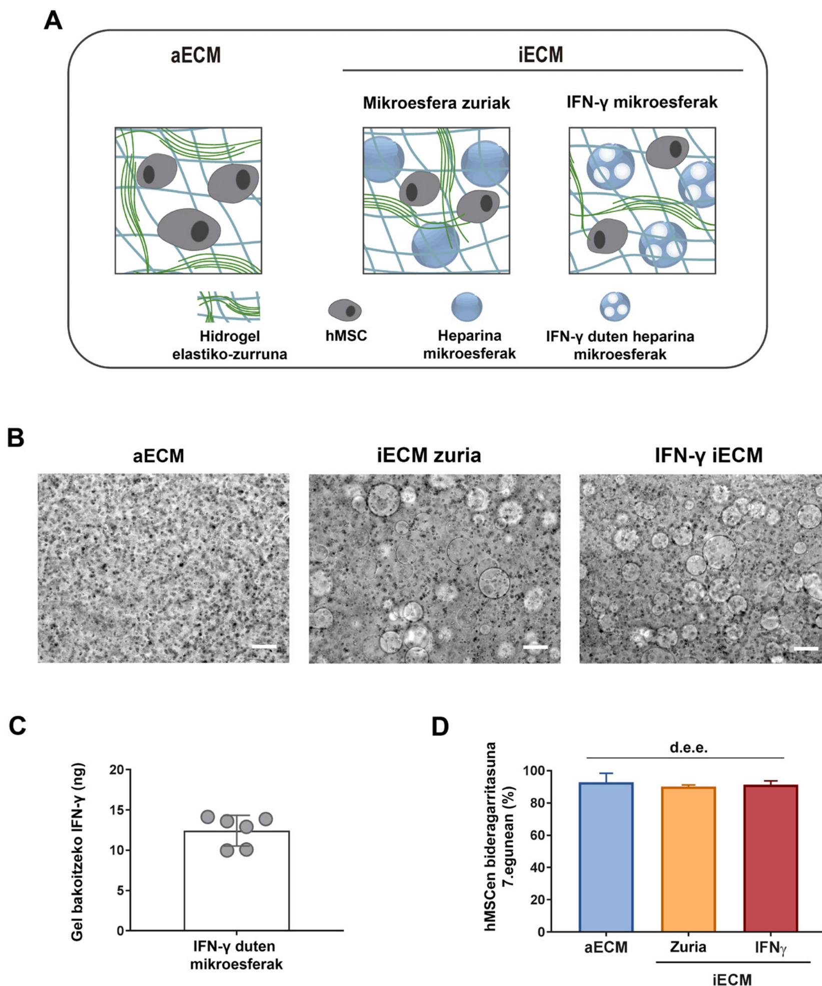
3. Irudia. aECM hidrogeletan kultibatutako hMSCen gene immunomodulatzaileen espresioa 3. egunean. (A) *IDO1*, **(B)** *GAL9*, **(C)** *PTGS2*, **(D)** *HGF*, **(E)** *IL10* eta **(F)** *IL1RN* geneen espresio normalizatua IFN-γ eta TNF-α zitokinekin baldintzatu ostean 3 egunez aECM hidrogeletan kultibatu ziren hMSCetan, 2Dtan kultibatutako zelulekin alderatuz. Normalizazioa egiteko *GAPDH* genea erabili zen. Estatistika: $^*p < 0.05$, $^{**}p < 0.01$ eta $^{***}p < 0.001$. Laburdurak: hMSC: giza zelula estromal mesenkimalak (*human mesenchymal stromal cells*). IFN-γ: γ interferoia. TNF-α: α tumorearen nekrosi faktorea. aECM: matrize extrazelular artifizial hidrogelak (*artificial extracellular matrix hydrogels*).

maskaratzen dutela da. Ildo horretan, etorkizuneko ikerketetan interesgarria izango litzateke bi baldintzapen estrategia horiek hMSCen ekintza immunomodulatzailea zein mekanismoen bidez bultzatzen duten aztertzea.



4. Irudia. aECM hidrogeletan kultibatutako hMSCen gene immunomodulatzaileen espresioa denboran zehar. **(A)** *IDO1*, **(B)** *GAL9*, **(C)** *PTGS2*, **(D)** *HGF*, **(E)** *IL10* eta **(F)** *IL1RN* geneen espresio normalizatua IFN-γ eta TNF-α zitokinekin baldintzatu ostean aECM hidrogeletan 0, 3 eta 7 egunez kultibatu ziren hMSCetan. Normalizazioa egiteko *GAPDH* genea erabili zen. Estatistika: *p < 0.05, **p < 0.01 eta ***p < 0.001, 3. eta 7.eguneko emaitzak alderatuz. Laburdurak: hMSC: giza zelula estromal mesenkimalak (*human mesenchymal stromal cells*). IFN-γ: γ interferoia. TNF-α: α tumorearen nekrosi faktorea. aECM: matrize extrazelular artifizial hidrogelak (*artificial extracellular matrix hydrogels*).

IDO1, *PTGS2* eta *GAL9* geneak oso garrantzitsuak direnez hMSCen immunomodulazioa gertatzeko, gure hurrengo helburua haien espresioa luzatzea izan zen. Horretarako, aECM hidrogelek eskaintzen duten 3Dtako kultibo biomimetikoa hanturazko baldintzapen iraunkorrarekin konbinatu genuen. Zehazki, aECM hidrogel elastiko zurrunetan IFN-γ zitokinarekin kargatutako heparina mikroesferak gehitu genituen, sistema multifuntzional berri bat sortuz: ECM hidrogel immunomodulatzaileak (iECM). aECM hidrogel moten artean desberdintasunik



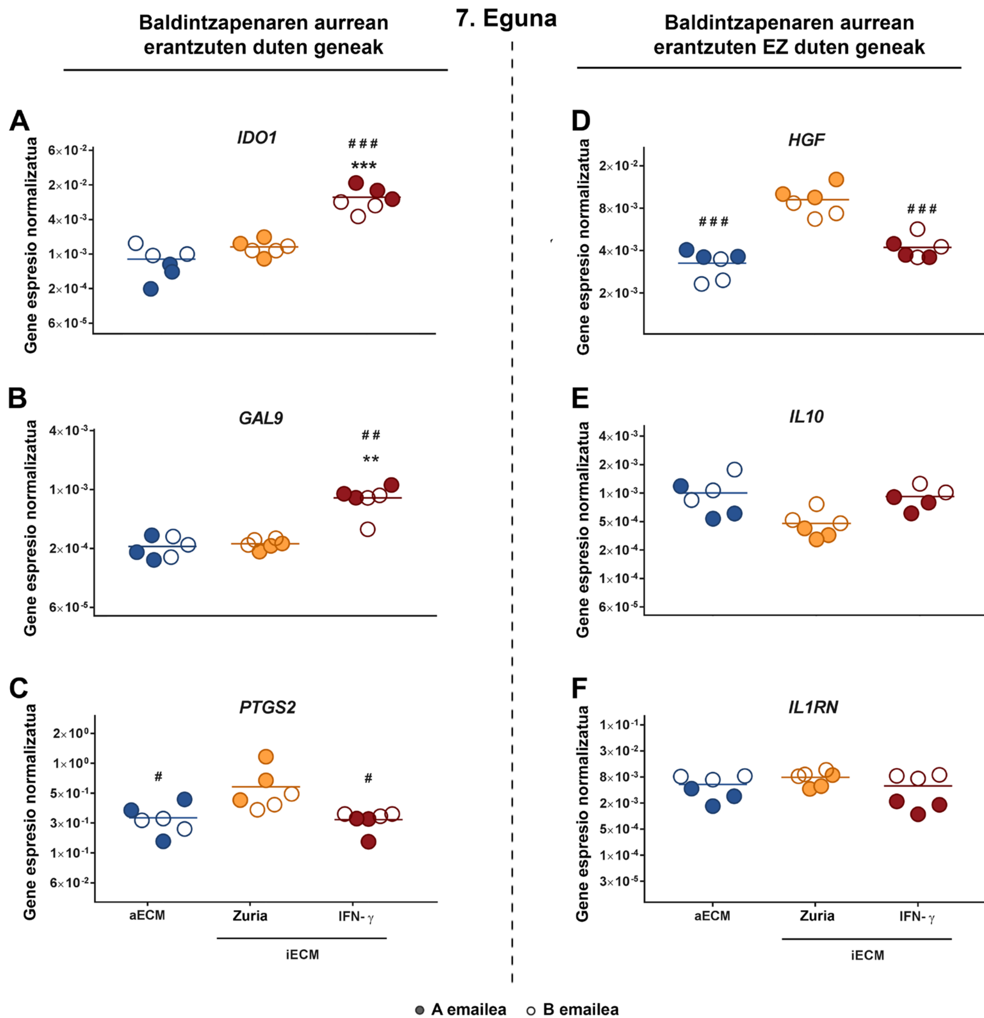
5. Irudia. iECM hidrogelen karakterizazioa. (A) iECM hidrogelen irudikapen eskematikoa. **(B)** iECM hidrogeletan kapsularatutako hMSCen mikroskopia irudiak. Eskala = 200 μm. **(C)** iECM hidrogeletan kargatutako IFN-γ kopurua. **(D)** hMSCen bideragarritasuna hanturazko baldintzapenaren ostean 3Dtan 7 egunez kultibatu ostean. Laburdurak: iECM: matrize extrazelular immunomodulatzaile hidrogelak (*immunomodulatory extracellular matrix hydrogels*). aECM: matrize extrazelular artifizial hidrogelak (*artificial extracellular matrix hydrogels*). hMSC: giza zelula estromal mesenkimalak (*human mesenchymal stromal cells*). IFN-γ: γ interferoia.

behatu ez genituenez, matrize elastiko zurruna aukeratu genuen iECM hidrogelak eratzeko, mekanikoki sendoena baita eta ondorioz, etorkizunean egingo diren *in vivo* ikerketetan inplantatzeko egokiena izango delako. Aipagarria da heparinak afinitate handia duela IFN-γ-rekin (disoziazio konstantea (KD) = 1 - 5 nM), mikroesferak zitokinarekin kargatzea ahalbidetu zuena (IFN-γ iECM, 5. Irudia A) [29]. Azpimarratzekoa da zitokina heparinara lotzean, haren degradazio proteolitikoa domeinu bakar batera murrizten dela eta horrek, IFN-γren potentzia erabat suspertzen duela [29, 30]. Horrez gain, heparinara hainbat faktore lotu daitezke [29, 31]. Hori dela eta, IFN-γrekin kargatutako mikroesferen eragina ikertzeaz gain, kargatu gabeko mikroesferak ikertu genituen ere (iECM zuria, 5. Irudia A), heparinara lotzen diren faktore ezberdinek MSCen ekintza immunomodulatzailea sustatzen duten aztertzeko. Mikroesferen gehiketak ez zuen kapsularatze prozesuan eraginik izan eta zelulen eta mikroesferen banaketa homogeneoa izan zen, 5. B irudian beha daitekeenez. Hidrogel bakoitzean (250 µL) zitokinaren 12,66 ± 2,33 ng kargatu ziren (5. Irudia C). Horrez gain, mikroesferek ez zuten zelulen bideragarritasunean eragin negatiborik izan, kapsularatu osteko 7. egunean frogatu zenez (5. Irudia D).

iECM hidrogelen ekintza aztertzeko, hMSCak IFN-γ eta TNF-α zitokinekin 16 orduz baldintzatu eta 7 egunez kultibatu ziren iECM edo aECM hidrogeletan. *IDO1* eta *GAL9* geneen espresioa nabarmenki handiagoa izan zen IFN-γ iECM hidrogeletan, hanturazko baldintzapenaren efektua luzatu zutela frogatuz (6. Irudia A-B). Bestalde, iECM zuriek *PTGS2* eta *HGF* geneen espresioa suspertu zuten (6. Irudia C-D), MSCen ekintza immunomodulatzailea sustatzen duten faktoreak heparinara lotzen direla iradokiz. *IL10* eta *IL1RN* geneen espresioan, ostera, ez ziren desberdintasunik antzeman (6. Irudia E-F), ziurrenik 3Dtako kultiboaren eragina nahikoa izan zelako haien espresioa mantentzeko. Datu hauen arabera, iECM hidrogelek MSCen ekintza immunomodulatzailea esanguratsuki suspertu eta luzatu zuten.

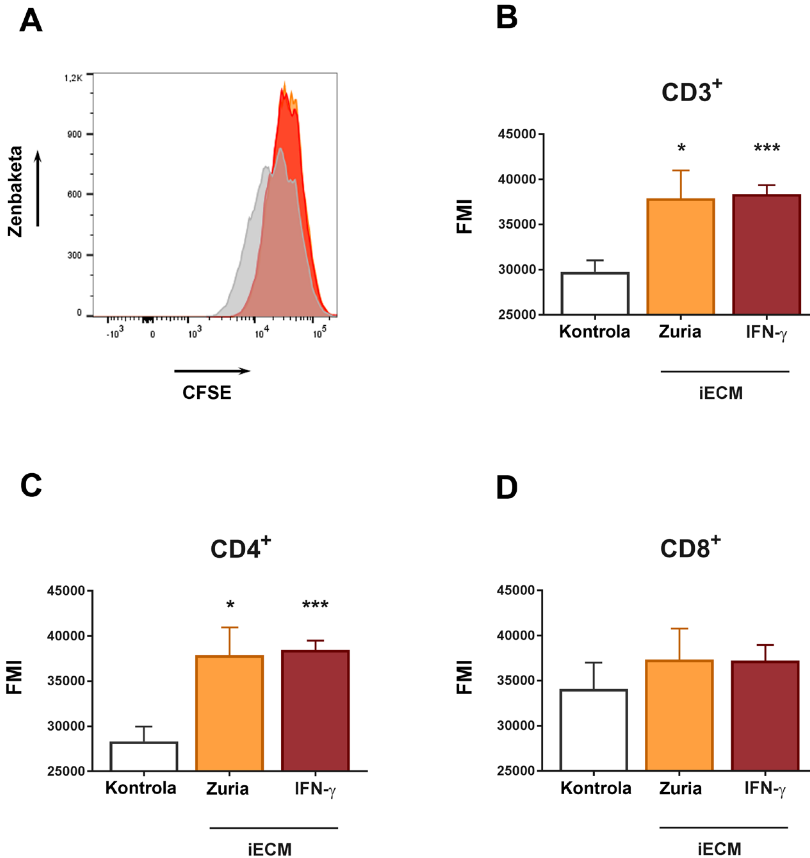
Azkenik, iECM hidrogelen funtzionalitatea frogatzeko, kapsularatutako hMSCak 4 egunez kultibatu ondoren, T zelulekin ko-kultibatu ziren 3 egunez (guztira, hMSCak iECM hidrogeletan 7 egunez kultibatu ziren). iECM hidrogeletan kapsularatutako zelulak, $CD3^+$ populazioaren proliferazioa inhibitzeko gai izan ziren (7. Irudia A-B), batez ere $CD3^+$ / $CD4^+$ linfozitoak gutxituz (7. Irudia C-D).

Ondorioz, iECM hidrogelek hMSCak ekoiztutako faktore immunomodulatzaileen difusioa eta askapena ahalbidetu zutela frogatu zen. Ezaugarri hori ezinbestekoa da, izan ere, horrelako biomaterialetan oinarritutako formulazio askoren tamaina handiak biomolekulen difusioa saihesten du [26]. Horrez gain, heparina mikroesferek ez zituzten zelulek ekoiztutako faktore



6. Irudia. iECM hidrogeletan kultibatutako hMSCen gene immunomodulatzaileen espresioa 7. egunean. (A) *IDO1*, **(B)** *GAL9*, **(C)** *PTGS2*, **(D)** *HGF*, **(E)** *IL10* eta **(F)** *IL1RN* geneen espresio normalizatua IFN-γ eta TNF-α zitokinekin baldintzatu ostean 7 egunez iECM edo aECM hidrogeletan kultibatu ziren hMSCetan. Normalizazioa egiteko *GAPDH* genea erabili zen. Estatistika: *$p < 0.05$, **$p < 0.01$ eta ***$p < 0.001$ aECM hidrogelekin konparatuz. #$p < 0.05$, ##$p < 0.01$ eta ###$p < 0.001$ iECM zuriekin alderatuz. Laburdurak: hMSC: giza zelula estromal mesenkimalak (*human mesenchymal stromal cells*). IFN-γ: γ interferoia. TNF-α: α tumorearen nekrosi faktorea. aECM: matrize extrazelular artifizial hidrogelak (*artificial extracellular matrix hydrogels*). iECM: matrize extrazelular immunomodulatzaile hidrogelak (*immunomodulatory extracellular matrix hydrogels*).

guztiak lotu, eta bestalde, ez zuten faktore horien hedapena fisikoki oztopatu. Garrantzitsua da aipatzea IFN-γren presentziak ez zuela eragin negatiborik izan ko-kultiboan, eta, beraz, zentzuzkoa da pentsatzea zitokinak seguruenik ez zuela sistematik ihes egin, edo, gutxienez, ez zela kopuru esanguratsu batean askatu.



7. Irudia. iECM hidrogeletan kapsularatutako hMSCen ekintza immunomodulatzailea. Hanturazko baldintzapenaren ostean hMSCak 4 egunez iECM hidrogeletan kultibatu ziren. Ondoren, iECMtan kultibatutako hMSCak CFSE markatzailearekin tindatutako T zelulekin ko-kultibatu ziren, 1:1.25 hMSC:T zelula proportzioan. Ko-kultiboa 3 egunez mantendu zen (guztira hMSCak 7 egunez kultibatu ziren iECM hidrogeletan). T zelulen proliferazioa CFSE markatzailearen diluzioarekin aztertu zen. **(A)** $CD3^{+}$ populazioaren histograma. **(B)** FMI $CD3^{+}$ populazioan. FMIren determinazioa **(C)** $CD4^{+}$ eta **(D)** $CD8^{+}$ populazioetan. Laburdurak: hMSC: giza zelula estromal mesenkimalak (*human mesenchymal stromal cells*). iECM: matrize extrazelular immunomodulatzaile hidrogelak (*immunomodulatory extracellular matrix hydrogels*). FMI: fluoreszentzia maximoaren intentsitatea. CFSE: sukzinimidil-karboxifluoreszeina (*carboxyfluorescein succinimidyl ester*).

3. Ondorioak

Azterlan honetan, bioinspiraturiko sistema anizdun bat diseinatu eta garatu genuen, 3Dtako kultibo biomimetikoa eta hanturazko baldintzapen iraunkorra konbinatuz hMSCen potentzial immunomodulatzailea sustatzeko. hMSCak 3Dtako hidrogeletan kultibatzean gene immunomodulatzaile garrantzitsuen espresioa suspertu zen. Bestalde, hidrogeletan IFN-γ zitokinarekin kargatutako heparina mikroesferak gehitzean, hanturazko baldintzapenaren menpe espresatzen diren geneen adierazpena sustatu eta luzatu zen, hala nola *IDO1* eta *GAL9*. Aldi berean, sistemak zelulek ekoiztutako faktore bioaktiboen hedapen egokia ahalbidetu zuen. Gaur egun, MSCen ekintza immunomodulatzailea sustatzeko estrategia berriak sakonki aztertzen ari direnez, gure emaitzek inplikazio garrantzitsuak dituzte zelula hauetan oinarritutako terapietan.

4. Eskerrak

A. Gonzalez-Pujanak Eusko Jaurlaritzari doktoratu aurreko laguntza eskertzen dio (PRE_2018_2_0133). Proiektu honek honako finantziazioa jaso du: Eusko Jaurlaritza (Talde kontsolidatuak, IT-907-16), Euskal Herriko Unibertsitatea (UPV/EHU), MINECO proiektua (SAF2017-82292-R), FEDER (UE), ICTS “NANBIOSIS”, National Institute of Dental & Craniofacial Research of the National Institutes of Health (R01DE013033, DM).

Erreferentzien zerrenda 182-183 orrialdeetan aurkitzen da.

4. Kapitulua

Hiru dimentsiotako kultiboak eta hanturazko baldintzapenak zelula estromal mesenkimalen erreferentzia geneen espresioa aldatzen dute denbora errealeko RT-qPCRan

Hiru dimentsiotako kultiboak eta hanturazko baldintzapenak zelula estromal mesenkimalen erreferentzia geneen espresioa aldatzen dute denbora errealeko RT-qPCRan

Ainhoa Gonzalez-Pujana[a,b*], Irene de Lázaro[c,d*], Kyle H. Vining[c,d], Edorta Santos-Vizcaino[a,b], Manoli Igartua[a,b], Rosa Maria Hernandez[a,b #], David J. Mooney[c,d #]

[a] NanoBioCel taldea, Farmazia Fakultatea, Euskal Herriko Unibertsitatea (UPV/EHU), Espainia.

[b] Sareko Ikerketa Biomedikorako Zentroa, Bioingenieritza, Biomaterial eta Nanomedikuntzan (CIBER-BBN), Espainia.

[c] John A. Paulson Ingeniaritza eta Zientzia Aplikatuen Fakultatea, Harvard Unibertsitatea, Cambridge, AEB.

[d] Biologian oinarritutako Ingeniaritzaren Wyss Institutua, Harvard Unibertsitatea, Cambridge, Massachusetts, AEB.

* Egileek era berean jardun zuten lan honetan. [#]Egile urgazleak: Rosa Maria Hernandez, David J. Mooney.

LABURPENA

Giza zelula estromal mesenkimalek (*human mesenchymal stromal cell* edo hMSC) potentzial terapeutiko handia dute hanturazko eta immunitate gaixotasunak tratatzeko, haien ekintza immunomodulatzailea dela eta. Ekintza hori aztertzeko, maiz, indoleamina 2,3-dioxigenasa 1 (*IDO1*) bezalako gene immunomodulatzaileen espresioa ikertzen da. Horretarako metodo nagusia denbora errealeko polimerasaren kate-erreakzio kuantitatiboaren (RT-qPCR) teknika da, bere sentsibilitate handia eta sinpletasuna direla eta. Denbora errealeko RT-qPCRaren bidez lortutako emaitzak normalizatzeko erreferentzia geneak erabiltzen dira. Erreferentzia gene horiek baldintza esperimental jakinetan egonkorrak izatea nahitaezkoa da emaitza zehatzak lortzeko. Halere, askotan alde batera uzten da erreferentzia geneen balioztatzea, ondorio okerrak ekar ditzakeena. Azterlan honetan, sistematikoki ikertu genuen hMSCen ekintza terapeutikoa sustatzeko erabiltzen diren 3 estrategien eragina hMSCen erreferentzia gene ohikoenen espresioan. Zehazki, estrategia horiek hurrengoak izan ziren: 3Dtako kapsularazioa, 3Dtako matrizearen ezaugarri mekanikoak eta hanturazko baldintzapena. Gure emaitzek erakutsi zutenez, 3Dtako kapsularazioak eta baita hanturazko baldintzapenak ere eragin handia izan zuten *ACTB* eta *UBC* erreferentzia geneen espresioan. Gene horiek *IDO1* genearen espresioa normalizatzeko erabili zirenean akats garrantzitsuak egin ziren *IDO1* mRNA mailen ebaluazioan, bere espresioa gutxietsiz edo gainestimatuz. Bestalde, zelulak kapsularatutako matrizearen ezaugarri mekanikoek ez zuten erreferentzia geneetan eraginik izan. Laburbilduz, gure ikerketak erreferentzia geneen egonkortasuna baldintza esperimental jakinetan balioztatzea ezinbestekoa dela azpimarratzen du, aztertu nahi diren itu geneen emaitza fidagarriak lortzeko.

Eztabaida

Zelula estromal mesenkimalen (*mesenchymal stromal cell* edo MSC) primerako ezaugarriak direla eta, oso baliagarriak dira zeluletan oinarritutako terapietan. MSCek fenotipo hipoimmunogenikoa aurkezten dute eta hiru leinu mesodermikoetan desberdintzatzeko ahalmena dute. Horrez gain, MSCek immunitate eta hanturazko erantzunak murriztu ditzakete haien eragin immunomodulatzaileari esker. Izatez, MSCek berezko immunitatea eta immunitate adaptatiboa erregulatzen dituztela behatu da, hainbat zelula immuneen oinarrizko funtzioak modulatuz. Zehazki, MSCek hainbat zelulen aktibazioa, heltzea, proliferazioa edo jarduera zitotoxikoa moldatzen dutela jakin da [1], haien artean, T, B eta zelula hiltzaile naturalak (*natural killer cells* edo NK) diren linfozitoak [2-4], zelula dendritikoak [5] eta monozito zein makrofagoak daudelarik [6,7]. MSCen ekintza immunomodulatzailea mekanismo desberdinen konbinazio konplexuaren emaitza bada ere, zelulek ekoizten dituzten faktore bioaktiboen eragin parakrinoak haien jarduera terapeutikoaren arduradun nagusitzat jotzen dira [8,9]. MSCek askatzen dituzten faktore ugarien artean interleukinak, entzima metabolikoak, hazkuntza faktoreak eta kimiokinak aurki ditzakegu, gaitz anitzen tratamenduan aplikagarriak direnak. MSCek faktore horiek zuzenean askatzeko ahalmena izateaz gain, zelulaz kanpoko besikulen (*extracellular vesicles* edo EV) bitartez transferitu ahal dituzte ere (1. irudia).

MSCak administratzeko aukera baliotsu bat zelulak hiru dimentsiotako (3D) hidrogel sistemetan kapsularatzea da. Estrategia honek kapsularatu gabeko zelulen injekzioaren arazoak gainditzen ditu, hala nola, administratutako zelulen atxikipen mugatua edota zelulen ezabatze arina hartzailearen sistema immunearen bitartez [10]. Hori dela eta, kapsularatutako MSCek *de novo* ekoizten dituzten faktore bioaktiboak era iraunkorrean askatzea ahalbidetzen da. Horrez gain, hidrogel matrizearen hainbat ezaugarri eraldatzea posiblea da. Hori, abantaila handia da zelulen bideragarritasuna eta funtzionalitatea hobetzen dituzten matrizeak lortzeko. Hala ere, itzulpen klinikoa lortzeko, kapsularatutako zelulen teknologian hainbat erronka gainditzea nahitaezkoa da oraindik.

Zelulak alginato-poli-L-lisina alginato (APA) mikroesferetan kapsularatzea asko ikertu da zelulak ostalariaren erantzun immunetik babesteko. Immunobabesak zelulek ekoizten dituzten faktore terapeutikoen askapen iraunkorra ahalbidetzen du [11]. Hala ere, kontuan hartu behar da sistemaren arrakasta inplantatutako zelulen jokabidearen menpe dagoela. Ondorioz, kapsularatutako zelulen portaera kontrolatzeko ezintasunak inplantearen funtzionalitatea

eta segurtasuna erabat mugatzen ditu [12-13]. Zelulen erantzun erratikoen artean, gehiegizko proliferazioa arazo nagusienetako bat da, zelula agregatu erraldoiak sor-tzen baitira. Agregatu horien nukleora elikagaien eta oxigenoaren difusioa oso mugatua dago, barneko zelulen heriotza eraginez [14]. Aldi berean, zelula apoptotikoek erantzun immunea eta hanturazko prozesuak sustatzen dituzten alarma molekulak askatzen dituzte [15]. Horrez gain, tamaina handiko agregatuek hidrogela haustu dezakete zelulen askapena eraginez, terapiaren segurtasuna zeharo mugatzen duena [16].

1. Irudia. MSCen ezaugarri immunomodulatzaileak. MSCak fenotipo hipoimmunogenikoa aurkezten dute. MSCen eragin immunoezabatzailea mekanismo desberdinen bidez gauzatzen da, haien artean, zelula-zelula arteko elkarrekintzak, MSCek gainazalean adierazten dituzten ligandoen bidez; eta bereziki, faktore bioaktibo eta EVen ekoizpena daudelarik. Laburdurak: MSCak: zelula estromal mesenkimalak (*mesenchymal stromal cells*). EVs: zelulaz kanpoko besikulak (*extracellular vesicles*). NK: zelula hiltzaile naturalak (*natural killer*). DC: zelula dendritikoak (*dendritic cells*). M2: M2 makrofagoak.

Bestalde, zelulen bideragarritasuna mantentzeko, funtsezkoa da matrize extrazelularraren (*extracellular matrix* edo ECM) antzeko ezaugarri mekanikoak eta biologikoak dituzten 3Dtako sistemak garatzea. Hala ere, mikroingurune konplexu hori imitatzeak erronka handia izaten jarraitzen du. Berez, zelulen biziraupena eta molekula bioaktiboen ekoizpena bultzatzen duten faktore fisiologiko ugari kontuan hartzea beharrezkoa da.

Faktore horietako batzuk asko aztertu dira era isolatuan, baina inoiz ez aldi berean. Adibidez, hori izan da matrizeen biofuntzionalizazioaren kasua. Biofuntzionalizazioa ECMean naturalki dauden peptidoak edo proteinak matrizean gehitzean datza. Zelulak molekula horiek antzemateko gai dira integrina izendatutako mintz-hartzaileen bidez. Hori dela eta, motibo horien presentziak zelulek biomaterial geldoekin elkarreragitea ahalbidetzen du. Elkarrekintza horien ondorioz, zelulen funtzio zuzena sustatzen duten kaskada intrazelularrak gertatzen dira. Hainbat ikerketek nabarmendu dute ezaugarri mekano-itsasgarriak dituzten matrizeen potentziala. Halere, integrina klase bakoitzaren afinitatea ez da berbera ECMeko motibo desberdinetarako. Beraz, funtzionalizazio estrategia eraginkorrak diseinatzeko, lehendabizi erabiliko den zelula mota zehatzak aurkezten dituen integrina klaseak aztertzea nahitaezkoa da. Gaur egun, integrinen espresioa fluxu-zitometria bezalako teknika konplexuen bidez ikertu daiteke. Hala eta guztiz ere, integrinen espresioa eta baita haien eragina zelulen atxikipen prozesu osoan ere aztertzen duten metodo errazak garatzea beharrezkoa da oraindik.

Horrez gain, mikroingurune naturaleko zitokinak aztertzea oso garrantzitsua da ere, MSCen gaitasun immunomodulatzailean eragin zuzena dutela jakin baita. Zehazki, MSCak erabili aurretik hanturazko zitokinekin estimulatzean, hala nola, γ interferoiarekin (IFN-γ) edo α tumorearen nekrosi faktorearekin (TNF-α), zelulen fenotipo immunoezabatzailea sustatzen dela frogatu da, molekula bioaktiboen askapena hobetuz. Alabaina, zelulen hanturazko aurre-baldintzapenaren eragina iragankorra da. Hori dela eta, epe luzeko efektu immunomodulatzailea beharrezkoa den aplikazioetan estrategia horren erabilera oso mugatua dago [17-19]. MSCen ahalmen immunomodulatzailea sarritan ikertu da indoleamina 2,3-dioxigenasa 1 (*IDO1*) bezalako geneen espresioa aztertuz denbora errealean buruturiko erretrotranskriptasa bidezko polimerasaren kate-erreakzio kuantitatiboaren (RT-qPCR) teknikaren bidez. Teknika horren emaitzak normalizatzeko erreferentzia geneak erabili ohi dira. Ildo horretan, oso ezaguna da erreferentzia geneak balioztatzea funtsezkoa dela baldintza esperimental jakinetan, emaitza

zehatzak lortzeko eta ondorio okerrak ekiditeko. Hori dela eta, gaur egun, MSCen eragin immunomodulatzailea sustatzeko aztertzen ari diren estrategien eragina erreferentzia gene ohikoenen gainean aztertzea premiazkoa da.

Azaldutakoa kontuan hartuz, gure hipotesia honako hau izan zen: MSCak kapsularatzen dituzten APA mikrokapsulak, biofuntzionalizazio estrategia egokia eta IFN-γ bidez lortutako hanturazko baldintzapena konbinatzen dituen sistema funtzio anizdun baten garapena oso baliagarria izango litzateke (1) MSCen portaera kontrolatzeko eta haien immunobabesa lortzeko inplantearen segurtasuna hobetuz, (2) zelulen bideragarritasuna sustatzeko eta (3) haien eragin immunoezabatzailea bultzatzeko.

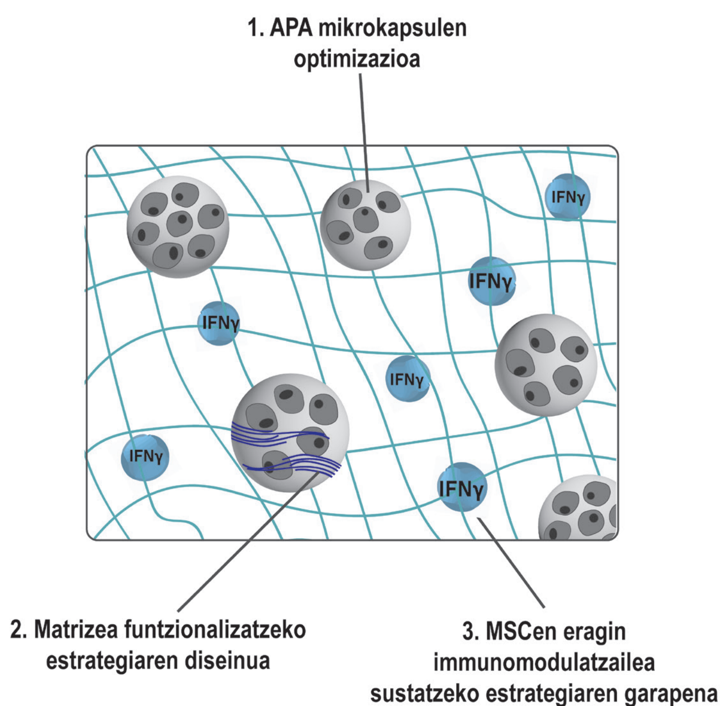
Halere, hain konplexua den sistema hori garatzeko, aldez aurretik elementu bakoitzaren eraginkortasuna balioztatzea ezinbestekoa da. Hori dela eta, tesi honen helburu nagusia hiru konponenteen inguruan ikuspegi sakon bat lortzea eta haien erabilera optimizatzea izan zen, osagai horiek hurrengoak izanik: (1) APA mikrokapsulak, (2) matrizearen biofuntzionalizazioaren diseinua eta (3) MSCen eragin immunomodulatzailea bultzatzen duen estrategia, IFN-γ hanturazko zitokinaren etengabeko baldintzapenean eta 3Dtako kultiboan oinarrituta (2. Irudia).

Tesi honen lehen urratsean, APA mikrosistemetan kapsularatutako zelulen erantzun erratikoak erregulatzea bilatu zen. Zelulen mikrokapsularatze prozesua gauzatzeko, erabilitako biomaterialen disoluzioa nahitaezkoa da, beraz, soluzio horiek osmolaritate fisiologikoa aurkeztu behar dute zelulentzat egokiak izateko [20]. Helburu horrekin, osmolaritate doitzaileak soluzioetara gehitzen dira, bi motatakoak izan daitezkeenak: elektrolitoetan oinarritutako doitzaileak edo doitzaile geldoak. APA mikrokapsulen eraketa prozesuan elkarrekintza elektrostatikoak gertatzen direnez, inguruan dauden elektrolitoen presentziak, batez ere katioi dibalenteenak, eragin handia izango du eratutako matrizean [21,22]. Beraz, lan honen hipotesia osmolaritate doitzaile mota desberdinen aukerak hidrogelen ezaugarri mekanikoak eraldatuko zituela izan zen, zelulen erantzunetan eragin zuzena izanez [23].

Osmolaritate doitzaileen eragina aztertzeko, bi soluzio lote diseinatu genituen, bakoitzak mikrokapsulak ekoizteko beharrezkoak diren soluzio guztiak zituena (%1,5 alginatoa, %0,05

poli-L-lisina (PLL), %0,1 alginatoa eta garbiketa soluzioak). Lote Biologikoan, sodioa, potasioa, kaltzioa edo fosfatoak bezalako elektrolitoak aukeratu ziren doitzaile gisa. Lote Teknologikoan, aldiz, manitola doitzaile inerte bezala hautatu zen. Lortutako loteekin, D1 MSCak kapsularatu ziren, aldez aurretik pSIN-EF2-Epo-Pur bektore lentiviralarekin genetikoki moldatuak izan zirenak eritropoetina askatzeko (D1-MSC-EPO) [24]. Horrela, mikrokapsula Biologikoak eta Teknologikoak lortu genituen. D1-MSC-EPO lerro zelularrak proliferazio ahalmen handia du [24,25], eta horregatik, matrizearen konfigurazio mekanikoak zelulen portaeran duen eragina aztertzeko eredu egokia da.

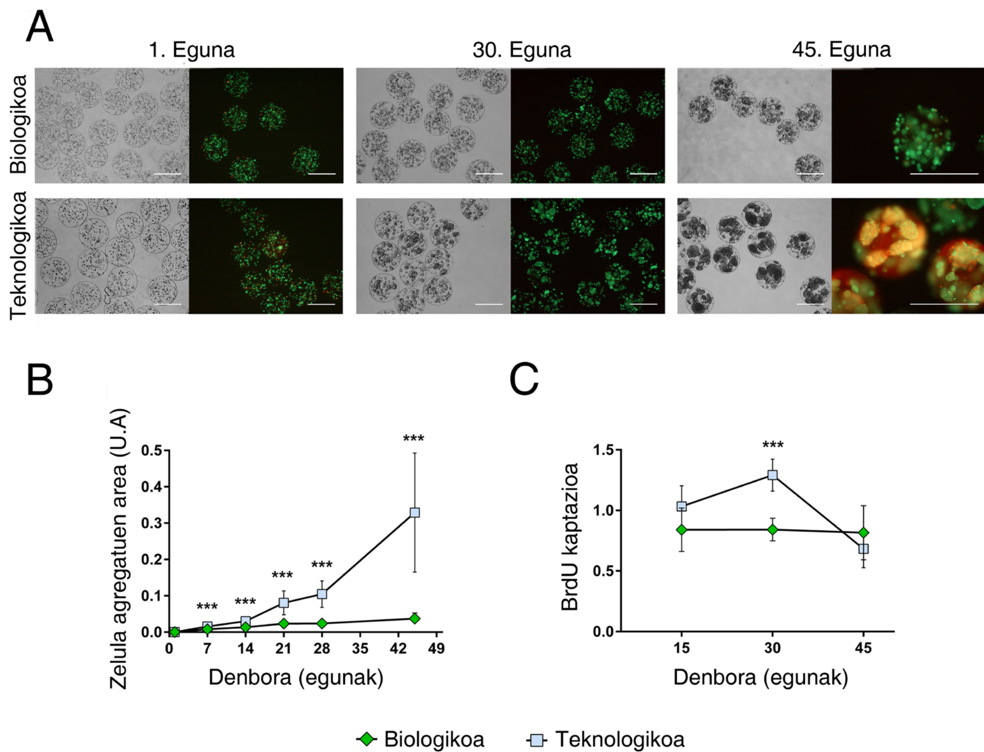
In vitro analisiek bi taldeen arteko desberdintasun esanguratsuak zehaztu zituzten. Mikrokapsula Teknologikoetan, zelula-agregatu erraldoien eraketa behatu genuen. Proliferazio abiadura handi hori, bromodesoxiuridinaren (BrdU) kaptazio entsegu batekin egiaztatu zen, timidina DNAren aitzindariaren analogoa dena (3. Irudia). Emaitza horien bidez azaldu ziren mikrokapsula talde desberdinetan kapsularatutako zelulek erakutsi zituzten portaera desberdinak bideragarritasunaren, metabolismoaren eta baita eritropoetinaren (EPO) askapenaren



2. Irudia. Tesi honen helburuen irudikapen eskematikoa.
APA: alginato-poly-L-lisina-alginato. MSC: zelula estromal mesenkimalak (*mesenchymal stromal cells)*.

entseguan ere. Hiru parametro horien igoerak eredu matematiko lineal bat jarraitu zuen talde Biologikoan, eta ostera, esponentziala Teknologikoan. Interesgarria da aipatzea proliferazioaren beherakada handia ikusi zela talde Teknologikoan 45. egunean, seguruenik zelulen bideragarritasuna asko gutxitu zelako momentu horretan. Berez, zelula-agregatu erraldoiek mikrokapsulen poroen kolapsoa gauzatu ahal dute [26], oxigenoaren eta elikagaien difusioa erabat murriztuz.

Antzemandako desberdintasunak konfigurazio mekaniko ezberdinen ondorioz gertatu ziren aztertzeko, mikrokapsulen ezaugarri mekanikoak ikertu ziren. Elkargurutzatzailearen (gure kasuan, kaltzioa) eta alginatoaren arteko loturak matrizeari gogortasuna ematen dio. Hori dela



3. Irudia. MSCen *in vitro* portaera desberdina izan zen APA mikrokapsulak formulatzeko osmolaritate doitzaile mota desberdinak erabiltzean. (A) Ikerketaren denbora puntu desberdinetan eremu argiko irudiaetan eta baita LIVE/DEAD tindaketarekin tratatutako zelulen irudi fluoreszenteetan behatu zenez, MSCen portaera erabat desberdina izan zen mikrokapsula mota bakoitzean. Irudi bakoitzeko eskalak 400 µm adierazten ditu. **(B)** Zelula-agregatuen area kuantifikatzean, estatistikoki esanguratsuak ziren desberdintasunak behatu ziren. **(C)** Bromodeoxiuridina (BrdU) kaptazioaren entseguak aurreko emaitzak egiaztatu zituen, zelulen proliferazio garrantzitsua erakutsiz talde Teknologikoan 30. egunean. Grafikoek batazbestekoa ± SD adierazten dute. Estatistika: ***$p < 0.001$. Laburdurak: MSC: zelula estromal mesenkimalak. U.A: unitate arbitrarioak.

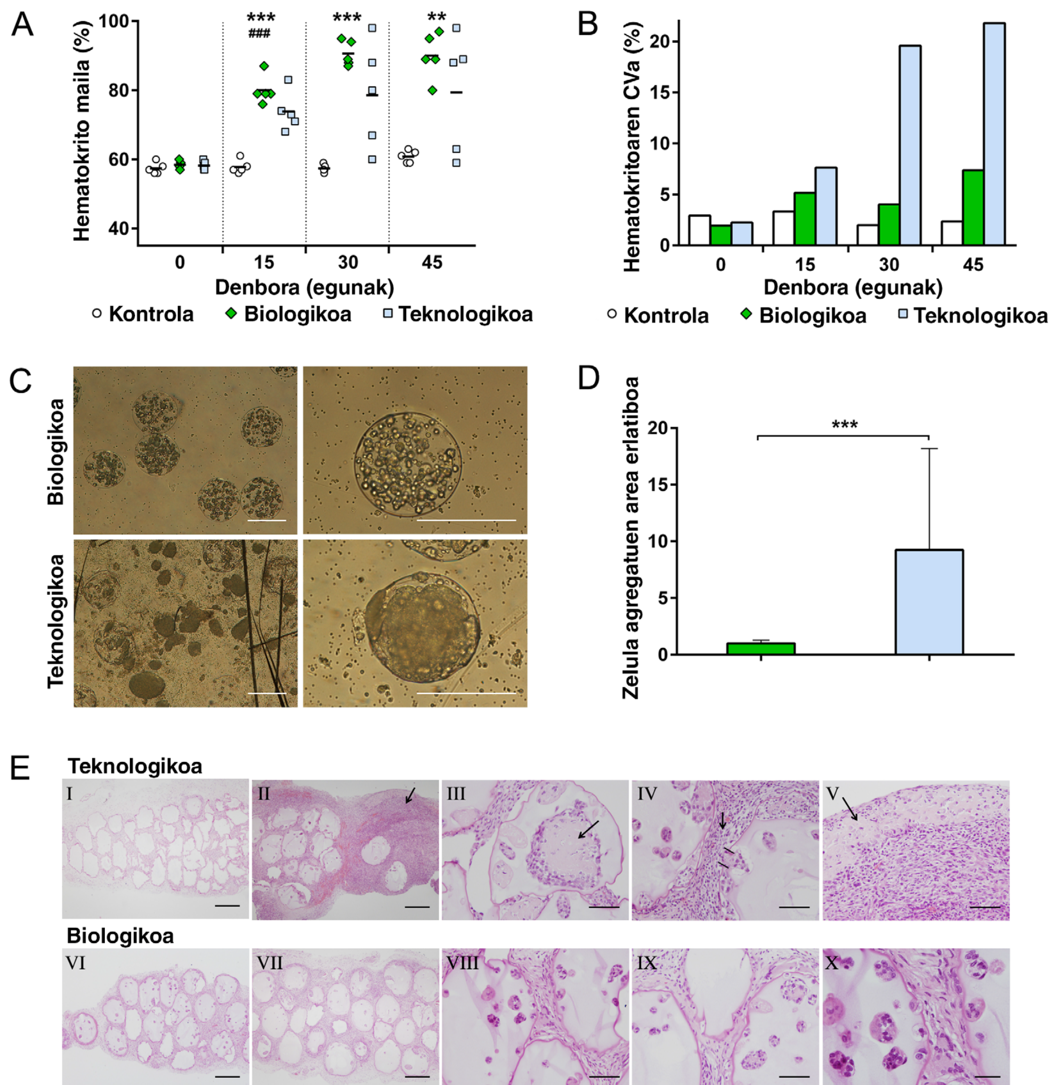
eta, mikrokapsulak alginato liasarekin tratatu ziren matrizea eratzen duen kaltzioa aska zezaten, eta katioiaren kopurua kit kolorimetriko baten bidez aztertu zen. Kapsula Biologikoetan kaltzio maila altuagoak behatu ziren, matrizearen gurutzaketa maila handiagoa zela eta hortaz, gogortasun handiagoa zuela iradokiz. Emaitza horien arabera, mikrokapsulen nukleo polimerikoa eratu ostean, estaldura prozesuan, kaltzio galera txikiagoa izan zen hidrogela katioi hori zuten soluzioekin kontaktuan jartzean. Horrenbestez, mikrokapsula Biologikoek kaltzio gehiago mantendu zuten, eta lortutako matrize gogorragoak proliferazioa murriztu zuen, zelulen portaeran kontrola ezarriz [27,28]. Kapsula Teknologikoetan, ostera, kaltzioaren galera handiagoa izan zen, matrize permisiboagoa eratuz [29,30]. Hori dela eta, talde horretan proliferazio handiagoa eta ondorioz, zelula agregatu erraldoien sorrera ahalbidetu zen. Beraz, osmolaritate doitzaileek ez zuten eragin zuzenik izan zelulen portaeran, baizik eta kapsula eratzeko prozesuan, zehazki, alginato matrizearen gurutzaketa mailan. Interesgarria da aipatzea mikrokapsula Teknologikoek erresistentzia osmotiko altuagoa erakutsi zutela. Horrek, mintz erdiiragazkorraren alginatoaren eta PLLaren arteko elkarreragina sendoagoa izan zela adierazten du. Honen azalpena honako hau izan daiteke: alde batetik, kapsula Teknologikoen nukleoak elkargurutzaketa maila baxuagoa erakutsi zuen eta ondorioz, alginato kate gehiago zeuden eskuragai PLLarekin elkarreragiteko. Bestalde, mikrokapsulen eraketa prozesuan ingurugiroan elektrolitorik ez egoteak bi elementu horien arteko elkarrekintza erraztu zuen. Iragazkortasunari dagokionez, ez zen desberdintasun esanguratsurik antzeman fluoreszeina isotiozianatoarekin (FITC) markatutako pisu molekular desberdinetako dextranoen difusioan. Berez, bi taldeek iragazkortasun egokia erakutsi zuten kapsularatze teknologiaren helburuak betetzeko.

Ondoren, matrizearen gurutzaketa mailak zelulen zatiketan duen eragina aztertu genuen. D1-MSC-EPO zelulak BrdUrekin tratatu ziren, sintetizatu berri den DNAra eransten dena. Horrez gain, DNAra lotzen den 7-aminoaktinomizina Drekin (7-AAD) tindatu ziren, zelulak zikloaren zein fasean dauden agerian utziz: G0/1 (atseden fasea), edo S/G2/M (zatiketa prozesuaren fase desberdinak). Laginak fluxu-zitometriaren bidez aztertzerakoan, 30. egunean proliferazio abiadura askoz handiagoa behatu zen sistema Teknologikoetan kapsularatutako zeluletan. Zelula gehienak G0/1 egoeran zeuden arren, mikrokapsula Biologikoetan (%55,3) zein Teknologikoetan (%57,9), gainontzeko zeluletan, desberdintasun garrantzitsuak hauteman ziren. Bereziki, talde Biologikoan, zelula asko BrdU gehitu aurretik zeuden S/G2/M egoera

berean mantendu ziren 48 orduz. Talde Teknologikoaren zelulak, ostera, material genetikoa bikoizteko eta zikloa osatzeko gai izan ziren, G0/1 fasera DNAra erantsitako BrdU markatzailearekin itzuliz. Emaitza horiek, matrize Teknologikoek, permisiboagoak izanda, zelulen proliferazio azkarragoa ahalbidetu zutela adierazten dute [26].

In vitro lortutako emaitzek mikrokapsulen efektu terapeutikoan eragina zuten zehazteko, *in vivo* entseguak burutu genituen (4. irudia). Horretarako, mikrokapsulak C57BL/6 sagu immunogaien larruazalpean aloinplantatu ziren eta une desberdinetan aurpegiko zainetik odol laginak bildu ziren. Odolaren hematokrito maila ikertzeko, mikrohematokrito metodo estandarra erabili zen. Mikrokapsula Biologikoetan kapsularatutako D1-MSC-EPO zelulek hematokrito profilak modu progresiboan handitu ahal izan zituzten taldeko sagu guztietan. Inplante Teknologikoak zituzten bi saguk ostera, 30. egunean, dagoeneko, hematokritoa igotzeari huts egin zioten, eta gainontzeko saguen artean aldakortasun handia behatu zen. Administrazioaren 45 egun ondoren, inplanteak berreskuratu ziren eta morfologikoki aztertu zirenean, mikrokapsula Teknologikoetan zelula agregatu erraldoiak ikusi ziren. Bestalde, kapsula Biologikoek itxura esferikoa eta osotasuna mantendu zuten. Kapsula Teknologiko gehienak, berriz, hautsi egin ziren, zelulak inguruko ehunera askatuz. Seguruenik, sistemaren mintzek ezin izan zuten jasan gehiegizko proliferazioaren ondorioz sortutako zelula agregatu erraldoiek eragiten zuten presioa. Analisi histologikoak emaitza hauek egiaztatu zituen. Talde Teknologikoan, kapsulen haustura maila handiagoa antzeman zen. Horrek, zelulen askapena bultzatu zuen, inguruko ehunak inbadituz eta tumoreen antzeko masak eratuz, hanturazko erantzun larria eragin zutenak.

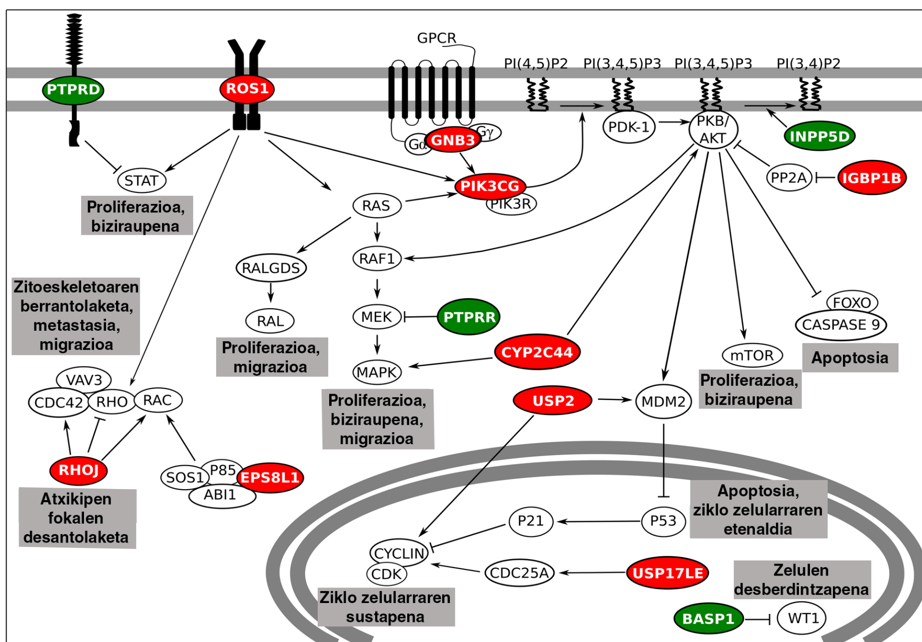
Azkenik, zelulen espresio genikoa aztertzeko, gene mikroarray ikerketa bat burutu genuen. Entsegu honen emaitzek zelulak kapsularatutako matrizearen ezaugarri mekanikoek gene espresioan eragin handia zutela zehaztu zuten (5. irudia). Sistema Teknologikoetan kapsularatutako zeluletan, proliferazio kaskada garrantzitsuak era nabarmenean aktibatu ziren, hala nola, PI3K / Akt / mTor edo Ras / Raf / MAPK bideak. Interesgarria da aipatzea bi bide horiek mutatuta edo anplifikatuta daudenean, proliferazio eta biziraupen seinaleak modu iraunkorrean aktibatzen direla behatu dela, tumoregenesia bultzatuz [31]. Emaitza horiek egiaztatuz, zitoeskeletoa berrantolatzeko prozesuan parte hartzen duten geneen espresioa suspertuta zegoela ikusi zen, tumore-supresore gene batzuen espresioa, aldiz, gutxituta zegoelarik. Datu



4. Irudia. *In vivo* ikerketetan EPOren askapen profil desberdinak behatu ziren MSCak kapsularatzen zituen mikrokapsula motaren arabera. (A-B) Hematokrito profilak era kontrolatuan igo ziren inplante Biologikoetan. Talde Teknologikoan, aldiz, dispertsioa oso handia izan zen erreplikatuen artean. Estatistika: Talde Kontrol eta Biologikoaren artean: $^{**}p < 0.01$ and $^{***}p < 0.001$. Talde Kontrol eta Teknologikoaren artean: $^{\#\#\#}p < 0.001$. **(C)** Esplanteen karakterizazio morfologikoak talde Teknologikoan zelula-agregatu erraldoiak eta hautsitako mikrokapsulak agerian utzi zituen. **(D)** Zelula-agregatuen area neurtzean, estatistikoki esanguratsuak ziren desberdintasunak behatu ziren bi mikrokapsula taldeen artean. Estatistika: $^{***}p < 0.001$. **(E)** Ikerketa histologikoak mikrokapsula hutsak erakutsi zituen efektu terapeutikorik izan ez zuten erreplikatu Teknologikoetan (I). Analisiak talde Teknologikoan honakoak erakutsi zituen: hantura handia (II), mikrotumoreak sortu zituzten zelula agregatuak (III), kapsulen haustura (IV) eta horren ondorioz, kapsula kanpoko tumoreen eraketa (V). Mikrokapsula Biologikoetan, ordea, ez zen horrelakorik aurkitu: hantura maila txikiagoa izan zen (VI-VII) eta kapsulen osotasuna behatu zen (VIII-X). Grafikoek batazbestekoa ± SD adierazten dute (n = 5 animali entseguetan, n = 30 zelulen area kuantifikatzeko). Irudien eskala: (C), (E) I,II, VI, VII = 400 μm. (E) III, IV, V, VIII, IX = 100 μm. (E) X = 50 μm.

horien arabera, D1-MSC-EPO zelulek mikrokapsula teknologikoetan ez zuten oztopo handirik aurkitu tumore moduko jokabidea garatzeko. Hortaz, gure ikerketak literaturarekin bat egiten du, matrizearen ezaugarri mekanikoek zelulen portaeran eragina dutela frogatuz, zelulak atxikitzeko ECMeko domeinuen gabezian [16, 32]. Ildo horretan, etorkizuneko ikerketek prozesu hori nola gertatzen den aztertu beharko lukete, zelula-mikroingurune elkarrekintzen inguruan ezagutza sakonagoa eskuratzeko.

Oro har, osmolaritate doitzaileak matrizearen konfigurazio mekanikoa eraldatzeko tresna baliagarri gisa erabil daitezkeela frogatu genuen, biomaterial edo elkargurutzatzaile mota edota proportzioa aldatu beharrik gabe. Sistemaren ezaugarri mekanikoek zelulen gene espresioan eragin handia izan zuten. Ondorioz, soluzio Teknologikoekin edo Biologikoekin eratutako mikrokapsuletan kultibatutako zelulen portaeran desberdintasun esanguratsuak antzeman ziren *in vitro* zein *in vivo*. Matrize Biologikoek zelulen proliferazioaren gainean kontrol estua ezarri zuen, zelula-agregatu erraldoien eraketa, zelulak kapsuletatik ateratzearen arriskua



5. Irudia. Gene espresioa desberdina izan zen mikrokapsula Biologiko eta Teknologikoetan, eta horren ondorioz, zelulen portaera bestelakoa izan zen. Zelula barneko kaskaden irudikapen eskematikoak, talde Teknologikoan *Fold change* delakoaren > 3 edo < -3 aldaketa izan zuten geneak erakusten ditu. Gorriz, espresio suspertuta erakutsi zuten geneak. Berdez, espresio gutxitua erakutsi zuten geneak.

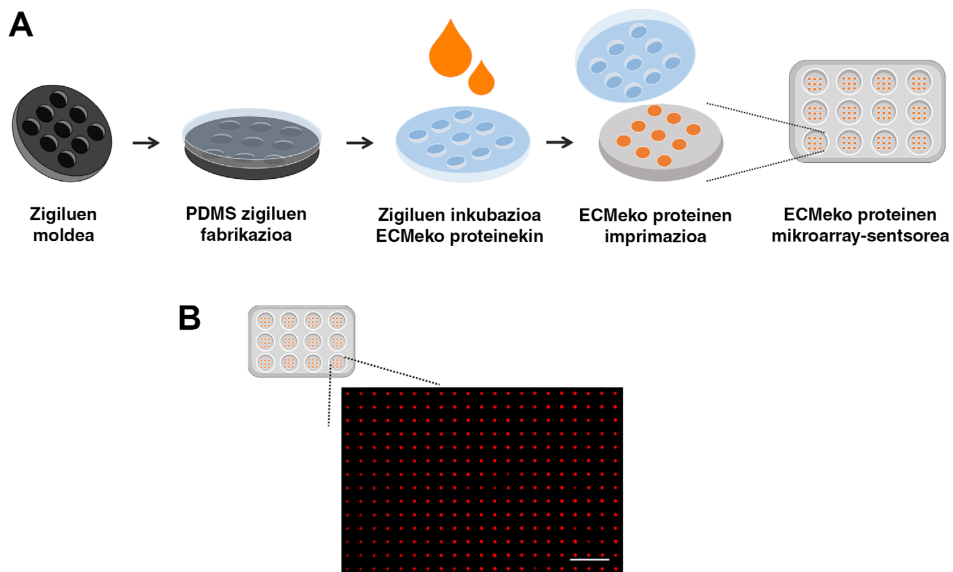
eta hanturazko erantzun larriak saihestuz. Beraz, gure emaitzek erakutsi zuten osmolaritate doitzaileen aukera egokiak MSCen portaera erregulatu ahal duela, faktore terapeutikoaren askapen iraunkorra bermatuz eta terapia seguruagoa lortuz.

Zelulen erantzun erratikoak kontrolatzeaz gain, kapsularaturako zelulen bideragarritasuna eta funtzionalitatea sustatzea ere funtsezkoa da. Hori dela eta, tesiaren bigarren urratsean, biofuntzionalizazio estrategia egokien garrantzian zentratu ginen. Horretarako, fibronektina (FN) mikroarray-biosentsore bat garatu genuen, zelulak banaka aztertzen dituena, haien integrinen profila ikertzeko eta zelulen eta biomaterialeen arteko elkarreragin dinamikoak karakterizatzeko, biofuntzionalizazio estrategia arrakastatsuak diseinatzeko tresna gisa.

Sentsorea fabrikatzeko, *Micro-contact Printing* (μCP) deritzon *patterning* edo egituraketa teknika zelulen kultiborako plaketara (*tissue culture plate* edo TCPra) egokitu genuen. Bertan, FN irla zirkularretan oinarritutako patroia inprimatu genuen, atxikipen entseguak errazteko helburuarekin. Lehenik, fotolitografiaren bidez, zigiluaren moldea lortu zen, 20 μmko zutabe zirkularrak dituzten polidimetilsiloxano polimeroaz (PDMS) osatutako zigiluak fabrikatzeko erabili zena. Bigarrenez, PDMS zigilu horiek TAMRA fluoroforoarekin markatutako behi-serum albumina (BSA) eta FN zituen PBS soluzio batekin inkubatu ziren, proteinaren inprimazio egokiaren kontrol positiboa lortzeko. Azkenik, tintatutako PDMSa TCP putzuetan zigilatu zen, 20 μm FN irlen patroi homogeneoa sortuz, ikerketa honetan erabilitako zelula mota guztien banako atxikipena lortzen duena (6. Irudia).

Ildo horretan, ezaugarri desberdinak dituzten lau zelula mota aztertu genituen biosentsorearen bidez, plataformaren aplikagarritasun erreala ikertzeko. Zehazki, bi zelula lerro erabili genituen: hamster kumearen giltzurrun fibroblastoak (*baby hamster kidney* edo BHK) eta sagu C_2C_{12} mioblastoak (C_2C_{12}), eta baita bi zelula primario mota ere: giza fibroblasto dermikoak (*human dermal fibroblast* edo hDF) eta giza gantz MSCak (*human adipose mesenchymal stromal cell* edo hMSCa).

Lehenik eta behin, zelulen atxikipen zinetika aztertu genuen. Horretarako, zelulak bi orduz inkubatu genituen biosentsorean eta FN irlen okupazioa (*dot array occupancy* edo DAO) denbora puntu desberdinetan kuantifikatu genuen. Zelula guztien kasuan, DAOa denboran



6. Irudia. ECMeko proteinen mikroarray biosentsorearen garapena. (A) ECMeko proteinen mikroarray-sentsorearen fabrikazio prozeduraren eskema. **(B)** Fibronektina-BSA-TAMRAren inprimazio patroia (eskalak 400 µm adierazten ditu). PDMS: polidimetillsiloxano polimeroa. ECM: matrize extrazelularra (*extracellular matrix*).

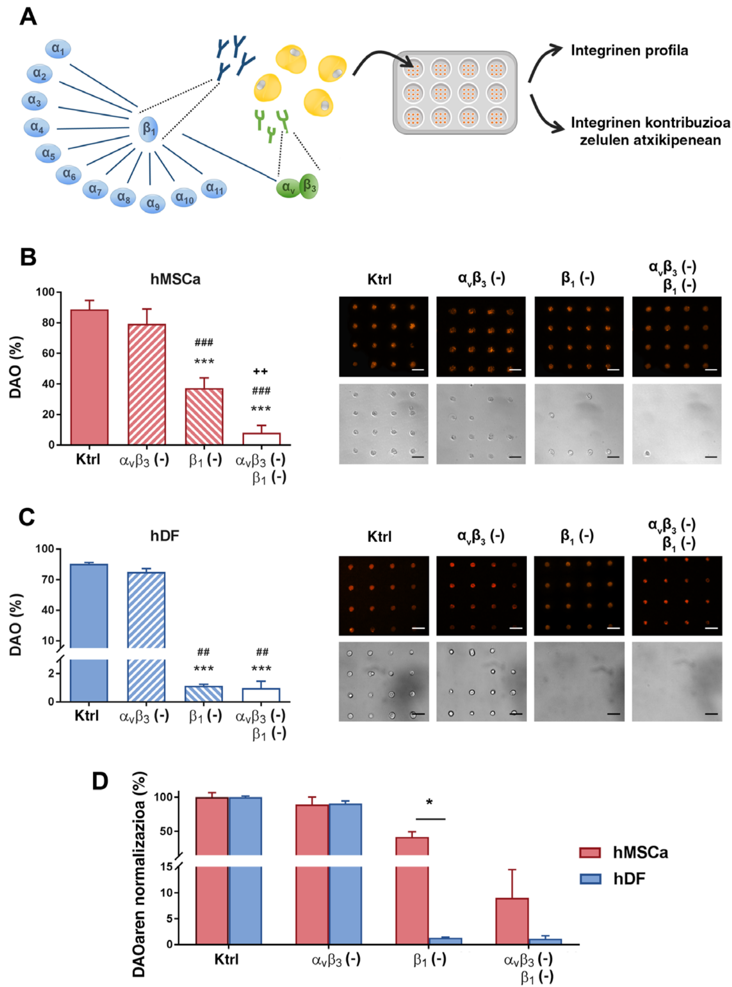
zehar handitzen zela behatu genuen, lautada batera iritsi arte. Ondoren, atxikipen kurbak lehen ordeneko zinetika batera doitu genituen, abiadura konstantea (k) eta t_{50} balioak eskuratuz. Azken hori, patroiaren irlen %50eko okupazioa ematen den denbora bezala definitzen da. Parametro horietaz baliatuz, bi zinetika desberdin identifikatu genituen: k eta t_{50} balioak esanguratsuki handiagoak izan ziren C_2C_{12} eta hMSCa zeluletan. Hori dela eta, substratuari BHK eta hDF zelulak baino askoz azkarrago atxikitu ziren. Emaitza horien arabera, atxikipen zinetikan eragin nagusia zuen faktorea zelula mota zela behatu genuen. Zelula eta substratuaren arteko elkarrekintzak batez ere integrinen bidez gauzatzen direnez [33], gure hipotesia, atxikipen zinetikan antzemandako desberdintasunak zelula mota bakoitzaren integrina profil desberdinagatik gertatzen zirela izan zen.

Hipotesi hori egiaztatzeko, gure hurrengo helburua zelulen atxikipenean integrin mota zehatzek duten eragina aztertzea izan zen (7. irudia). Horretarako, hMSCa eta hDF zelulekin burutu genituen hurrengo entseguak, bi arrazoi nagusi direla eta: alde batetik, zinetika desberdina aurkezteagatik eta bestalde, ikerketa honen lau zelula moten artean erakargarrienak direlako ikuspegi terapeutiko batetik, giza zelula primarioak baitira [34-36]. Garrantzitsuagoa

dena, 2. Irudiak erakusten duen sistema funtzio anizduna kontuan hartuz, zelula horiek oso interesgarriak ziren biofuntzionalizazio estrategia egokiak diseinatu nahi genituelako alde batetik MSCentzat, gure zelula terapeutikoak baitira, eta bestalde, baita hDF zelulentzat ere, kanpoaldeko hidrogela biodegradagarria dela eta larruazalpean administratzen dela suposatuz, ostalariaren fibroblastoek ehunaren berriztapena sustatu ahal dutelako. Lehendabizi, zelulak integrinen aurkako antigorputzekin inkubatu genituen. Zehazki $\alpha_v\beta_3$ integrina blokeatzen duten antigorputzak edo β_1 azpi-unitatea aurkezten duten integrina guztiak blokeatzen dituzten antigorputzak erabili genituen. Integrina horiek, hMSCa eta hDF zeluletan FNra lotzen diren hartzaile nagusiak izateagatik aukeratu ziren [37,38]. Ondoren, zelulak biosentsorean inkubatu ziren eta DAOa kuantifikatu zen. Bi zelula moten kasuan, $\alpha_v\beta_3$ integrinaren blokeoak ez zuen DAOan eraginik izan. Ostera, β_1 azpiunitatea aurkezten dituzten integrina guztiak blokeatzean, FN irlen okupazioak behera egin zuen nabarmenki. Bi zelula motak zuzenean konparatzeko datuak normalizatu ziren. Konparaketa horretan, β_1 azpi-unitatearen blokeoak hDF zeluletan eragin handiagoa zuela antzeman genuen. Zehazki, β_1 azpi-unitatea duten integrinak blokeatzean, hMSCen %40a atxiki zen substratuari. hDF zelulen kasuan, aldiz, %2aren inguru atxiki zen soilik. Gure emaitzek aztertutako zelulek integrina profil desberdinak dituztela iradokitzen dute. Alde batetik, hDF zeluletan β_1 azpi-unitatea duten integrinen espresioa nagusia dela behatu zen. hMSCetan, ostera, β_1 integrina horiek garrantzitsuak izan arren, haien blokeoa beste integrina batzuen espresioarekin nolabait orekatuta dagoela ikusi zen, esaterako, α_v klaseko integrinen espresioarekin. Horretaz gain, aipagarria da entsegu honek plataformaren sentsibilitatea egiaztatu zuela, integrina bakarra ($\alpha_v\beta_3$) edo integrina ugari (β_1 domeinua duten guztiak) blokeatzean DAOan desberdintasun esanguratsuak antzeman zuelarik [39].

Bestalde, biosentsorea integrina klase bakoitzak zelulen atxikipen prozesuaren sendotzean zein heinean parte hartzen duen zehazteko gai izan zen. Gure plataformarekin entsegua egin aurretik, maiz erabiltzen den immunozitokimika teknikak integrina mota bakoitzak atxikipena sendotzean duen kontribuzioari buruzko informaziorik ematen ez duela baieztatu genuen. Hori dela eta, immunozitokimikaren bidez ez genuen desberdintasunik antzeman $\alpha_v\beta_3$ integrina edo β_1 azpi-unitatea duten integrina guztiak blokeatzean. Gure biosentsorearekin egindako askapen azterketak, aldiz, zelulen eta substratuaren arteko elkarrekintzari buruzko ikuspegi sakonagoa eman zuen. $\alpha_v\beta_3$ edo β_1 domeinua aurkezten zuten integrinak blokeatu ondoren,

zelulak biosentsorean inkubatu ziren eta DAOa kuantifikatu zen. Ondoren, zelulak askatzeko indarra aplikatu genuen likido aspirazioaren bidez, eta DAOa berriro zenbatu zen. Aurreko entseguetan, $\alpha_v\beta_3$ blokeatzeak kontrolarekin aldenduz, DAOaren kuantifikazioa aldatzen ez zuela behatu genuen arren, esperimentu honetan, $\alpha_v\beta_3$ blokeatzean, FN patroiarekiko zelulen



7. Irudia. hMSCa eta hDF zelulek integrina profil desberdinak erakutsi zituzten. (A) Prozedura esperimentalaren irudikapen eskematikoa. $\alpha_v\beta_3$ edota β_1 integrinen blokeoaren eragina **(B)** hMSCa eta **(C)** hDF zeluletan. Grafikoek batazbestekoa ± SD irudikatzen dute (n = 4 erreplikatu talde bakoitzeko). Estatistika: $^{***}p < 0.001$ kontrol taldearekin alderatuz. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ $\alpha_v\beta_3$ (-) taldearekin konparatuz. $^{++}p < 0.01$ β_1 (-) taldearekin alderatuz. Irudi bakoitzaren eskalak 50 μm adierazten ditu. **(D)** hMSCa eta hDF zelulen atxikipena normalizatu eta konparatu zen. Grafikoek batazbestekoa ± SD irudikatzen dute (n = 4 erreplikatu talde bakoitzeko). Estatistika: $^{*}p < 0.05$. Laburdurak: DAO: inprimatutako irlen okupazioa (*dot array occupancy*). hMSCa: giza gantz zelula estromal mesenkimalak (*adipose-derived human mesenchymal stromal cells*). hDF: giza fibroblasto dermikoak (*human dermal fibroblasts*). Ktrl: Kontrol taldea.

atxikipena ez zen kontrol taldearena bezain sendoa izan, aztertutako bi zelula motetan. α_v eta β_1 integrinen artean dagoen *crosstalk*-delako elkarreraginak emaitza horiek azaldu ditzake. Izatez, behin substratuari lotuta, α_v integrinek $\alpha_5\beta_1$ integrinen *clustering* edo multzokatzea eragiten dutela jakin da, zelulen atxikipena indartuz [40]. Horrenbestez, $\alpha_v\beta_3$ren blokeoak, integrinen arteko elkarrekintza oztopatu ahal du, zelulen atxikipena substratura ahulduz. Horretaz gain, β_1 azpi-unitatearen blokeoak zelulen atxikipena ahuldu zuen ere, domeinu hori daukaten integrinen multzoak, atxikipena sendotzean paper garrantzitsua dutenak, ez baitziren sortu. Aurretik aipatu den bezala, bi zelula motek portaera berdina erakutsi zuten. Hori jakinda ere, hDFen kasuan, β_1 azpi-unitatea duten integrinen blokeoak, zelulen atxikipenean eragin handiagoa izan zuen $\alpha_v\beta_3$ integrinaren blokeoarekin alderatuz; hMSCa zeluletan gertatu ez zena. Horrek, hMSCa zelulekin konparatuz, hDFetan β_1 integrinen eragina handiagoa dela iradokitzen du. Emaitza horiek aurreko entseguetan lortutako datuak egiaztatzen dituzte, eta aldi berean, bat datoz literaturarekin, non hDFetan $\alpha_4\beta_1$ eta $\alpha_5\beta_1$ integrinen kontribuzioa funtsezkoa dela nabarmentzen den [37].

Azkenik, gure plataforma balioztatzeko, integrina horien espresioa fluxu-zitometriaren bidez aztertu genuen. Teknika horrek, hDF zelulek $\alpha_v\beta_3$ren espresio baxuagoa eta $\beta_1$en adierazpen handiagoa aurkezten zutela erakutsi zuen hMSCa zelulekin alderatzean, gure biosentsorearekin lortutako hiru ondorio nagusiak egiaztatuz: (1) α_v klaseko integrinak, β_1 azpi-unitatea aurkezten duten integrinak, $\alpha_5\beta_1$ esaterako [37], baino azkarrago lotu ziren FNra, zelulen atxikipen prozesuari hasiera emanez. Hori kontuan hartuz, C_2C_{12} eta hMSCa zeluletan behatutako zinetika azkarragoak, zelula horietan α_v klaseko integrinen dentsitate handiagoa zegoela adierazten du, BHKekin eta hDFekin aldenduz. (2) hMSCa eta hDF zelulek integrina espresio profil desberdinak aurkeztu zituzten. Zehazki, β_1 integrinen kontribuzioa nagusi izan zen hDF zeluletan, eta garrantzitsua baino ez bakarra hMSCa zeluletan. (3) $\alpha_v\beta_3$ eta β_1 integrinen papera hMSCa zelulen atxikipenaren sendotzean antzekoa izan zen. hDF zeluletan, ostera, β_1 integrinen kontribuzioa esanguratsuki handiagoa izan zen.

Integrinen espresioa fluxu-zitometria bezalako teknika konplexuen bidez ikertu ahal den arren, prozedura horrek ez du informaziorik ematen atxikipen prozesu osoan duten kontribuzioaren inguruan. Bestalde, garrantzitsua da aipatzea horrelako teknikak erabiltzeko, zelulak substratutik askatu behar direla, zelulen atxikipen prozesuaren menpe dauden *pathway*

edo bide zelularrak konprometituz. Gure biosentsoreak arazo horiek gainditzen ditu, ECM naturala birsortuz zelulen atxikipena mikroingurune egokian aztertzeko, eta integrinen profilaketa zein haien eginkizuna atxikipen prozesuaren sendotzean aztertuz. Hori dela eta, biosentsoreak, materialen biofuntzionalizazio arloan funtsezkoa den informazioa eskaintzen du.

Ildo horretan, oso garrantzitsua da erabiliko den zelula mota jakinean integrinen profila eta haien kontribuzioa atxikipen prozesuan ikertzea biofuntzionalizazio estrategia egokiak diseinatzeko [41]. Egoera hori agerian uzten duen adibide bat, matrizeak funtzionalizatzeko RGD (arginina-glizina-aspartiko azidoa) tripeptidoaren erabilera da. RGD FNren eta beste ECMeko proteina ugariren parte da, zelulen atxikipena sustatzeko behar den ezinbesteko gunea duelarik [11,42]. Integrina anitzek RGD gunera lotzen diren arren, peptidoarekiko afinitatea ez da berdina integrina mota guztietan. Adibidez, $\alpha_v\beta_3$ eta $\alpha_5\beta_1$ integrinak RGDra lotzen badira ere, bigarrenak PHSRN (Pro-His-Ser-Arg-Asn) sinergia gunea ere behar du zelulen atxikipena gauzatzeko, mikroingurune naturalean RGDtik gertu dagoena [37,43,44]. Era berean, $\alpha_4\beta_1$ integrinak ere PRARI (Pro-Arg-Ala-Arg-Ile) sekuentzia sinergikoara lotzen dira [44]. Ondorioz, gure datuek iradokitzen dute hMSCa zelulen kasuan, PHSRN edo PRARI bezalako sinergia guneak matrizeetan eranstea onuragarria izan daitekeen arren, RGDren presentziarekin nahikoa dela zelulen atxikipena eragiteko. hDFetan, aldiz, RGDrekin batera, peptido sinergikoak matrizetan gehitzea ezinbestekoa da, β_1 azpi-unitatea duten integrinen dentsitate handia eta $\alpha_v\beta_3$ integrinen kontribuzio txikiagoa kontuan hartuta.

2. Irudiak erakusten duen sistema funtzio anizduna kontuan hartuz, gure biosentsorearekin lortutako datuek MSCak kapsularatzen dituzten APA mikrokapsulak funtzionalizatzeko RGD domeinua eranstea estrategia aproposa izango litzatekeela agerian uzten dute. Bestalde, kanpoaldeko hidrogelaren biofuntzionalizazioa desberdina izan daiteke. Hidrogel hori biodegradagarria dela eta larruazalpean administratzen dela suposatuz, ostalariaren fibroblastoen inbasioa eta ondorioz, ehunaren berriztapena sustatzeko, FN edo kolageno bezalako ECMeko proteina osoak gehitzea aukera egokiagoa izango litzateke.

Oro har, gure biosentsoreak, ikertu nahi den substratuarekiko zelula mota jakin baten afinitatea sakonki aztertzeko aukera ematen du, ez da FNra soilik mugatzen. Horrez gain, antigorputz egokiak aukeratuz, klase desberdinetako integrinak hainbat zelula motetan paraleloki

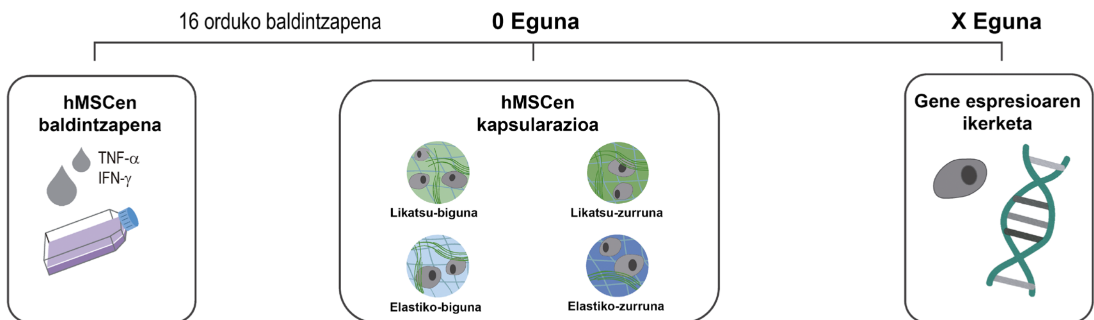
aztertzea ahalbidetzen du. Biosentsore honen potentziala haren sentsibilitatean, zehaztasunean, sinpletasunean eta kostu txikian oinarritzen da. Hori dela eta, interesgarria izango da material biomimetikoen arloan jarduten duten ikertzaile guztientzat.

APA mikrokapsuletan MSCen portaera kontrolatu eta haien integrinen profila aztertu ondoren, sistemaren 3. elementuan zentratu ginen eta MSCen eragin immunomodulatzailea sustatzen duen estrategia berri bat diseinatu genuen. Zehazki, sistema multifuntzional bat garatu genuen zelulen 3Dtako kultibo biomimetikoa eta hanturazko baldintzapen iraunkorra konbinatuz. MSCen ekintza immunomodulatzailea aztertzeko, gene erregulatzaileen panel zabal baten espresioa ikertu genuen denbora errealeko RT-qPCRren bidez. Panel horretan, erantzun immunoezabatzailearen maila desberdinetan jarduten duten faktoreak kodifikatzen dituzten geneak aztertu genituen, emaitza adierazgarriak lortzeko asmoz.

Lehenik eta behin, hanturazko baldintzapenak hezur muineko giza MSC (*human MSC* edo hMSC) primarioetan duen eragina aztertu genuen. Horretarako, IFN-γ eta TNF-α zitokinekin $\approx$ 16 orduz inkubatu genituen zelulak, eta ondoren, bi dimentsiotan (2D) kultibatu genituen, TCPetan zehazki. Indoleamina 2,3-dioxigenasa 1 (*IDO1*), prostaglandina sintetasa 2 (*PTGS2*) eta galaktina-9 (*GAL9*) geneen espresioa esanguratsuki handitu egin zen. Emaitza horiek literaturarekin bat datoz, bi zitokina hauen konbinazioak hMSCen fenotipo immunoezabatzailea bultzatzen duela dioenak [19], hMSCen faktore bioaktiboen ekoizketa sustatuz [45]. Hala ere, hanturazko baldintzapen hori iragankorra zela frogatu genuen, 2Dtako kultiboan 3 egun igaro ondoren, aipatutako geneen espresioa era nabarmenean murriztu baitzen. Baldintzapen horrek beste gene batzuetan eraginik izan ez zuenez, gure gene panela bi multzotan banatu genuen: hanturazko baldintzapenaren aurrean erantzun zuten geneak: *IDO1*, *GAL9*, *PTGS2*; eta erantzun ez zutenak: hazkuntza faktore hepatozitikoa (*hepatocyte growth factor* edo *HGF*), interleukina-10 (*IL10*), eta interleukina-1 hartzailearen antagonista (*interleukin-1 receptor antagonist* edo *IL1RN*).

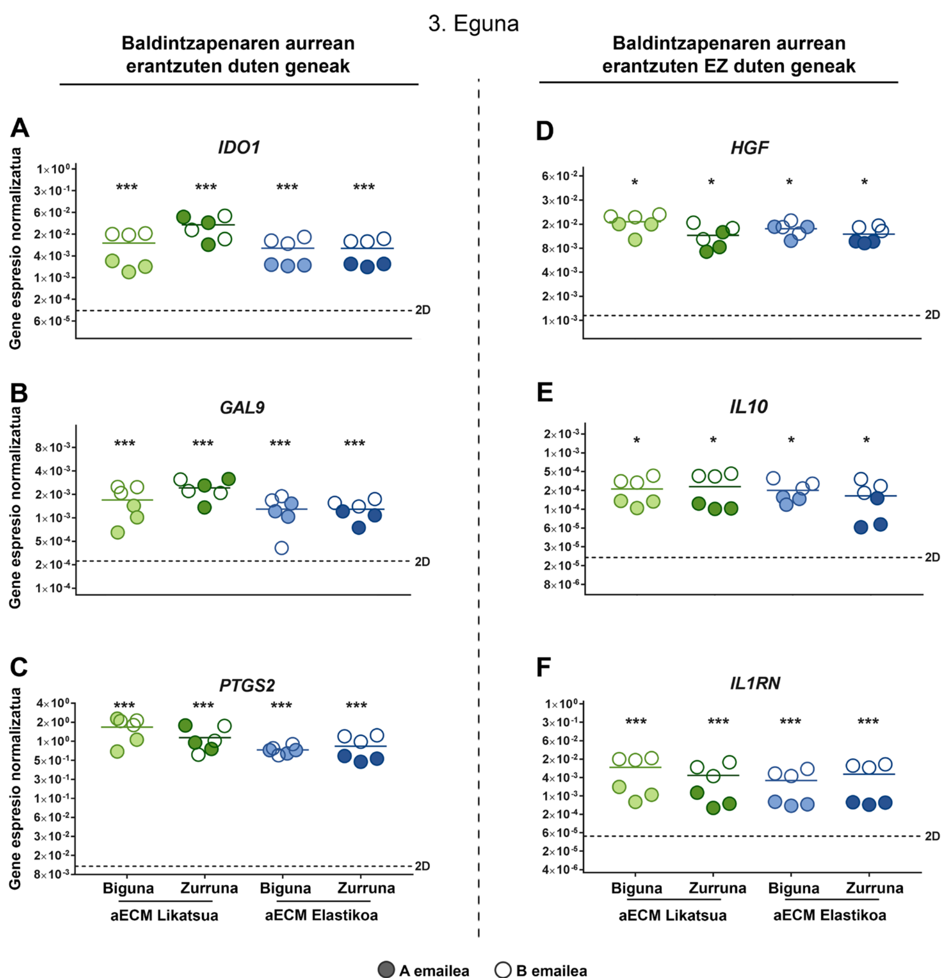
Aldez aurretik aipatu den bezala, 3Dtako hidrogel biomimetiko batek gene immunomodulatzaile horien adierazpena mantenduko zuelaren hipotesiaren inguruan jardun genuen. Hipotesi hori frogatzeko, aldez aurretik 16 ordu inguru hanturazko zitokinekin baldintzaturiko hMSCak ECM artifizial (aECM) bezala izendatutako hidrogeletan kapsularatu genituen (8.

Irudia). aECM hidrogelen matrizea alginatoz eta I. motako kolageno fibrilarrez osatutako sare interpenetratzailea da, ECM naturalaren arkitektura imitatzeko helburuarekin diseinatu zena. Berez, I. motako kolagenoan aberatsak diren inguruneek hMSCen biziraupena sustatzen dutela jakina da [46]. aECM hidrogelen lau bariante erabili ziren: likatsu-biguna, likatsu-zurruna, elastiko-biguna eta elastiko-zurruna. Elastikotasuna eta zurruntasuna modu independentean eraldatu ziren, alginatoaren gurutzaduraren modua eta magnitudea aldatuz. Hidrogel likatsuek, estres-erlaxazio erantzun arina erakusten dute gurutzaketa ioniko itzulgarria dutelako azido guluroniko ugari dituzten blokeetan (G blokeetan). Gurutzaketa kobalente iraunkorra gauzatzen duten taldeak gehitzean, norborenoa (Nb) eta tetrazina (Tz) hain zuzen, sarea elastikoa bihurtzen da. Horren azalpena, talde horiek matrizean sartzean, Diels-Alderren alderantzizko erreakzio elektroi hartzaile bio-ortogonala ematen dela da, dagoeneko existitzen diren gurutze-lotura ionikoak “klikatuz” [47, 48]. Zelulen kapsularaketaren 3 egun ondoren, hidrogel matrizeak hMSCen ekintza immunomodulatzailea nabarmenki bultzatu zuen, 2Dtan kultibatutako zelulekin alderatuta. Berez, gure panelean zeuden gene immunomodulatzaile guztien espresioa handitu egin zen, hanturazko baldintzapenaren aurrean erantzun edo ez (9. Irudia). Emaitza horiek 3Dtako kultibo biomimetikoaren eragin garrantzitsua agerian utzi zuten, hanturazko baldintzapenaren efektuaren independentea izan zena.



8. Irudia. hMSCen eragin immunomodulatzailea sustatzeko estrategiaren garapenean jarraitu zen prozedura esperimentala. hMSCak γ interferoiarekin (IFN-γ) eta α tumorearen nekrosi faktorearekin (TNF-α) 16 orduz baldintzatu ondoren, hidrogel desberdinetan kapsularatu ziren (3D). Denbora puntu desberdinetan, zelulen gene espresioa ikertu zen denbora errealeko RT-qPCR teknikaren bidez. Laburdurak: hMSC: giza zelula estromal mesenkimalak (*human mesenchymal stromal cells*). IFN-γ: γ interferoia. TNF-α: α tumorearen nekrosi faktorea.

Hala ere, 7. egunean, hanturazko baldintzapenaren aurrean erantzun zuten geneen espresioa nabarmenki murriztu zen. Baldintzapen horri erantzuten ez zioten geneen espresioa, ostera, mantendu egin zen, matrizearen efektua iraunkorra zela iradokiz. Horrela, azaldu ahal da lehenengo egunetan hanturazko baldintzapenaren eta 3Dtako matrizearen eraginak sinergikoak izan zirela eta 7. egunerako efektua murriztu zela, matrizearen efektua iraunkorra izan arren, hanturazko zitokinekin burututako baldintzapenaren izaera iragankorra baita.



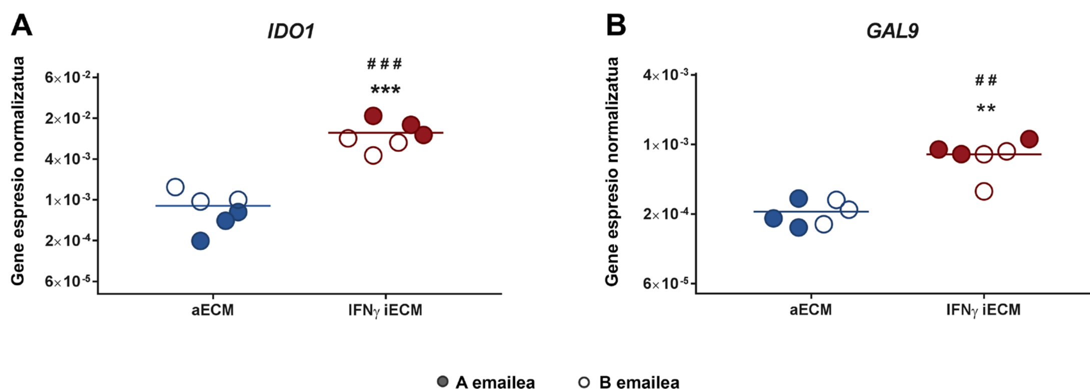
9. Irudia. aECM hidrogeletan kapsularatutako hMSCen gene immunomodulatzaileen espresioa 3. egunean. **(A)** *IDO1*, **(B)** *GAL9*, **(C)** *PTGS2*, **(D)** *HGF*, **(E)** *IL10* eta **(F)** *IL1RN* geneen espresio normalizatua aECM hidrogeletan kultibatutako hMSCetan, hanturazko baldintzapenaren eta kapsularazioaren 3 egun ostean. Normalizazioa egiteko *GAPDH* genea erabili zen. Estatistika: *$p < 0.05$, **$p < 0.01$ eta ***$p < 0.001$ 2Detan kultibatutako zelulekin alderatuz. Laburdurak: aECM: matrize extrazelular artifizial hidrogelak (*artificial extracellular matrix hydrogels*). hMSC: giza zelula estromal mesenkimalak (*human mesenchymal stromal cells*).

Interesgarria da aipatzea hMSCen gene immunomodulatzaileen espresioan ez zirela desberdintasun esanguratsurik antzeman ezaugarri mekaniko desberdinak zituzten lau aECM hidrogel moten artean. Gure datuen arabera, hanturazko zitokinekin egindako baldintzapenaren eta 3Dtako kultibo biomimetikoaren efektu sinergikoak matrizearen ezaugarri mekanikoen eragina maskaratzen du. Honen harira, beharrezkoa da oraindik aECM hidrogelek hMSCen ekintza immunomodulatzailea zein mekanismoaren bidez sustatzen duten zehaztea.

Hanturazko baldintzapenaren aurrean erantzuten duten geneen espresioa luzatzeko, aECM hidrogelek eskaintzen duten 3Dtako kultibo biomimetikoa hanturazko baldintzapen iraunkorrarekin konbinatu genuen. Etengabeko hanturazko ingurunea lortzeko asmoz, aECM hidrogel elastiko zurrunetan IFN-γ zitokinarekin kargatutako heparina mikroesferak gehitu genituen. aECM matrizeen lau bariante mekanikoen artean desberdintasunik antzeman ez genituenez, hidrogel elastiko zurruna aukeratu zen mekanikoki sendoena zelako eta, beraz, etorkizunean egingo diren ikerketetan inplantatzeko egokiena izango delako. Ondorioz, sistema multifuntzional berri bat lortu genuen: ECM hidrogel immunomodulatzaileak (iECM) (10. Irudia). Azpimarratzekoa da IFN-γ heparinara lotzean, haren degradazio proteolitikoa domeinu bakar batera murrizten dela eta horrek, zitokinaren potentzia erabat suspertzen duela [49, 50].

10. Irudia. γ Interferoiarekin (IFN-γ) kargatutako iECM hidrogelen irudikapen eskematikoa. Laburdurak: iECM: matrize extrazelular immunomodulatzaile hidrogelak (*immunomodulatory extracellular matrix hydrogels*). hMSC: giza zelula estromal mesenkimalak (*human mesenchymal stromal cells*).

7. Eguna



11. Irudia. iECM hidrogeletan kapsularatutako hMSCen gene immunomodulatzaileen espresioa 7. egunean. (A) *IDO1* eta **(B)** *GAL9* geneen espresio normalizatua iECM hidrogeletan kultibatutako hMSCetan, hanturazko baldintzapenaren eta kapsularazioaren 7 egun ostean. Normalizazioa egiteko *GAPDH* genea erabili zen. Estatistika: $^{**}p < 0.01$ eta $^{***}p < 0.001$. Laburdurak: aECM: matrize extrazelular artifizial hidrogelak (*artificial extracellular matrix hydrogels*). iECM: matrize extrazelular immunomodulatzaile hidrogelak (*immunomodulatory extracellular matrix hydrogels)*. hMSC: giza zelula estromal mesenkimalak (*human mesenchymal stromal cells*).

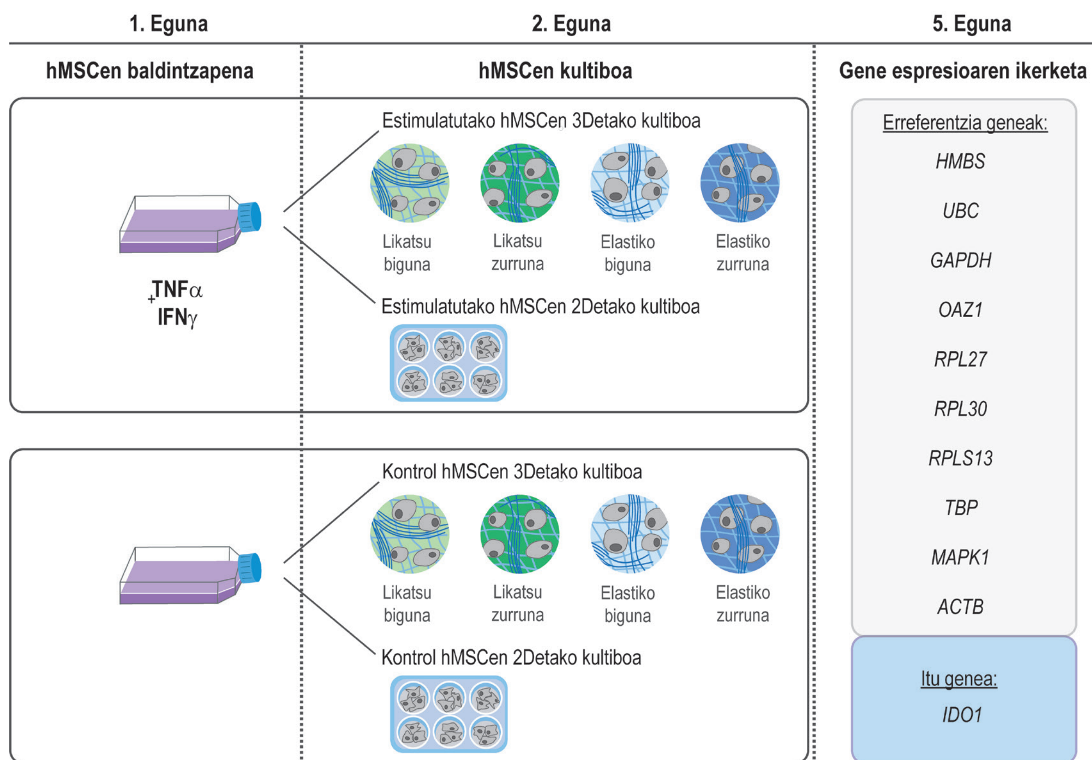
iECM hidrogelek hanturazko baldintzapenaren efektua luzatu zutela frogatu genuen eta, beraz, *IDO1* eta *GAL9* bezalako gene immunomodulatzaile garrantzitsuen espresioa mantendu egin zen 7. egunean (11. irudia). Horrez gain, iECM matrizeek hMSCen ekintza immunoezabatzailea ahalbidetu zuten: T zelulen proliferazioa inhibitu egin zen linfozitoak iECM hidrogeletan kapsularatutako hMSCekin ko-kultibatzean. Emaitza horrek agerian uzten du garatutako sistemak hMSCak ekoiztutako faktore immunomodulatzaileen difusioa eta askapena ahalbidetu zuela. Ezaugarri hori funtsezkoa da, horrelako biomaterialetan oinarritutako formulazio askoren tamaina handiak biomolekulen difusioa saihesten baitu [51]. Bestalde, heparina mikroesferek ez zituzten zelulek ekoiztutako faktore guztiak lotu, eta horretaz gain, ez zuten faktore horien hedapena fisikoki oztopatu. Aipagarria da ere, IFN-γren presentziak ez zuela eragin negatiborik izan ko-kultiboan, eta, beraz, zentzuzkoa da pentsatzea zitokinak seguruenik ez zuela sistematik ihes egin, edo, gutxienez, ez zela kopuru esanguratsu batean askatu.

Oro har, 3Dtako kultibo biomimetikoaren eta hanturazko baldintzapen iraunkorraren konbinazioak hMSCen ekintza immunomodulatzailea sustatu zuela frogatu zen, zelulek ekoiztutako faktore bioaktiboen hedapen egokia ahalbidetu zuen heinean. Emaitza horiek inplikazio garrantzitsuak dituzte hMSCetan oinarritutako terapietan, haien potentzial terapeutikoa bultzatzeko estrategia berriak sakon aztertzen ari direlako gaur egun.

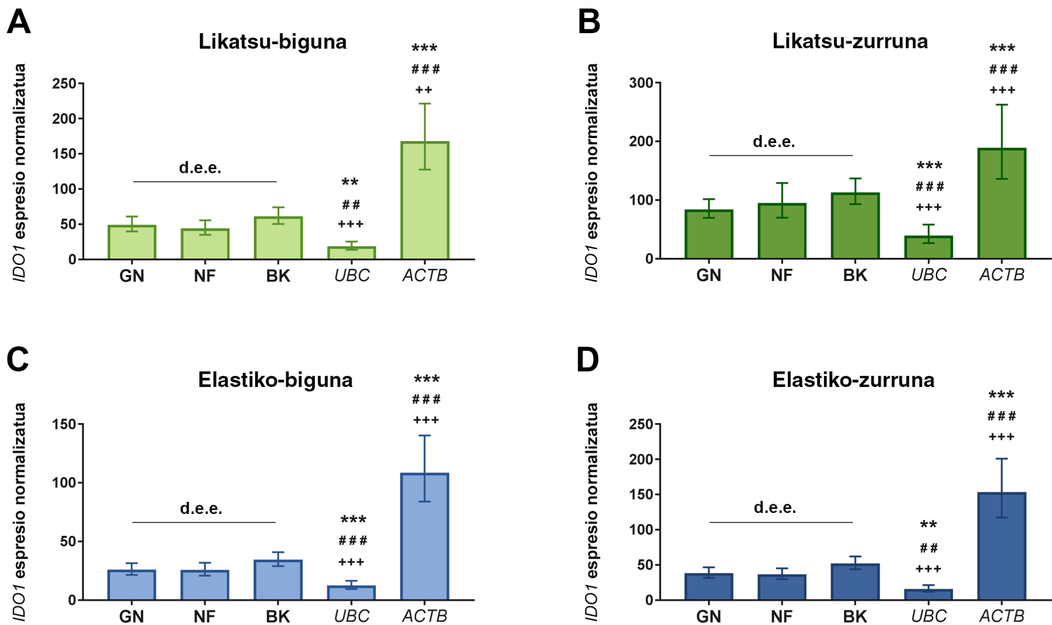
Aurreko lanean, hMSCen eragin immunomodulatzailea zehazteko geneen espresioaren ebaluaketa funtsezkoa izan zen. Denbora errealeko RT-qPCRa da horretarako metodo nagusia, bere sentsibilitate handia eta sinpletasuna direla eta. Hala ere, maiz, datuak normalizatzeko erabiltzen diren erreferentzia geneak baldintza esperimental jakinetan balioztatzea alde batera uzten da, emaitza eta ondorio okerrak ekar ditzakeena. Hori dela eta, doktorego tesiaren azken urratsean, 3Dtako kapsularaketak, matrizearen ezaugarri mekanikoek eta hanturazko baldintzapenak hMSCen erreferentzia gene ohikoen espresioan duten eragina sistematikoki ikertu genuen. Hutsune hori betetzea premiazkoa da, hMSCen ekintza immunomodulatzailea suspertzeko azterlan ugariek zelulak baldintzatzen baitituzte.

Hasteko, erreferentzia geneen egonkortasuna aztertu genuen egoera esperimental desberdinetan. Horretarako, IFN-γ eta TNF-α zitokinekin baldintzaturiko hMSCak eta baldintzaturik gabeko kontrol zelulak 2Dtan (TCPetan) edo 3Dtan (aurretik deskribatutako lau aECM hidrogel mota desberdinetan) kultibatu ziren (12. Irudia). Egonkortasun ikerketa hori, BestKeeper (BK), NormFinder (NF) eta geNorm (GN) algoritmoen bidez egin zen. Haien arabera, *RPS13* eta *RPL30* proteina erribosomikoak gene egonkorrenen artean zeuden. Datu horiek bat egiten dute de Jonge eta kideek egindako meta-analisi batean lortutako emaitzekin, non zelula mota anitzekin eta baldintza esperimental ugariekin egindako azterketan, *RPS13* eta *RPL30* erreferentzia gene egonkorren sailkapenean lehen eta laugarren postuan zeuden, hurrenez hurren [52]. Bestalde, NF algoritmoak *ACTB* eta *UBC* gene ezegonkor gisa kalifikatu zituen, 0,35 eta 0,36 egonkortasun balioak eskainiz, hurrenez hurren; aztertutako beste erreferentzia geneen balioetatik oso urrun. BK eta GN algoritmoek emaitza hori egiaztatu eta *ACTB* eta *UBC* hautagai ezegonkorragoen artean sailkatu zituzten. *ACTB* 12 erreferentzia gene erabilienen artean egon arren [52], hainbat ikerketek bere ezegonkortasuna frogatzen dute baldintza esperimental ugarietan [53-55].

ACTB eta *UBC* erreferentzia geneen ezegonkortasuna dela eta, gure konfigurazio esperimental jakinetan *IDO1* genearen espresioa haiekin normalizatzean emaitza okerrak lortzen zirela frogatu genuen. Denbora errealeko RT-qPCRarekin eskuratutako datuak, $2^{-\Delta\Delta CT}$ metodoaren bidez aztertu genituen, Livak metodoa bezala ezagutzen dena [56]. Lehendabizi, 3Dtako kapsularazioaren eragina ikertu genuen. Analisiak, *ACTB*ren espresioaren beherakada eta *UBC*ren igoera antzeman zuen 3Dtan kultibatutako hMSCetan, 2Dtan kultibatutako zelulekin alderatzean. Hori dela eta, datuak *ACTB*rekin normalizatzean *IDO1* genearen gainestimazioa behatu zen, eta berriz, *UBC* erabiltzean, *IDO1*ren espresioa gutxietsi zen (13. Irudia). Emaitza horiek argitaratutako ikerketekin bat datoz, non GN eta NF algoritmoek *ACTB*



12. Irudia. hMSCen erreferentzia geneen egonkortasuna aztertzeko jarraitu zen prozedura esperimentala. hMSCak IFN-γ eta TNF-α hanturazko zitokinekin 16 orduz baldintzatu ondoren, hidrogel desberdinetan kapsularatu ziren (3D) edo kultiborako plaketan (TCP: tissue culture plate) kultibatu ziren (2D). Prozedura berdina jarraitu zen baldintzatu ez ziren kontrol hMSCekin. Kultiboaren 3 egun ondoren, 10 erreferentzia geneen espresioa, eta baita *IDO1* itu genearen espresioa ere ikertu zen denbora errealeko RT-qPCR teknikaren bidez. Laburdurak: hMSC: giza zelula estromal mesenkimalak (*human mesenchymal stromal cells*). IFN-γ: γ interferoia. TNF-α: α tumorearen nekrosi faktorea.

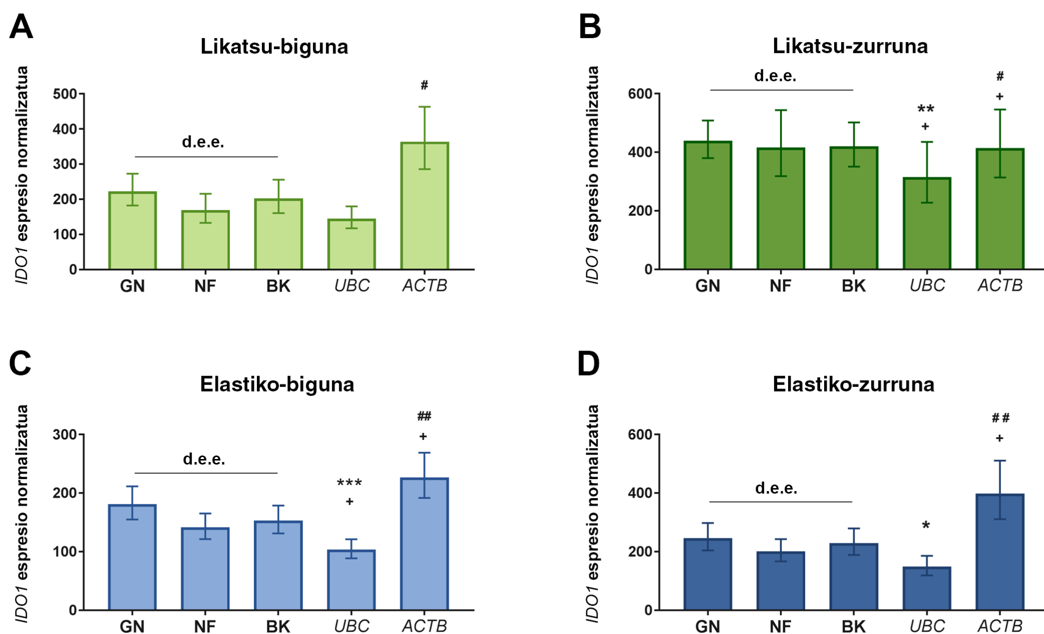


13. Irudia. 3Dtako kultiboaren eragina hMSCen erreferentzia geneen egonkortasunean. *IDO1* itu genearen espresioa hidrogel **(A)** likatsu-bigunetan **(B)** likatsu-zurrunetan **(C)** elastiko-bigunetan eta **(D)** elastiko-zurrunetan kapsularatutako hMSCetan, 2Dtan kultibatutako hMSCekin normalizatuz. Erabilitako erreferentzia geneak GN eta NF algoritmoek proposatutako gene konbinazioak, BK indizea, *UBC* eta *ACTB* geneak izan ziren. Grafikoek batazbestekoa ± SD irudikatzen dute (n = 3 erreplikatu talde bakoitzeko). Estatistika: $^{*}p < 0.05$, $^{**}p < 0.01$ eta $^{***}p < 0.001$ GN algoritmoarekin konparatuz. $^{\#\#}p < 0.01$ eta $^{\#\#\#}p < 0.001$ NF algoritmoarekin alderatuz. $^{++}p < 0.01$ eta $^{+++}p < 0.001$ BK algoritmoarekin konparatuz. Laburdurak: hMSC: giza zelula estromal mesenkimalak (*human mesenchymal stromal cells*). NF: NormFinder. GN: geNorm. BK: BestKeeper. d.e.e.: desberdintasun esanguratsurik eza.

genea 3Dtan kultibatutako hezur-muineko MSCetan hiru erreferentzia gene ezegonkorragoen artean identifikatu zuten [53]. Horretaz gain, Liu eta kideek *ACTB* erreferentzia gene ezegonkorrena bezala sailkatu zuten presio hidrostatiko dinamikoa jasaten duten MSCetan, mekanobiologiako ikerketetan kontrol egokia ez dela ondorioztatuz [57].

Ondoren, erreferentzia geneen egonkortasuna ebaluatu genuen gure aldaera mekaniko zehatzetan. Berez, eztabaida honetan lehenago aipatu den bezala, azterlan ugariek deskribatu dute matrizearen ezaugarri mekanikoek zelulen biologian daukaten eragina. Interesgarria da *ACTB*ren eta *UBC*ren espresioa egonkor mantendu zela lau aECM hidrogel desberdinetan kapsularatutako hMSCetan.

Azkenik, *ACTB* eta *UBC* geneen egonkortasuna IFN-γ eta TNF-α hanturazko zitokinekin baldintzaturiko hMSCetan ikertu genuen. 3Dtan kapsularatutako zeluletan behatutako joera bera antzeman genuen kasu honetan, nahiz eta desregulazioa hain nabaria ez izan. Zehazki, *ACTB*ren espresioa baxuagoa eta *UBC*rena altuagoa izan ziren zitokinekin baldintzaturiko hMSCetan, baldintzatu gabeko kontrol zelulekin konparatuz. Horrek, aipatutako erreferentzia geneen bidez *IDO1* normalizatzean, bere espresioaren gainestimatzea edo gutxiestea eragin zuen, hurrenez hurren (14. Irudia). Argitaratu berri den ikerketa batek gure emaitzak egiaztatzen ditu, miRNA klaseko erreferentzia gene batzuen egonkortasun eskasa frogatu zuelarik IFN-γrekin baldintzaturiko MSCak askatutako EVen azido nukleikoen edukia kuantifikatzeko [58].



14. Irudia. Hanturazko baldintzapenaren eragina hMSCen erreferentzia geneen egonkortasunean. *IDO1* itu genearen espresioa baldintzaturiko hMSCetan hidrogel **(A)** likatsu-bigunetan **(B)** likatsu-zurrunetan **(C)** elastiko-bigunetan eta **(D)** elastiko-zurrunetan kapsularatuta, baldintzatu ez ziren kontrol hMSCekin normalizatuz. Erabilitako erreferentzia geneak GN eta NF algoritmoek proposatutako gene konbinazioak, BK indizea, *UBC* eta *ACTB* geneak izan ziren. Grafikoek batazbestekoa ± SD irudikatzen dute (n = 3 erreplikatu talde bakoitzeko). Estatistika: *$p < 0.05$, **$p < 0.01$ eta ***$p < 0.001$ GN algoritmoarekin konparatuz. #$p < 0.05$ eta ##$p < 0.01$ NF algoritmoarekin alderatuz. +$p < 0.05$ BK algoritmoarekin konparatuz. Laburdurak: hMSC: giza zelula estromal mesenkimalak (*human mesenchymal stromal cells*). NF: NormFinder. GN: geNorm. BK: BestKeeper. d.e.e.: desberdintasun esanguratsurik eza.

Orokorrean, gure datuek agerian uzten dute 3Dtako kapsularaketak eta hanturazko baldintzapenak eragin handia dutela hMSCetan maiz erabiltzen diren erreferentzia gene batzuen egonkortasunean, hala nola, *UBC* eta *ACTB* geneetan. Hori dela eta, erreferentzia gene horien erabilera gure egoera esperimental jakinetan, *IDO1* itu genearen mRNA mailak gainestimatzea edo gutxiestea eragin zuen denbora errealeko RT-qPCR teknikaren bidez egindako azterketetan. Emaitza horiek, hMSCen erreferentzia geneak balioztatzea funtsezkoa dela nabarmentzen dute haien ekintza immunomodulatzailea suspertzeko baldintzatzen direnean.

Laburbilduz, doktorego tesi honetan, 3Dtako hidrogel sistemen optimizazioan jardun gara. Zehazki, zelulen erantzun erratikoetan kontrola ezartzeko, biofuntzionalizazio estrategia egokiak diseinatzeko eta MSCen ekintza immunomodulatzailea sustatzeko ikerketa desberdinak burutu dira. Oro har, gure emaitzekin, zelulak kapsularatzeko 3Dtako sistemen garapenean aurrerapauso bat eman dugula esan dezakegu, MSCetan oinarritutako terapietan inplikazio garrantzitsuak dituena.

Erreferentzien zerrenda 246-249 orrialdeetan aurkitzen da.

Ondorioak

Doktoretza tesi honetako ikerketa esperimentaletan lortutako emaitzetatik honako ondorioak eratorri ahal dira:

1. Alginato-poli-L-lisina-alginato mikrokapsulen formulazioan, osmolaritate doitzaileen aukera egokiak zelula estromal mesenkimalen (*mesenchymal stromal cell* edo MSC) portaera kontrolatu zuen, biomaterial edota elkargurutzatzaile mota edo proportzioa aldatu beharrik gabe. Lortutako sistemak zelulen proliferazioaren gainean kontrol estua ezarri zuen, *in vivo* faktore terapeutikoen askapen iraunkorra bermatuz eta terapia seguruagoa lortuz.

2. Zelula zehatz batentzat biofuntzionalizazio estrategia aproposena zein den iradoki dezakeen matrize extrazelularreko proteinetan oinarritutako mikroarray biosentsore bat garatu zen. Tresna analitiko honek, zelulen atxikipen-zinetika ebaluatzeaz gain, integrinen profilaketa eta haien eragina atxikipenaren sendotzean ikertzea ahalbidetu zuen.

3. Hanturazko baldintzapen iraunkorra eta hiru dimentsiotako (3D) kultibo biomimetikoa konbinatzen dituen sistema funtzio anizduna garatu zen. Sistema horrek MSCen gene immunomodulatzaile garrantzitsuen espresioa sustatu eta luzatu zuen eta aldi berean, zelulek ekoiztutako faktore bioaktiboen hedapen egokia ahalbidetu zuen.

   Horrez gain, gure ikerketek 3Dtako kapsularaketak eta hanturazko baldintzapenak MSCetan erabiltzen diren erreferentzia gene ohikoenen egonkortasunean eragin handia dutela frogatu zuten, hala nola, *UBC* eta *ACTB* geneetan. Erreferentzia gene horiek erabiltzean, *IDO1* gene ituaren espresio mailen emaitza okerrak lortu ziren denbora errealeko RT-qPCR teknikaren bidez egindako analisietan. Gure datuek nabarmentzen dute MSCen erreferentzia geneak balioztatzea funtsezkoa dela haien eragin immunomodulatzailea suspertzen duten baldintzapen estrategiak erabiltzean.

APPENDIX
IRUZKINA

Alginate microcapsules for drug delivery

Alginates and Their Biomedical Applications, Springer Series in Biomaterials Science and Engineering 11. Springer Nature Singapore (2018) 67-100.
DOI: https://doi.org/10.1007/978-981-10-6910-9_3

Ainhoa Gonzalez-Pujana[a,b], Edorta Santos-Vizcaino[a,b], Gorka Orive[a,b,*], Jose Luis Pedraz[a,b], Rosa Maria Hernandez[a,b,*]

[a] NanoBioCel Group, Laboratory of Pharmaceutics, School of Pharmacy, University of the Basque Country (UPV/EHU), Spain.
[b] Biomedical Research Networking Centre in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Spain.
* Corresponding author: Rosa Maria Hernandez.

ABSTRACT

Currently, conventional drug delivery systems do not provide adequate therapeutic profiles for the management of multiple diseases. In this regard, cell encapsulation technology emerges as a suitable alternative. Undoubtedly, one of the most employed biomaterials for this purpose is alginate, since it presents multiple advantages that favor the development of this technology. Importantly, the thorough study concerning the purification and modification of the polymer has led to biocompatible alginates, a vital advancement for the correct function of the system. Furthermore, the possibility to entrap different cell types together with the plausibility of engineering cells to produce disparate therapeutic biomolecules has given rise to numerous applications. That is the case of relevant and prevalent diseases nowadays such as diabetes, cancer or neurological diseases. Intensive research in the field has resulted in promising preclinical studies in animal models that have instigated the conduction of several clinical trials. Nonetheless, addressing some current challenges regarding aspects such as biosafety or biofunctionalization seems to be a prerequisite before the clinical translation.

1. Introduction

Noncommunicable diseases (NCDs), also known as chronic diseases, are those not passed from person to person, usually associated to long duration and slow progression. NCDs include cardiovascular diseases, cancer, chronic respiratory diseases, diabetes or neurodegenerative diseases. According to the World Health Organization, NCDs represent one of the major health challenges of the 21st century, considering both patient suffering and the subsequent socioeconomic burden [1, 2]. Their management is complex, as many of them demand a tight regulation of therapeutic factors based on physiological requirements. Hence, conventional drug administration offers poor control over this type of pathologies, leading in many cases to non-efficient treatments and undesirable effects. Consequently, over the last decades new strategies have been thoroughly studied in order to develop new drug delivery systems.

One of the technologies that has shown great potential to become a viable therapeutic option for chronic diseases is cell microencapsulation. In this strategy, cells that produce therapeutically active biomolecules are enveloped in a polymeric matrix. The resulting microspheres are usually coated with a polycation in order to form a semipermeable membrane. Thus, the system allows the ingress of nutrients and oxygen and the egress of therapeutic factors. Furthermore, the passage of immune cells and antibodies is restricted, leading to immunoprotection of the encapsulated cells (Fig. 1). Hence, the transplantation of encapsulated cells may represent a valuable approach to enable sustained delivery of therapeutic biomolecules to specific targets. Moreover, because of the permselective membrane, the co-administration of immunosuppressive therapies may be reduced, diminishing the severe side effects related to these drugs [3, 4].

This biotechnology gives rise to myriad opportunities for application and meets, *a priori*, the requirements for an adequate treatment that would drastically improve efficacy, as well as patient compliance and comfort. Therefore, the field of cell microencapsulation is currently under intensive research to face the challenge of clinical translation. A multidisciplinary approach is crucial for the development of the technique, given that cell encapsulation combines areas such as biology, medicine, pharmaceutical technology or surface chemistry. Specifically, material science is of paramount importance in this field; indeed, the choice of

the biomaterial may make a real difference in the eventual success of the biosystem. In this sense, it is essential that the applied material presents suitable mechanical properties as well as adequate biocompatibility in order to respect cell homeostasis and viability.

To date, many polymers have been utilized with that purpose, including natural materials such as alginate, chitosan, agarose, collagen or cellulose and synthetic like poly(ethylene glycol) (PEG), polyurethane or polyvinyl alcohol. However, recently concerns have arisen on the use of synthetic polymers due to their impossibility to trigger physiologic cellular responses and their difficulty to form gels *in situ* following cell-friendly protocols. This fact has resulted in a shift of interest to natural materials [5]. In particular, alginate remains the most widely employed polymer nowadays. Although other biomaterials may become a good alternative in the future, currently there is a consensus that only alginate has been exhaustively studied so as to be qualified as safe for application in humans [6]. Moreover, the intrinsic properties of alginate make it suitable for the needs of this biotechnology and confer multiple advantages to the system. Thus, alginate represents a promising biomaterial in cell microencapsulation.

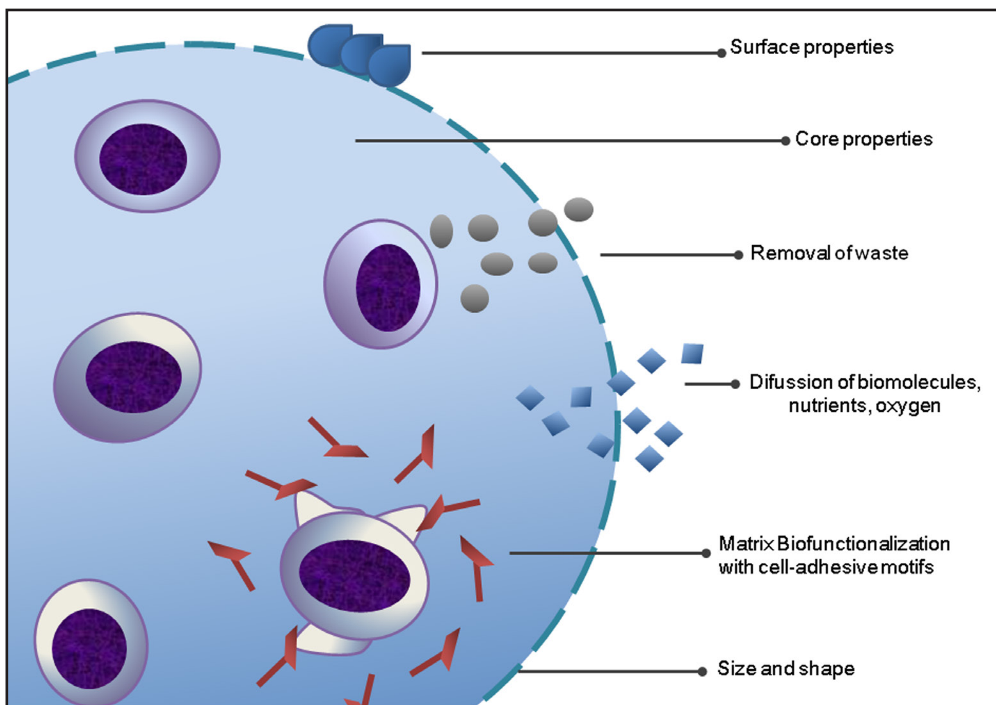


Fig. 1. Scheme representing the main properties of alginate microcapsules.

2. Cell microencapsulation technology

Cell microencapsulation emerged in 1933 when Bisceglie demonstrated that tumor cells remained viable after being encapsulated and transplanted in pig's abdominal cavity [7]. Three decades later, in 1964, Chang and colleagues encapsulated erythrocyte hemolysates in nylon microspheres and proposed the use of semipermeable membranes as immunoisolating barriers [8]. It was not until 1980 that Lim and Sun proved the therapeutic application of the method by transplanting alginate microcapsules containing Langerhan cells in diabetic rats and returning the animals to normoglycemia for 2-3 weeks [9].

Since then, the use of a great variety of suitable biomaterials has been studied in detail. Nevertheless, after almost four decades of investigation, sodium alginate is still today the most widely used biopolymer for cell encapsulation. Alginate is a linear unbranched block copolymer composed of (1,4)-linked β-D-mannuronate (M) and its C5-epimer α-L-guluronate (G) arranged to form homopolymeric (GGG-blocks and MMM-blocks) or heteropolymeric block structures (MGM or GMG-blocks). Alginates present the advantageous property of easily forming hydrogels by ionic cross-linking with divalent cations. The process occurs following the so called "egg-box model" [10]: the divalent cation binds to the G-blocks of two adjacent alginate polymer chains forming a three-dimensional (3D) gel network (Fig. 2A) [11]. It was believed that G-blocks were the only to participate in the cross-linking reaction [12], however, MGM-blocks may also contribute to hydrogel formation [13] (Fig. 2B). Nonetheless, G content is still considered as the major factor involved in determining gel elasticity, porosity and stability [14].

Moreover, alginates have different affinity for cationic cross-linkers, increasingly: calcium (Ca^{+2}), strontium (Sr^{+2}), Barium (Ba^{+2}) [15]. Consequently, the gel-forming ion may also influence final properties of microcapsules such as swelling and stability [13]. Following this premise, it is possible to choose the adequate agent to obtain microcapsules with the desired properties. The most frequently used cross-linking agent is Ca^{+2} for its non-toxicity compared to other cations. In particular, $CaCl_2$ is usually employed as a Ca^{+2} source since its high solubility in aqueous solutions leads to a rapid ionotropic gelation [16]. Barium is also often used as a cross-linker, as it is known to form microcapsules with high mechanical strength [17].

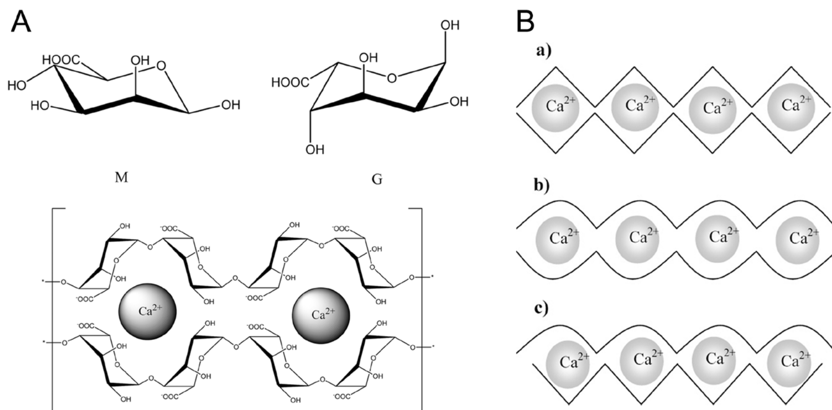


Fig 2. (A) Structure of β-D-mannuronic acid (M) (top left) and α-L-guluronic acid (G) (top right). Representation of the "egg-box" model binding of α-L-guluronic acid blocks to calcium ions in alginic acid (bottom). Reproduced from Ref. [11] with permission of The Royal Society of Chemistry©. **(B)** Graphical description of the 3 possible junctions in alginate gels. (a) GG/GG junctions, (b) MG/MG junctions, and (c) mixed GG/MG junctions. Reprinted with permission from ref. [13] ©2005 American Chemical Society.

Alginates also form gels by photo cross-linking [18], thermal gelation [19] or even a combination of these methods with cationic cross-linkers [20]. However, these procedures may not tolerate cell survival to the same extent ionic cross-linking does. Hence, ionotropic gelation represents a good alternative to entrap cells in alginate hydrogels under mild conditions [21]. Consequently, sol-gel processes based on the original extrusion method developed by Lim and Sun [9] are the most widely employed to manufacture alginate capsules. Cell-entrapment within the hydrogel starts by suspending cells in an aqueous solution of the polymer, giving rise to the sol flowing phase. The suspension is then extruded into a solution of the cross-linking agent, with the subsequent sol-gel transition that forms the microbeads.

Although the obtained beads may protect allogeneic cells, alginates are too porous to provide xenograft immunoprotection [6, 22]. Therefore, microbeads are usually coated with a polycation that controls the molecular weight cut-off of the biosystem by forming a semipermeable membrane. This permselective barrier allows the diffusion of the therapeutic factor, oxygen, nutrients, and cellular waste while it protects the implant from the host immune system and mechanical stress. In this regard, poly-L-lysine (PLL) [23, 24] and poly-L-ornithine (PLO) [25, 26] are the most frequently employed polycations. As positively charged ions, when implanted in the organism they may trigger a strong immunological reaction [27-30]. For this reason, a second coating of diluted alginate is usually added to mask the positive

charges and improve graft biocompatibility. Therefore, when performing both coatings alginate-poly-L-lysine-alginate or alginate-poly-L-ornithine-alginate (APA) microcapsules are obtained. Nevertheless, it has been reported that those two coatings are not multilayered, but instead blend forming an external layer of PLL and alginate that surrounds the inner calcium-alginate core [31]. If the degree of interaction between the molecules is not sufficient, unbound PLL may be exposed at the surface of microcapsules, fact that might explain the immune response that APA microcapsules cause and that opens the debate about the importance of the second coating based on alginate [32, 33].

Thus, coatings are still nowadays one of the most limiting factors of cell microencapsulation technology due to issues regarding biocompatibility, mechanical strength and stability [34]. Therefore, new coating strategies are being extensively explored to overcome these limitations [35]. Chitosan [36-38], modified chitosan [39], poly(methylene-co-guanidine) [30, 40] or the application of diblock copolymers of PEG and PLL [41, 42] have been some of the alternative approaches.

3. Alginate microcapsules as platform for sustained drug delivery

Cell microencapsulation within alginate matrices represents a promising tool for secretory cell dysfunction management. When implanted in the body, the tissue and vasculature that surrounds the capsule provide the cells with oxygen and nutrients, supporting cell viability, and as a result, functionality. Thus, the immobilized cells are able to produce the therapeutic factor *de novo* in a sustained way from weeks to months, which may match treatment duration with disease longevity [43]. Consequently, the single application of the treatment would remarkably improve patient comfort and compliance. Hence, it may overcome the huge problem of adherence, particularly important in diseases where an exhaustive regulation of drug delivery is mandatory to achieve treatment efficacy.

This implantation of cells is possible because of the immunoisolation that the semipermeable membrane confers to the system. Indeed, it has been widely demonstrated that microencapsulation protects allografts [44]. Furthermore, due to the shortage of donor tissues for patients, xenograft transplantation has emerged as a proper alternative. Despite the host's

immune response towards xenogeneic cells is more aggressive, the semipermeable membrane of microcapsules has been able to prolong xenograft survival [45, 46]. However, there are still unsolved issues such as the release of xenogeneic epitopes, which induce the formation of encapsulated tissue specific antibodies [47] or proinflammatory cytokines [48] that could lead to graft rejection. For this reason, different approaches have been carried out in order to find a solution. Examples are incorporating the CXCL12 chemokine to the alginate, which can repel effector T cells while recruiting immune-suppressive regulatory T cells [49] or conjugating to the hydrogel a peptide inhibitor for the cell surface IL-1 receptor (IL-1R) thus blocking the interaction between the entrapped cells and the cytokines [50]. May these issues be overcome, chronic co-administration of immunosuppressant drugs could be significantly reduced [51]. This fact would have a major impact from a therapeutic standpoint since the severe side effects produced by these drugs may be avoided. To date, almost 50 significant adverse effects have been related to the use of immunosuppressant therapy, thus, immunoisolation may improve significantly the life quality of patients [52].

In addition, microcapsules are 3D scaffolds that mimic more efficiently the natural microenvironment than 2-dimensional (2D) hydrogels. Another advantage of this 3D structure is that the surface to volume ratio is increased compared to conventional bulk hydrogels. This improves nutrient and oxygen supply [53], overcoming a problem that in many cases leads to graft failure [54].

3.1 Advantages alginates offer in cell encapsulation

3.1.1 A natural and biocompatible polymer

One of the advantages of using alginate for cell microencapsulation is the abundance because of its natural origin. In particular, the most commonly used alginates derive from brown algae such as *Laminaria digitata*, *Laminaria hyperborea*, *Macrocystis pyrifera*, *Ascophyllum nodosum*, *Laminaria japonica*, *Durvillea Antarctica*, *Eclonia maxima*, *Lessonia nigrescens*, and *Sargassum spp* [55]. Alginates with more defined chemical structures, and therefore, physical properties, can be obtained by bacterial biosynthesis. In particular, alginate is produced by two genera: *Pseudomonas* and *Azotobacter*. The relatively easy modification of bacteria and the recent advances in regulation of the polymer biosynthesis may enable manufacture of

tailor-made alginates with high value biomedical applications [56].

As natural polymers, alginates may incorporate contaminants such as heavy metals, proteins, endotoxins or polyphenolic compounds. Moreover, additional impurities could be introduced during the industrial extraction processes of raw alginates [57]. Their presence compromises the biocompatibility of the graft. In particular, a recent study pointed to endotoxins, or in other words pathogen-associated molecular patterns (PAMPs), as responsible for the proinflammatory responses in the host when microcapsules are implanted [58]. PAMPs are small molecular motifs found on pathogens that initiate immune responses to eliminate pathogenic bacteria and thus protect the host from infections. Cells of the innate immune system recognize PAMPs via Toll-like receptors (TLRs) and other pattern recognition receptors (PRRs). Some of the PAMPs that raw alginates contain are peptidoglycan, lipoteichoic acid or flagellin and they predominantly activate TLR2, 5, 8, and 9 [58]. When present in alginates, PAMPs lead to immune activation with the subsequent cytokine release. The majority of them are small enough to pass the membrane, having a deleterious effect on cell function and viability. They may also limit the diffusion of nutrients, oxygen, therapeutic molecules and the waste products of metabolism [59]. As a consequence of cell death, intracellular components are released. These molecules are damage or danger associated molecules (DAMPs) which also evoke an inflammation cascade when recognized by PRRs, mainly via TLRs [53]. Therefore, it is believed that DAMPs also play a role in the responses against encapsulated cells (Fig. 3A) [6].

In order to avoid these problems, purification of alginates has been intensively studied. In fact, it has been reported that for manufacturing biocompatible alginate microcapsules that are suitable for cell transplant, the use of ultrapurified alginate is mandatory [60]. Efforts have given rise to different methods to obtain pure alginates that meet clinically useful criteria [61]. This ultra purified, “clinical-grade” alginate has been proven to reduce the foreign body reaction in many *in vivo* studies (Fig. 3B) [62-64]. Indeed, nowadays there are commercially available highly purified alginates that evoke no immune reaction when injected subcutaneously to mice [12]. Moreover, the modification of alginates has been suggested as a tool to mitigate the foreign body response and achieve a better biocompatibility [65]. In particular, recently, the modification of alginates with triazole-thiomorpholine dioxide (TMTD) led to a long-term

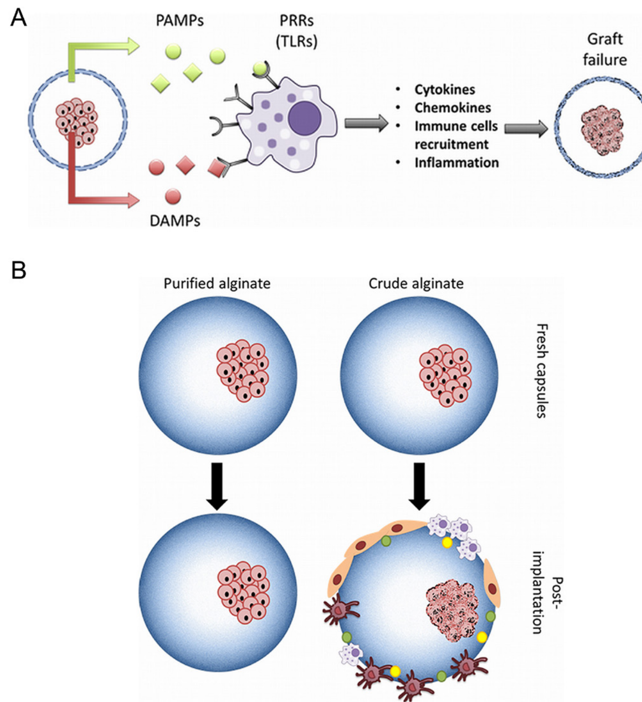


Fig 3. (A) PAMP and DAMP release from microencapsulated cells. **(B)** Microcapsules produced with purified or crude alginate. Figure as originally published in [53].

functionality of the graft in immunocompetent animals with no need of immunosuppression [66]. Further, in a recent study, a platform was designed to predict whether the purification is efficacious [67]. Importantly, alginate purification is not only beneficial to avoid the immune rejection of the implant, but also to improve viability of the encapsulated cells [68].

3.1.2 Rapid hydrogel formation and scalability

As previously mentioned, alginates form hydrogels by ionic cross-linking with divalent cations such as Ca^{+2}. This property makes alginates the most widely employed biomaterial for cell microencapsulation for two main reasons: (i) the synthesis of the capsules under mild conditions is enabled, (ii) the fact that it results in a hydrogel provides the encapsulated cells with an adequate microenvironment.

Unlike many other polymers, alginates present advantageous gelling properties. Being a thermally stable polymer, the hydrogel formation occurs at room temperature, allowing the immobilization of cells under safe and mild conditions that promote their viability and thus,

ensures the production of the therapeutic factor [16]. In contrast to alginate, a large number of polymers are not suitable for this technology as harsh chemicals or high temperatures are required for the gel forming, leading to death of the implanted cells [69]. Moreover, the rapid gelation shortens the encapsulation process, avoiding the manipulation of the encapsulated cells for long periods of time. This advantage, together with the relatively uncomplicated method and the simple equipment that is required, gives rise to an easy and fast manufacture of the capsules. For this reason, alginate microcapsules present the additional advantage of scalability. The high throughput encapsulation systems facilitate the production of a vast number of microcapsules for biomedical applications [70-73]. Nonetheless, for a further development of the methodology and a better understanding of the lab-to-lab variations in the process, it is mandatory to define and standardize some particular characteristics. To date, five different parameters that influence the final properties of the capsule have been claimed to be compulsory for an adequate description of the system: the applied polymer, permeability, surface properties, biocompatibility and storage conditions [74].

On the other hand, the resulting hydrogel forms a 3D network that is capable of mimicking the basic structural properties of the natural extracellular matrix (ECM). Indeed, the high water content, necessary for physiological processes, and mechanical properties closely match the ones of soft tissues in the body [75]. Moreover, the biological interaction between hydrogels and cells can be easily modified through multiple extracellular matrix peptides or proteins (see section 5.1). Therefore, biological, chemical and mechanical properties and even the degradation kinetics can be tailored depending on the application. As a result, such hydrogels are recognized to meet the requirements of bioencapsulation [76, 77].

3.2 Therapeutic factors delivered through cell encapsulation

The major advantage of cell encapsulation lies beneath the adaptability of the biosystem. The optimization of this technology is giving rise to promising treatments for multiple disorders, since the secretion of a wide range of therapeutic factors is possible by simply selecting the appropriate cell type or by bioengineering cells so that they produce the biomolecule of interest. To date, many studies have focused their attention in specific therapeutically active molecules and their delivery through cell microcapsules. Table 1 classifies some of the most studied ones according to their nature.

3.2.1 Hormones

Hormones are secretory products that act in specific target cells where they elicit physiologic, morphologic or biochemical responses. Since they regulate fundamental bodily and biochemical processes, their use in clinic is extensive. Indeed, being considered as important biological molecules in terms of clinical utility, hormones as therapeutics are currently under intensive research and cell encapsulation represents a valuable strategy for their delivery.

Undoubtedly, one of the most thoroughly studied hormones in cell encapsulation is insulin. Type 1 diabetes requires a strict exogenous insulin supplementation. However, the current treatments, such as insulin injections or subcutaneous pumps, are not able to reproduce physiological profiles of the protein, which results in secondary complications. To overcome this issue, multiple studies have analyzed the possibility of cell microencapsulation as a tool for insulin delivery [62, 78]. Encapsulation of β-cells from human and even xenogeneic donors (pigs, rats) has given rise to intensive research. A recent study showed that

Table 1. Examples of therapeutic factors delivered through cell encapsulation. EPO: erythropoietin, GLP1: Glucagon-like peptide-1, BDNF: Brain-derived neurotrophic factor, GDNF: Glial cell line-derived neurotrophic factor, VEGF: Vascular endothelial growth factor.

Recipient	Administration Site	Application	Ref.
Insulin			
Xenogeneic	Peritoneal cavity	Diabetes	[77]
Isogeneic/Allogeneic	Omentum pouch	Diabetes	[26]
Allogeneic	Peritoneal cavity	Diabetes	[78]
Xenogeneic	Peritoneal cavity	Diabetes	[81]
Allogeneic / Xenogeneic	Peritoneal cavity	Diabetes	[83]
EPO			
Allogeneic	Subcutaneous tissue	Chronic anemia	[85, 87]
GLP-1			
Xenogeneic	Brain	Alzheimer's disease	[89]
Xenogeneic	Brain	Amyotrophic lateral sclerosis	[90]
Xenogeneic	Coronary artery branches	Heart failure	[91]
Xenogeneic	Brain	Traumatic brain injury	[92]
BDNF			
Xenogeneic	Cochlea	Auditory neuron degeneration	[96, 97]
GDNF			
Allogeneic	Brain (striatum)	Parkinson's disease	[99]
VEGF			
Xenogeneic	Brain	Alzheimer's disease	[101]
Therapeutic antibodies			
Allogeneic	Subcutaneous tissue	Cancer	[108]

alginate-encapsulated human islet cells implanted in the peritoneal cavity of non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice corrected hyperglycemia immediately [79]. This metabolic correction was maintained until the end of the study (5 weeks post-transplantation). Moreover, the majority of the implanted capsules were retrieved and their functionality analysis revealed a rapid, potent and dose-dependent insulin response (Fig. 4). Another study demonstrated the long-term viability of encapsulated rat islets transplanted in the omentum pouch [26]. This site was suggested as a viable transplantation site because of its high vascularization, which provides a suitable environment for an adequate supply of nutrients and oxygen. Furthermore, the co-encapsulation of islets with mesenchymal stromal cells (MSCs) has resulted in an improved islet function [80]. MSCs are known to elicit beneficial effects on the graft by reducing inflammatory damage and immune rejection, and thus, improve the islet transplantation outcome [81]. Nonetheless, the shortage of deceased human donors together with the risk of retroviral transmission and the more aggressive immune rejection from xenogeneic donors [82] represent a drawback. For this reason, some alternative sources have been investigated. An intriguing alternative is the encapsulation of non-endocrine cells that have been genetically engineered to produce insulin [83]. Another interesting approach is the use of stem cells, considering their ability to differentiate towards insulin-producing β-cells. The stem cell-derived insulin-producing cells may overcome the issue of donor limitation and its combination with alginate encapsulation holds great promise for type 1 diabetes treatment [84-86]. In fact, when compared to 2D constructs, the 3D alginate capsules have been proven to promote the expression of primary maturation markers and the insulin delivery [71]. In a promising study, glucose responsive mature β-cells derived from human embryonic stem cells were able to provide a long-term glycemic control in diabetic immunocompetent C57BL/6J mice [66]. The functionality of the graft continued for the 174 days of study, when implants were retrieved. Interestingly, the analysis of explants revealed viable insulin-producing cells.

Erythropoietin (EPO) is a glycoprotein that enhances the stimulation and maintenance of erythropoiesis (red blood cells maturation) as well as erythrocyte differentiation. Consequently, its delivery has gained relevance in the treatment of chronic anemia, and cell encapsulation has emerged as novel technology with this purpose [87]. This approach eliminates the need for repeated parenteral administrations of EPO, while it allows the continuous secretion of

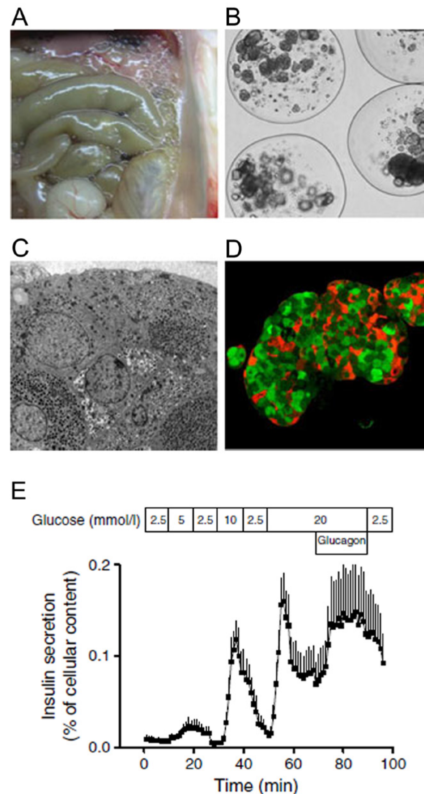


Fig. 4. Analysis of encapsulated human beta cell implant in NOD/SCID mice at post-transplant week 5. (A) Free-floating capsules in the peritoneal cavity were retrieved as single units without fibrosis or cellular overgrowth **(B)**. They contained well-granulated endocrine islet cells **(C)** that stained positively for insulin (green) and glucagon (red) **(D)**. Their insulin release was examined in perifusion at 10 min episodes of varying glucose concentration with and without 10 nmol/l glucagon. Insulin release rate is expressed as a function of the cellular insulin content before perifusion **(E)**. Data represent mean ± SEM from 5 independent experiments. Reprinted from ref. [79], with kind permission from Springer Science+Business Media.

the drug, and thus, avoids instability. An optimized capsule model achieved a controlled EPO delivery during 300 days *in vivo* without the implementation of immunosuppressive therapies [88]. Posterior studies confirmed the efficacy of the system by showing the maintenance of implanted cell viability and the sustained delivery of EPO, which resulted in the elevation of hematocrit levels in animal models [89, 90].

Glucagon-like peptide-1 (GLP-1) is an endogenous insulin-stimulating hormone secreted in response to food intake from the gastrointestinal tract. Furthermore, GLP-1 receptors are present in the mammalian brain and their activation leads to neuroprotective and neurotrophic effects. Thus, this factor may be effective for the treatment of multiple disorders. Considering

its high potential, numerous studies have investigated the entrapment of GLP-1 producing cells in alginate matrices. The applications are diverse: Alzheimer's disease (AD) [91, 92], Amyotrophic lateral sclerosis (ALS) [93], damaged myocardium [94] or brain injury [95]. The latter has gained especial interest because of the recent clinical trials addressing it.

3.2.2 Neurotrophic factors

Neurotrophic factors play a key role in the maintenance of normal neuronal function in adults and in neuronal survival and differentiation during the stages of development. The local up-regulation of these factors has been detected close to the site of lesions such as acute brain injury and neurodegenerative diseases [96]. Since direct injections of different factors have been demonstrated to improve recovery, the transplantation of neurotrophic factor-producing cells close to the affected area may serve as an attractive treatment for these pathologies.

An interesting approach has been the implantation of choroidal plexus (CP) cells. CP cells are known to secrete multiple biologically active neurotrophic factors, and thus, their encapsulation has derived in promising systems that enable the local delivery of these biomolecules for restoration of brain tissue [96, 97]. This strategy has been successful in pathologies such as AD. AD is the most common form of dementia, characterized by the presence of extensive deposition of amyloid-β peptide (Aβ), abnormally phosphorilated tau and neuronal loss. In a recent study, alginate microcapsules containing CP cells were implanted overlying the cerebral cortex of rats with exogenously induced Aβ memory impairment. Animals presented a significant recovery, which was attributed to the decrease in apoptosis, and the increase in neurogenesis, resulting in improved long-term memory [98].

Another example is the brain-derived neurotrophic factor (BDNF) delivery to prevent deafness-induced auditory neuron degeneration. As it has already been proven, the administration of exogenous neurotrophins to the deaf cochlea is a successful treatment; nevertheless, the benefits are rapidly lost when the therapy stops. Therefore, alginate microcapsules have been studied as a good alternative for achieving a sustained release of the factor. In a recent research work, encapsulated BDNF producing Schwann cells showed significant survival-promoting effects on the auditory neurons of deaf guinea pigs. Further, it was suggested that this treatment in combination with a cochlear implant might enhance and extend the

benefits of the latter [99]. This advantageous effect was demonstrated in a posterior work. Specifically, it was observed that when cell-based therapy is combined with a cochlear implant, the enhanced auditory neuron survival effects are translated in important benefits with respect to electrical stimulation thresholds [100]. Altogether, the results suggest that this technology may have important clinical benefits in this area.

Another promising strategy has been the encapsulation of glial cell line-derived neurotrophic factor (GDNF) and nerve growth factor (NGF) producing cells for the treatment of neurodegenerative diseases such as Parkinson's disease (PD). PD is a neurodegenerative central nervous system (CNS) disorder that is characterized by a debilitating motor impairment. This affectation is closely related to the gradual loss of dopaminergic neurons in the *substantia nigra*, which leads to a progressive decrease in dopamine levels in the striatum. PD is currently treated with levodopa o dopaminergic agonists, however, their effectiveness is temporary and the course of the pathology leads to a point where the loss of neurons is so remarkable that these therapies become ineffective. A valuable therapeutic alternative might be directly treating the cause of decreased levels of dopamine, which is the loss of dopaminergic neurons, instead of balancing them with the administration of the neurotransmisor. For this reason, encapsulation of trophic factors-releasing cells may allow the local and sustained delivery of these neurotrophic factors that protect neurons, avoiding their dysfunction and loss [101]. With this aim, different studies have obtained promising results. In particular, cell encapsulation enabled the continuous delivery of GDNF in the striatum of parkinsonian rats over 6 months. Therefore, a significant behavioral improvement was observed in the animals [102]. Moreover, the brain implantation of cell microcapsules for the delivery of neurotrophic factors has been shown to promote the survival of co-grafted cells for long time-periods. In particular, baby hamster kidney (BHK) cells modified to release NGF were implanted approximately 1.5 mm away from co-grafted unencapsulated rat chromaffin cells in hemiparkinsonian rats. The survival of chromaffin cells was significantly enhanced, resulting in important improvements in animal rotational behavior [103, 104].

Vascular endothelial growth factor (VEGF) has also been delivered through alginate microcapsules for its application in PD because of its neuroprotective effects [105]. VEGF is a potent angiogenic factor; for this reason, encapsulated cells secreting it have been employed

with many other goals. One of the approaches has been the use of microencapsulated VEGF secreting cells in AD. Results in mice indicated that the therapy improved cognition as well as reduced apoptotic cell death. Aβ clearance was promoted, as a consequence of the neovascularization produced by VEGF, and hyperphosphorilated-tau expression decreased, being both main factors associated to this degenerative dysfunction [106]. Additionally, this treatment enhanced cellular proliferation in the hippocampal dentate gyrus, representing a novel strategy for the treatment of brain amyloidosis [107]. Among many other applications, a completely different strategy is to use the VEGF secretion as a therapy in bone defects, since this growth factor enhances osteoblast differentiation and moreover, angiogenesis seems to be a prerequisite for bone rehabilitation. In this sense, a recent study showed that VEGF delivery promoted the differentiation of bone marrow MSCs, and thus potentiated bone regeneration [108]. VEGF secreting alginate capsules have also been applied to improve wound healing and angiogenesis in xenogeneic acellular dermis matrix (ADM) transplants [109] or as a promising therapy for the survival of the ischemic skin flaps [110]. Considering the wide variety of applications, an angiogenesis-on-a-chip system has been proposed for the evaluation and quantification of the pro-angiogenic potential of factors such as VEGF secreted from encapsulated cells. Thus, the platform emerges as a potentially valuable preclinical tool that provides quantitative information on encapsulated cell behavior, giving rise to an easier and less time-consuming method in comparison to *in vivo* angiogenesis assays [111].

3.2.3 Antitumor factors

Therapeutic antibodies are nowadays employed for the treatment of multiple diseases. Therefore, encapsulated cells that produce them hold a great potential for many applications in drug delivery. One approach is the utilization of these systems for cancer management, since the interaction of antibodies with cells from the immune system modulates their response. This modulation may occur by tampering with receptors involved in immune inhibition or by over-stimulating receptors that promote the cellular immune response. In particular, the encapsulation of hybridoma cells has been widely studied as a platform for antibody delivery [112]. For instance, microcapsules containing hybridomas that produced anti-CD137 and anti-OX40 antibodies elicited an efficacious antitumor response by enhancing tumor-specific cellular immunity [113]. Recently, the capacity of encapsulated cells producing bispecific

antibodies against carcinoembryonic antigens, which are present in most colon carcinomas, has been proven [114].

Another alternative is the secretion of endostatin, which is an endogenous antiangiogenic peptide that has shown potent antitumor activity. The approach of encapsulating endostatin-producing cells has been studied for more than 15 years. In fact, in 2000, a promising study already showed the efficacy of the treatment obtaining considerable survival benefits in the immunocompetent BT4C brain tumor model [115]. Posterior studies confirmed the antiangiogenic effect of endostatin by showing a significantly reduced tumor vascularization. Nonetheless, the effect on tumor growth was not observed [116].

The hypoimmunnogenic and immunomodulatory properties of MSCs make them an interesting alternative for cancer management. In particular, alginate encapsulated MSCs showed a 3-fold decrease in cytokine expression compared to entrapped cell lines [117]. Furthermore, MSCs present inherent tumor trophic migratory properties and the possibility to be modified to express diverse therapeutic factors renders them optimal vehicles for the targeted delivery to isolated tumors and metastatic disease [118]. In a relevant example, MSCs were modified to secrete the angiogenesis inhibitor hemopexin-like protein (PEX). Their administration adjacent to glioblastoma tumors resulted in a relevant reduction not only in tumor volume (87%), but also in tumor weight (83%) [117].

4. Clinical trials

The promising results obtained in experimental animal models have led to the conduction of several clinical trials in order to move this technology towards clinical translation. This section gathers some relevant examples of clinical trials concerning cell encapsulation.

4.1 Diabetes

Overall, encapsulation of insulin producing-cells holds a great potential. For this reason, this platform has been studied in clinical trials over the last twenty years. The first human clinical trial with alginate-encapsulated islet transplantation dates back to 1994. In this work, encapsulated cadaveric human islets were implanted intraperitoneally. Capsules were able to

establish a glycemic control for nine months in a type 1 diabetes patient who was on anti-rejection medications [119]. Since this successful approach, different clinical trials have been performed. Such is the case of Calafiore *et al*., who carried out a study where alginate-PLO microencapsulated islets were intraperitoneally transplanted without the use of immunosuppression. The results did not show side effects of the grafting procedures or any evidence of immune sensitization, confirming the technique as a powerful tool for immunoprotection. Moreover, patient's necessity of exogenous insulin intake decreased approximately to half of the pre-transplantation consumption levels [120, 121]. Tuch and colleagues carried out a trial with four patients in which allogeneic islets were transplanted without immunosuppression. Neither insulin requirement nor glycemic control was altered and C-peptide, an indirect measurement of insulin levels, was undetectable by 1-4 weeks. However, in the particular case of a recipient that received three separate islet infusions the analysis of C-peptide revealed its presence up to the next 2.5 years [122]. Recently, Jacobs-Tulleneeers-Thevissen *et al*. transplanted in a patient allogeneic islets encapsulated in Ca^{+2}/Ba^{+2} alginate microcapsules. Three months after transplantation capsules were retrieved and cells remained glucose-responsive. However, the transplant showed insufficient biocompatibility [79].

In another report, Sernova Corp. announced the CellPouch® System, a commercial product of the macroencapsulation device that is currently ongoing a Phase I/II clinical trial in diabetic patients were measures of safety and efficacy are the primary and secondary endpoints [123]. Likewise, the company Living Cell Technologies (LCT) offers the DIABECELL® product, which is currently in late-stage clinical trials. In 1996, LCT initiated a novel study addressing the efficacy of xenogeneic islets. In particular, porcine islets were encapsulated and implanted in the peritoneal cavity of patients without the use of immunosuppressive therapies. Nine and a half years after transplantation, laparotomy of one of the patients showed the presence of microcapsules in the peritoneal cavity. Although the majority contained necrotic islets, impressively, some of them still remained viable [124]. This fact demonstrated the potential of the approach, and in consequence, the company has continued the research to achieve a correct glycemic control without the need immunosuppressants [125]. In a Phase I/II safety study tolerability of the implant was confirmed [126]. Moreover, the trial showed proof of principle of efficacy demonstrating improvement in blood glucose control, even permitting the discontinuation of insulin injections entirely for up to 32 weeks. Later, a Phase IIa dose finding

trial was performed [127], followed by a Phase IIb safety and efficacy study [128], which resulted in a clinically significant reduction of insulin dose and unaware hypoglycemia. As of now, a recent newsletter from the website announced the launch of Phase IIb/III clinical trials [48].

4.2 Intracerebral hemorrhage (ICH)

Regarding ICH, a Phase I/II clinical trial has been conducted to evaluate the safety of its treatment with encapsulated cells that secrete GLP-1 [129]. The goal of this therapy is to improve the outcome after ICH surgery by enabling the local delivery of the neuroprotective and anti-inflammatory factor. Thus, the treatment may promote the healing of the secondary neuronal injury that occurs in the first weeks after the bleeding. With this purpose, stroke patients with space-occupying intracerebral hemorrhage were selected. After surgical evacuation of the hematoma, alginate microcapsules containing allogeneic mesenchymal cells transfected to produce GLP-1 were implanted in patients. Grafts were removed by a second surgery after 14 days of treatment. Preliminary results revealed that neither side effects from the surgical intervention, nor implant-related side effects were shown in the interim evaluation of the first 11 patients. Furthermore, 30% of the implanted cells were viable and maintained their secretory capacity after explantation [130].

4.3 Neurological diseases

The LCT company is also carrying out clinical trials regarding neurological diseases. In particular, the aim is to encapsulate clusters of neonatal porcine CP cells in alginate matrices. As already mentioned, CP cells produce a wide variety of neurotrophic and neuroprotective factors that support brain health. This product, branded NTCELL®, is intended for the treatment, without the implementation of immunosuppression, of different neurological diseases such as PD, AD, ALS or Huntington disease. After obtaining promising preclinical data in a model of PD [131], the company conducted a Phase I/IIa clinical trial [132]. The study, completed in June 2015, investigated the safety and clinical effect of the capsules in four patients that had been diagnosed with PD at least 5 years before. The implants were safe and well tolerated and improved the clinical symptoms of PD in all of the patients, maintaining the effect for 26 weeks post-implant. In March 2016, a Phase IIb study commenced with the purpose of confirming the most effective dose [133]. The company has claimed that if the obtained results

are positive, they will apply for provisional consent to launch NTCELL as the first disease modifying treatment for PD in 2017 [134].

5. Complementary strategies

The multiple clinical trials conducted in the field demonstrate the applicability and potential of cell encapsulation. Nonetheless, the sole alginate capsule does not fulfill all the requirements the cells need to survive and accomplish a correct physiological activity. The same happens with crucial aspects such as the host immune response and the safety issues of the implant. Consequently, supplementary strategies need to be implemented to broaden the possibilities of the system and thus, to develop a successful technology in the clinical area.

5.1 Matrix biofunctionalization

In their natural niches, cells receive and process information from the ECM [135]. Specifically, cells bind to ECM molecules via integrins giving rise to biochemical and mechanical signals that regulate myriad cellular processes such as proliferation, migration or differentiation. Regarding cell encapsulation in alginate matrices, it is noticeable that this polymer by itself does not promote cell adhesion. Thus, controlling the intra-capsular microenvironment by the inclusion of appropriate ligands is of paramount importance to maintain the viability and correct function of the entrapped cells.

The biofunctionalization of alginates with ECM motifs can be achieved by incorporating full-length ECM molecules as fibronectin, collagen or laminin [34]. Nonetheless, a promising alternative involves the isolation of functional domains from these large ECM molecules and their use as short-peptide sequences to form biofunctional matrices. These cell adhesive peptides have the advantage of being relatively stable and can be easily attached to the hydrogel [136]. On this point, carboiimide chemistry has been the most commonly applied method for alginate functionalization. However, lately, new options have been suggested as efficient procedures for coupling bioactive molecules to alginates, such as the partial periodate oxidation followed by reductive amination [137]. The most widely employed motif is the arginine-glycine-aspartic acid (RGD), present in fibronectin, laminin or collagen, among

other ECM proteins. RGD has been extensively used in the field of cell microencapsulation with promising results [138-141]. Interestingly, these studies have highlighted critical aspects such as the ligand density, which has been described as a crucial factor to consider when elaborating the biomimetic capsules. Indeed, cytoskeleton organization may differ when encapsulating cells in alginate matrices with differing degree of substitution (DS). Since DS is defined as the total number of RGD peptides per alginate chain, different alginate types can be obtained. In particular, Fig. 5 shows a study in which cells were entrapped in four different alginates: DS 0 (No modified alginate), DS 1 (0.112 mM), DS 5 (0.5 mM), and DS 10 (1.12 mM) [141]. Moreover, the necessity of evaluating the role of RGD for each cell line has also been stated, since the effects of the peptide-sequence may vary. Other isolated ECM moieties that have been employed in alginate encapsulation are the laminin derived-peptides YIGSR (Tyr-Ile-Gly-Ser-Arg) and IKVAV (Ile-Lys-Val-Ala-Val) [73, 142]. Considering the importance of matrix functionalization, several novel approaches are being carried out [143, 144]. An interesting example is the introduction of galactosylated chitosan (GC) in the alginate core. GC provides cells with multiple binding domains that promote the cell-matrix interactions, improving viability and specific cell functions [144].

A different concept is the inclusion of growth factor-binding domains. Multiple signaling molecules that elicit essential cellular responses are present in the ECM given their non-covalent interactions with heparin sulfate proteoglycans. Therefore, the covalent tethering of heparan sulfate-containing molecules provides the hydrogel with binding domains that may

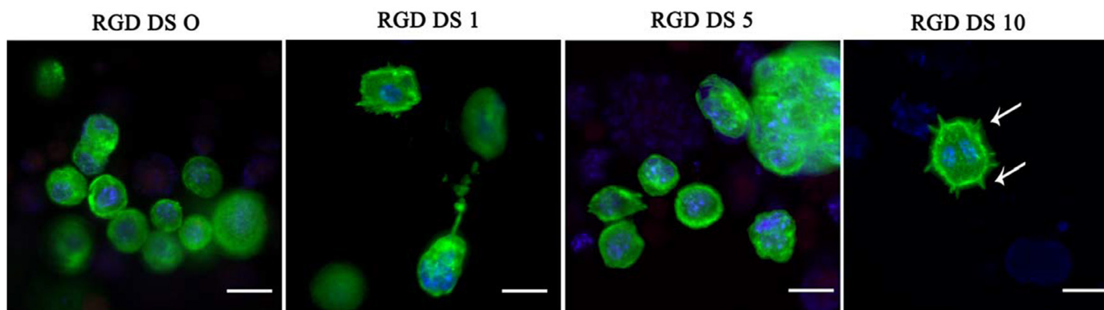


Fig. 5. *In vitro* cytoskeleton organization in cells encapsulated in microcapsules elaborated with alginates with different RGD substitution degrees. The cells immobilized in APA microcapsules were stained with phalloidin Alexa Fluor 488 for F-actin (green) and Hoechst for nucleus (blue). Scale bars = 520 μm. Reproduced from ref. [141] by permission of John Wiley & Sons Ltd.

sequestrate different growth factors for their posterior controlled release [145]. Therefore, the encapsulated cell microenvironment may mimic more efficiently the ECM, thus improving cell fate.

5.2 Reducing inflammation

The implantation of cell microcapsules involves a surgical procedure that in all cases is followed by a tissue repair response. This wound healing process is intensified due to the introduction of a foreign material that contains alien cells. Consequently, inflammation arises as a protective attempt to eliminate the prejudicial stimuli and initiate a healing process. Inflammation comprises vasodilatation, augmented blood flow and increased permeability, which permits the migration of proteins and blood cells from the circulation to the damaged tissue. This immune response may limit the success of the system by leading to the pericapsular fibrotic overgrowth (PFO), a fibrotic cell layer that surrounds the system and thus, reduces nutrient and oxygen supply endangering cell survival.

One of the strategies to mitigate this unsolved issue has been the co-administration of anti-inflammatory drugs. The *in vivo* screening of different anti-inflammatory molecules has pointed out to dexamethasone (DXM) and curcumin as the most effective drugs to inhibit reactive oxygen species (ROS) and early inflammatory proteases. Indeed, the co-encapsulation of cells with the latter has shown to reduce fibrosis and improve cell function [146]. The effect of DXM has also been studied. In an interesting approach, different composite drug delivery systems were designed to combine APA cell microcapsules with DXM-loaded poly(lactic-co-glycolic acid) (PLGA) microspheres. The combination showed enhanced performance of the system [147, 148], resulting in a promising alternative to prevent inflammation and thus, improve the long-term efficacy of the graft, even when implanting xenogeneic cells [148]. More recently, the co-encapsulation of pancreatic islets with the anti-inflammatory drug pentoxifylline (PTX) was demonstrated to improve the resistance of these cells against host immune cells such as lymphocytes [149]. Similarly, antagonists of inflammation-mediators have also been entrapped in cell microcapsules. With the aim to reduce the inflammation induced by the high mobility group box 1 (HMGB1), an antagonist of its receptor in immune cells, HMGB1 A box, was co-encapsulated. Results revealed that the amount of TNF-α

secreted from macrophages was significantly attenuated. Moreover, an *in vivo* study showed a significant improvement in the survival rate of implanted cells [150].

Another alternative is the modification of the capsule with different motifs that exert an anti-inflammatory effect. That is the case of sulfated alginates, which used as a secondary coating on alginate-PLL microcapsules or mixed in the gel core of non-coated microbeads, resulted in the reduction of inflammatory cytokines including TNFα, IL-6, IL-8 or IL-1β [151]. In another example, the attachment of an inhibitory peptide for cell surface IL-1β enabled the maintenance of cell viability in presence of a combination of different cytokines such as IL-1β or TNFα. Contrarily, cells encapsulated in unmodified hydrogels were unable to survive to the exposure of these cytokines [50].

A newer strategy to reduce inflammation, and consequently PFO, is the co-encapsulation of effector target cells with MSCs. MSCs are known to be hypoimmunogeneic and to exert an immunomodulatory effect because of the production of factors such as prostaglandin E2 or nitric oxide that modulate the immune response. [152-154]. A recent study demonstrated the beneficial effects of co-encapsulated MSCs in an aggressive xenotransplantation model of mice by showing a dose-dependent reduction of PFO with the subsequent improvement on graft survival [155]. This approach has resulted beneficial in different applications including pancreatic islet transplantation, where MSCs present a high potential to overcome some of the current limitations of the system by suppressing inflammatory damage and immune mediated rejection [81]. Moreover, it is possible to take advantage of the important characteristics of MSCs to modulate the neuro-inflammatory response. Therefore, it has been suggested that alginate encapsulation of MSCs may not only provide an auxiliary strategy to improve biocompatibility when co-encapsulated with other effector cells, but also a valuable treatment for CNS trauma [156]. Equivalently, Sertoli cells also present similar immunoregulatory properties. In fact, they have been demonstrated to deliver biomolecules associated with trophic and anti-inflammatory effects that synergistically act on multiple fronts [157]. Altogether, these factors have been proven to normalize glucose homeostasis in an experimental diabetes model [46] or to reduce striatal inflammation in Huntington disease, prolonging mice lifespan and improving quality of life [158].

5.3 Enhancing biosafety

Despite cell encapsulation holds great potential, the fact that it implies the use of living cells leads to safety concerns, particularly considering that in many cases these cells have been genetically modified. Moreover, once microcapsules are administered, the determination of their position and integrity is complex. In order to address this issue, different approaches have been proposed in order to monitor or inactivate the implants.

Diverse non-invasive imaging techniques have been developed with the aim to track the implants. Cell encapsulation is an asset for monitoring, as the contrast agents are contained in the hydrogel instead of directly labeling the cells. This reduces toxicity and promotes cell survival and function. Widely used modalities are X-ray/Computed Tomography (CT) [159, 160], Magnetic resonance imaging (MRI) [161] or ultrasound imaging [162]. For instance, in a recent study, alginate microcapsules with a self-assembled gold nanoparticle (AuNPs) coating resulted in distinctive contrast and enabled the identification by using a conventional small animal X-ray micro-CT scanner. In particular, AuNPs were modified with the cationic 2-(methacryloyloxy)ethyl trimethylammonium chloride (METAC) polymer in order to enable the electrostatic interaction of AuNPs with the negatively charged alginate microcapsules. The resulting PMETAC_SH-Au nanoparticles were coated onto preformed alginate microcapsules (PMETAC_SH-Au MCs) with successful imaging results (Fig. 6A) [160].

The limitation of these techniques is that they do not provide information about cell viability or functionality. To overcome this issue reporter genes have been employed [163-165]. They are typically based on bioluminescence and/or fluorescence and provide us with a helpful tool to perform non-invasive, quantitative and real-time live image determinations. Regarding imaging techniques, recent approaches have also studied the possibility of analyzing the host immune response while the capsules are still implanted [166, 167]. This progress may represent a pivotal advantage, as it would permit to adjust the therapy if needed. In other words, in cases when a significant immune response is detected, the pertinent therapy could be administered in order to alleviate acute inflammation. Thus, the transplantation regimen would improve, restricting the possibilities of graft failure. This advance is specially relevant considering that nowadays it is not possible to analyze the host immune response unless biopsies are obtained.

A determinant aspect regarding the safety of the system is the possibility to inactivate the graft. This inactivation might be useful when the therapy reaches its final goal or when the system causes undesirable effects. To this end, an interesting approach is the inclusion of suicide genes in the genome of the encapsulated cells. Suicide genes induce apoptosis upon external drug administration, which allows the inactivation of the implant when necessary. Interesting studies were carried out regarding ganciclovir (GCV) mediated inactivation in encapsulated cells bearing TGL triple-fusion reporter gene, which codifies for the suicide gene Herpes Simplex virus type 1 thymidine-kinase (HSV1-TK), green fluorescent protein (GFP) and Firefly Luciferase ($SFG_{NES}TGL$) [163, 165]. The *in vivo* studies demonstrated the potential of the procedure by providing information about the enclosed cells at desired time points in a non-invasive way, including the additional advantage of cell inactivation if required (Fig. 6B-D). The design of inducible systems may also dramatically improve safety. Recently, inducible hepatocyte growth factor (HGF) secreting human umbilical cord blood (hUCB) derived MSCs were produced via TALEN-mediated genome editing. The TetOn-HGF/hUCB-MSCs were encapsulated in alginate microcapsules and demonstrated an improvement in angiogenesis in a mouse hind-limb ischemia model, proving that these inducible cells are able to deliver the therapeutic factor in a controlled and effective manner [168]. Therefore, these strategies may take control over the therapeutic effects exerted by the implanted cells leading to safer treatments.

5.4 Improving administration and extraction

The retention of microcapsules within the tissue they are implanted and the suitable retrieval of the whole implant are necessary premises in this technology. For this reason, several attempts have been carried out in order to optimize administration and extraction protocols. Different systems have been proposed to envelop the alginate cell-microcapsules, such as calcium phosphate cements [169, 170], mesh bags [130] or hydrogel-based scaffolds [90]. The latter presents advantageous properties that permit to select between an invasive administration (as a preformed scaffold) or a non-invasive injection (as an *in situ* formed scaffold). Moreover, the scaffolds may be multifunctionalized by combining in the hydrogel the alginate microcapsules and other molecules such as anti-inflammatory drugs [148].

In addition, the area of implantation is generally a highly hypoxic and inflammatory zone that jeopardizes the graft survival. On this point, the β-air device has emerged as an ingenious approach to solve this problem [171]. Preliminarily, a rat-type β-air device was designed to be implanted under the skin or into the pre-peritoneal cavity, areas of easy access. It consisted of two main components separated by an oxygen-permeable membrane: an islet module that

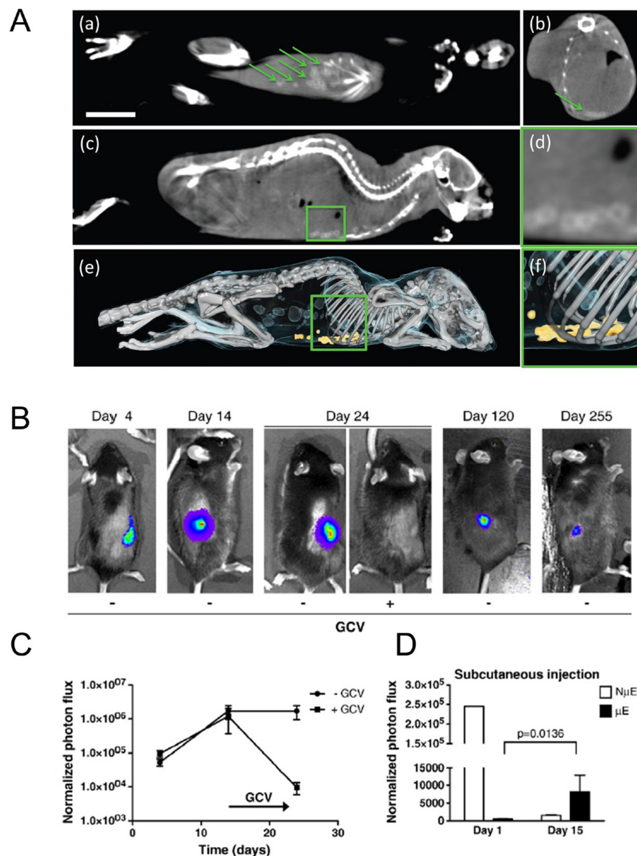


Fig. 6. (A) X-ray micro-CT reconstructed data of a mouse injected with PMETAC_SH-Au-MCs (4.7 g L^{-1}) (CTDI 28 mGy) in (a) coronal (b) transverse and (c) sagittal views with the magnification of the detail in the square shown in (d). Arrows indicate microcapsules (MCs). 3D rendering of the same mouse with the MCs artificially colored in yellow (e) and the detail in the square magnified in (f). Scale bar = 1 cm. Reproduced from Ref. [160] with permission of The Royal Society of Chemistry. **(B-D)** Behavior of C_2C_{12}-TGL microencapsulated cells *in vivo*. Microencapsulated C_2C_{12}-TGL cells exhibited light emission after being subcutaneously injected in C57BL/6J mice. Mice treated with 150 mg/kg/day ganciclovir (GCV) for a week showed almost no signal (B-C). Luciferase activity could be found in cells within the microcapsules 255 days after injection (B). Quantification of light emission demonstrates an increase in the normalized photon flux during the first 2 weeks after implantation (C-D), probably due to vascularization of microcapsule plugs. Non-microencapsulated C_2C_{12}-TGL cells displayed a marked decrease in light emission between days 1-15 (D). Conversely, microencapsulated myoblasts increased the emission during that period (D). μE: Microencapsulated cells. NμE: Non-microencapsulated cells. Reprinted from ref. [163], ©2010, with permission from Elsevier.

contained the alginate-encapsulated cells and a gas chamber connected to an external air pump and an outlet port by two transcutaneous silicone tubes. When implanted in rodents, the macrochamber, refueled with oxygen, normalized glycemic control for periods up to 6 months. Moreover, the tissue surrounding the implant did not present signs of inflammation and showed visual evidence of vasculature. The success of the approach led to a porcine model of the β-air device. Equipped with xenogeneic rat islets, the device was implanted in diabetic Sinclair minipigs. Once again, the restoration of normoglycemia was achieved [172]. Altogether, the hallmarks of the β-air device are the sufficient oxygenation of the implant, a substantial immune barrier, especially relevant for xenotransplants, and a minimally invasive surgical procedure.

6. Concluding remarks

Alginate is the most widely employed biomaterial for cell encapsulation as it provides the technology with pivotal advantages. In particular, the intrinsic characteristics of alginates make them biocompatible and allow the rapid and simple gelation under mild conditions, giving rise to scalable encapsulation methods. The obtained alginate microcapsules represent a potential alternative for the treatment of multiple chronic diseases that nowadays lack of an adequate management with conventional drug-delivery systems. That is the case of prevalent disorders such as diabetes, Alzheimer’s disease, Parkinson’s disease, chronic anemia or cancer. Over the last decades, important advances have been achieved in the field; however, there are still major challenges to face in order to move towards a definitive clinical translation. To this end, several attempts are being carried out, which include the search for suitable and biocompatible coatings [35], the biofunctionalization of the matrices, the optimization of safety measures such as inducible Tet-on/off systems [168], the synthesis of capsules with suitable size and shape [173], or the selection of the most appropriate administration site [34]. May these hurdles be overcome, therapeutic cell encapsulation would significantly evolve becoming an invaluable tool in the medical practice.

7. Acknowledgments

A. Gonzalez-Pujana thanks the Basque Government (Department of Education, Universities and Research) for the PhD fellowship. This project was partially supported by the Basque Government (Consolidated Groups, IT-907-16) and the University of the Basque Country UPV/EHU (UFI11/32).

8. References

1. World Health Organization (2014) Global status report on noncommunicable diseases. doi:ISBN 978 92 4 156485 4.

2. Shakir R (2015) Neurodegenerative noncommunicable diseases (Neurology NCDs). Where are we now?. J Neurol Sci 356(1-2):1-2. doi:10.1016/j.jns.2015.07.005.

3. Hunt NC, Grover LM (2010) Cell encapsulation using biopolymer gels for regenerative medicine. Biotechnol Lett 32(6):733-742. doi:10.1007/s10529-010-0221-0.

4. Chang TM (2005) Therapeutic applications of polymeric artificial cells. Nat Rev Drug Discov 4(3):221-235. doi:10.1038/nrd1659.

5. Mazzitelli S, Capretto L, Quinci F et al (2013) Preparation of cell-encapsulation devices in confined microenvironment. Adv Drug Deliv Rev 65(11-12):1533-1555. doi:10.1016/j.addr.2013.07.021.

6. de Vos P, Lazarjani HA, Poncelet D et al (2014) Polymers in cell encapsulation from an enveloped cell perspective. Adv Drug Deliv Rev 67-68:15-34. doi:10.1016/j.addr.2013.11.005.

7. Bisceglie V (1934) Über die antineoplastische Immunität. Zeitschrift für Krebsforschung 40(1):122-140.

8. Chang TM (1964) Semipermeable Microcapsules. Science 146(3643):524-525.

9. Lim F, Sun AM (1980) Microencapsulated islets as bioartificial endocrine pancreas. Science 210(4472):908-910.

10. Grant GT, Morris ER, Rees DA et al (1973) Biological interactions between polysaccharides and divalent cations: The egg-box model. FEBS Lett 32(1):195-198. doi:10.1016/0014-5793(73)80770-7.

11. Marriott AS, Bergstrom E, Hunt AJ et al (2014) A natural template approach to mesoporous carbon spheres for use as green chromatographic stationary phases. RSC Adv 4(1):222-228. doi:10.1039/C3RA44428G.

12. Lee KY, Mooney DJ (2012) Alginate: properties and biomedical applications. Prog Polym Sci 37(1):106-126. doi:10.1016/j.progpolymsci.2011.06.003.

13. Donati I, Holtan S, Morch YA et al (2005) New hypothesis on the role of alternating sequences in calcium-alginate gels. Biomacromolecules 6(2):1031-1040. doi:10.1021/bm049306e.

14. Andersen T, Auk-Emblem P, Dornish M (2015) 3D Cell Culture in Alginate Hydrogels. Microarrays (Basel) 4(2):133-161. doi:10.3390/microarrays4020133.

15. Haug A, Smidsrod O (1965) The effect of divalent metals on the properties of alginate solutions. Acta Chemica Scandinavica 19:341-351.

16. Leong J, Lam W, Ho K et al (2016) Advances in fabricating spherical alginate hydrogels with controlled particle designs by ionotropic gelation as encapsulation systems. Particuology 24:44-60. doi:10.1016/j.partic.2015.09.004.

17. Morch YA, Donati I, Strand BL et al (2006) Effect of Ca2+, Ba2+, and Sr2+ on alginate microbeads. Biomacromolecules 7(5):1471-1480. doi:10.1021/bm060010d.

18. Jeong SI, Jeon O, Krebs MD et al (2012) Biodegradable photo-crosslinked alginate nanofibre scaffolds with tuneable physical properties, cell adhesivity and growth factor release. Eur Cell Mater 24:331-343.

19. Zhao S, Cao M, Li H et al (2010) Synthesis and characterization of thermo-sensitive semi-IPN hydrogels based on poly(ethylene glycol)-co-poly(epsilon-caprolactone) macromer, N-isopropylacrylamide, and sodium alginate. Carbohydr Res 345(3):425-431. doi:10.1016/j.carres.2009.11.014.

20. Kingsley DM, Dias AD, Corr DT (2016) Microcapsules and 3D customizable shelled microenvironments from laser direct-written microbeads. Biotechnol Bioeng 113(10):2264-2274. doi:10.1002/bit.25987.

21. Gasperini L, Mano JF, Reis RL (2014) Natural polymers for the microencapsulation of cells. J R Soc Interface 11(100):20140817. doi:10.1098/rsif.2014.0817.

22. Orive G, Hernandez RM, Gascon AR et al (2003) Cell encapsulation: promise and progress. Nat Med 9(1):104-107. doi:10.1038/nm0103-104.

23. Qian D, Bai B, Yan G et al (2016) Construction of doxycycline-mediated BMP-2 transgene combining with APA microcapsules for bone repair. Artif Cells Nanomed Biotechnol 44(1):270-276. doi:10.3109/21691401.2014.942458.

24. Hu J, Li H, Chi G et al (2015) IL-1RA gene-transfected bone marrow-derived mesenchymal stem cells in APA microcapsules could alleviate rheumatoid arthritis. Int J Clin Exp Med 8(1):706-713.

25. Ibarra V, Appel AA, Anastasio MA et al (2016) This paper is a winner in the Undergraduate category for the SFB awards: Evaluation of the tissue response to alginate encapsulated islets in an omentum pouch model. J Biomed Mater Res 104(7):1581-1590. doi:10.1002/jbm.a.35769.

26. Pareta R, McQuilling JP, Sittadjody S et al (2014) Long-term function of islets encapsulated in a re-designed alginate microcapsule construct in omentum pouches of immune-competent diabetic rats. Pancreas 43(4):605-613. doi:10.1097/MPA.0000000000000107.

27. Anderson JM, Rodriguez A, Chang DT (2008) Foreign body reaction to biomaterials. Semin Immunol 20(2):86-100. doi:10.1016/j.smim.2007.11.004.

28. Rokstad AM, Brekke OL, Steinkjer B et al (2013) The induction of cytokines by polycation containing microspheres by a complement dependent mechanism. Biomaterials 34(3):621-630. doi:10.1016/j.biomaterials.2012.10.012.

29. Orning P, Hoem KS, Coron AE et al (2016) Alginate microsphere compositions dictate different mechanisms of complement activation with consequences for cytokine release and leukocyte activation. J Control Release 229:58-69. doi:10.1016/j.jconrel.2016.03.021.

30. Rokstad AM, Brekke OL, Steinkjer B et al (2011) Alginate microbeads are complement compatible, in contrast to polycation containing microcapsules, as revealed in a human whole blood model. Acta Biomater 7(6):2566-2578. doi:10.1016/j.actbio.2011.03.011.

31. Tam SK, Dusseault J, Polizu S et al (2005) Physicochemical model of alginate-poly-L-lysine microcapsules defined at the micrometric/nanometric scale using ATR-FTIR, XPS, and ToF-SIMS. Biomaterials 26(34):6950-6961. doi:10.1016/j.biomaterials.2005.05.007.

32. Tam SK, Bilodeau S, Dusseault J et al (2011) Biocompatibility and physicochemical characteristics of alginate-polycation microcapsules. Acta Biomater 7(4):1683-1692. doi:10.1016/j.actbio.2010.12.006.

33. Juste S, Lessard M, Henley N et al (2005) Effect of poly-L-lysine coating on macrophage activation by alginate-based microcapsules: assessment using a new in vitro method. J Biomed Mater Res A 72(4):389-398. doi:10.1002/jbm.a.30254.

34. Santos E, Pedraz JL, Hernandez RM et al (2013) Therapeutic cell encapsulation: ten steps towards clinical translation. J Control Release 170(1):1-14. doi:10.1016/j.jconrel.2013.04.015.

35. Demont A, Cole H, Marison IW (2016) An understanding of potential and limitations of alginate/PLL microcapsules as a cell retention system for perfusion cultures. J Microencapsul 33(1):80-88. doi:10.3109/02652048.2015.1134686.

36. Zhang W, Zhao S, Rao W et al (2013) A Novel Core-Shell Microcapsule for Encapsulation and 3D Culture of Embryonic Stem Cells. J Mater Chem B Mater Biol Med 2013(7):1002-1009. doi:10.1039/C2TB00058J.

37. Zheng G, Liu X, Wang X et al (2014) Improving stability and biocompatibility of alginate/chitosan microcapsule by fabricating bi-functional membrane. Macromol Biosci 14(5):655-666. doi:10.1002/mabi.201300474.

38. Yang HK, Ham DS, Park HS et al (2016) Long-term Efficacy and Biocompatibility of Encapsulated Islet Transplantation With Chitosan-Coated Alginate Capsules in Mice and Canine Models of Diabetes. Transplantation 100(2):334-343. doi:10.1097/TP.0000000000000927.

39. Hillberg AL, Oudshoorn M, Lam JB et al (2015) Encapsulation of porcine pancreatic islets within an immunoprotective capsule comprising methacrylated glycol chitosan and alginate. J Biomed Mater Res B Appl Biomater 103(3):503-518. doi:10.1002/jbm.b.33185.

40. Orive G, Hernandez RM, Gascon AR et al (2003) Development and optimisation of alginate-PMCG-alginate microcapsules for cell immobilisation. Int J Pharm 259(1-2):57-68. doi:10.1016/S0378-5173(03)00201-1.

41. Spasojevic M, Paredes-Juarez GA, Vorenkamp J et al (2014) Reduction of the inflammatory responses against alginate-poly-L-lysine microcapsules by anti-biofouling surfaces of PEG-b-PLL diblock copolymers. PLoS One 9(10):e109837.doi:10.1371/journal.pone.0109837.

42. Spasojevic M, Bhujbal S, Paredes G et al (2014) Considerations in binding diblock copolymers on hydrophilic alginate beads for providing an immunoprotective membrane. J Biomed Mater Res A 102(6):1887-1896. doi:10.1002/jbm.a.34863.

43. Orive G, Santos E, Poncelet D et al (2015) Cell encapsulation: technical and clinical advances. Trends Pharmacol Sci 36(8):537-546. doi:10.1016/j.tips.2015.05.003.

44. Wang T, Adcock J, Kuhtreiber W et al (2008) Successful allotransplantation of encapsulated islets in pancreatectomized canines for diabetic management without the use of immunosuppression. Transplantation 85(3):331-337. doi:10.1097/TP.0b013e3181629c25.

45. Sun Y, Ma X, Zhou D et al (1996) Normalization of diabetes in spontaneously diabetic cynomologus monkeys by xenografts of microencapsulated porcine islets without immunosuppression. J Clin Invest 98(6):1417-1422. doi:10.1172/JCI118929.

46. Luca G, Arato I, Mancuso F et al (2016) Xenograft of microencapsulated Sertoli cells restores glucose homeostasis in db/db mice with spontaneous diabetes mellitus. Xenotransplantation. doi:10.1111/xen.12274.

47. Scharp DW, Marchetti P (2014) Encapsulated islets for diabetes therapy: history, current progress, and critical issues requiring solution. Adv Drug Deliv Rev 67-68:35-73. doi:10.1016/j.addr.2013.07.018.

48. Qi M (2013) Transplantation of Encapsulated Pancreatic Islets as a Treatment for Patients with Type 1 Diabetes Mellitus. Advances in Medicine 2014:429710. doi:10.1155/2014/429710.

49. Chen T, Yuan J, Duncanson S et al (2015) Alginate encapsulant incorporating CXCL12 supports long-term allo- and xenoislet transplantation without systemic immune suppression. Am J Transplant 15(3):618-627. doi:10.1111/ajt.13049.

50. Su J, Hu BH, Lowe WL,Jr et al (2010) Anti-inflammatory peptide-functionalized hydrogels for insulin-secreting cell encapsulation. Biomaterials 31(2):308-314. doi:10.1016/j.biomaterials.2009.09.045.

51. Orive G, Gascon AR, Hernandez RM et al (2003) Cell microencapsulation technology for biomedical purposes: novel insights and challenges. Trends Pharmacol Sci 24(5):207-210. doi:10.1016/S0165-6147(03)00073-7.

52. Olabisi RM (2015) Cell microencapsulation with synthetic polymers. J Biomed Mater Res A 103(2):846-859. doi:10.1002/jbm.a.35205.

53. Paredes Juarez GA, Spasojevic M, Faas MM et al (2014) Immunological and technical considerations in application of alginate-based microencapsulation systems. Front Bioeng Biotechnol 2:26. doi:10.3389/fbioe.2014.00026.

54. Marchioli G, van Gurp L, van Krieken PP et al (2015) Fabrication of three-dimensional bioplotted hydrogel scaffolds for islets of Langerhans transplantation. Biofabrication 7(2):025009. doi:10.1088/1758-5090/7/2/025009.

55. McHugh DJ (2003) A Guide to the Seaweed Industry; Food and Agriculture Organization of the United Nations.

56. Hay ID, Ur Rehman Z, Moradali MF et al (2013) Microbial alginate production, modification and its applications. Microb Biotechnol 6(6):637-650. doi:10.1111/1751-7915.12076

57. Hernandez RM, Orive G, Murua A et al (2010) Microcapsules and microcarriers for in situ cell delivery. Adv Drug Deliv Rev 62(7-8):711-730. doi:10.1016/j.addr.2010.02.004.

58. Paredes-Juarez GA, de Haan BJ, Faas MM et al (2013) The role of pathogen-associated molecular patterns in inflammatory responses against alginate based microcapsules. J Control Release 172(3):983-992. doi:10.1016/j.jconrel.2013.09.009.

59. Dufrane D, Gianello P (2012) Macro- or microencapsulation of pig islets to cure type 1 diabetes. World J Gastroenterol 18(47):6885-6893. doi:10.3748/wjg.v18.i47.6885.

60. Montanucci P, Terenzi S, Santi C et al (2015) Insights in Behavior of Variably Formulated Alginate-Based Microcapsules for Cell Transplantation. Biomed Res Int 2015:965804. doi:10.1155/2015/965804

61. Sondermeijer HP, Witkowski P, Woodland D et al (2016) Optimization of alginate purification using polyvinylidene difluoride membrane filtration: Effects on immunogenicity and biocompatibility of three-dimensional alginate scaffolds. J Biomater Appl 31(4):510-520. doi:10.1177/0885328216645952.

62. Calafiore R, Basta G (2014) Clinical application of microencapsulated islets: actual prospectives on progress and challenges. Adv Drug Deliv Rev 67-68:84-92. doi:10.1016/j.addr.2013.09.020.

63. Kim AR, Hwang JH, Kim HM et al (2013) Reduction of inflammatory reaction in the use of purified alginate microcapsules. J Biomater Sci Polym Ed 24(9):1084-1098. doi:10.1080/09205063.2012.735100.

64. Basta G, Calafiore R (2011) Immunoisolation of pancreatic islet grafts with no recipient's immunosuppression: actual and future perspectives. Curr Diab Rep 11(5):384-391. doi:10.1007/s11892-011-0219-6.

65. Dolgin E (2014) Encapsulate this. Nat Med 20(1):9-11. doi:10.1038/nm0114-9.

66. Vegas AJ, Veiseh O, Gurtler M et al (2016) Long-term glycemic control using polymer-encapsulated human stem cell-derived beta cells in immune-competent mice. Nat Med 22(3):306-311. doi:10.1038/nm.4030.

67. Paredes-Juarez GA, de Haan BJ, Faas MM et al (2014) A technology platform to test the efficacy of purification of alginate. Mater 7(3):2087-2103. doi:10.3390/ma7032087.

68. Langlois G, Dusseault J, Bilodeau S et al (2009) Direct effect of alginate purification on the survival of islets immobilized in alginate-based microcapsules. Acta Biomater 5(9):3433-3440. doi:10.1016/j.actbio.2009.05.029.

69. Bhujbal SV, de Vos P, Niclou SP (2014) Drug and cell encapsulation: alternative delivery options for the treatment of malignant brain tumors. Adv Drug Deliv Rev 67-68:142-153. doi:10.1016/j.addr.2014.01.010.

70. Swioklo S, Ding P, Pacek AW et al Process parameters for the high-scale production of alginate-encapsulated stem cells for storage and distribution throughout the cell therapy supply chain. Process Biochemistry. doi:10.1016/j.procbio.2016.06.005.

71. Richardson T, Kumta PN, Banerjee I (2014) Alginate encapsulation of human embryonic stem cells to enhance directed differentiation to pancreatic islet-like cells. Tissue Eng Part A 20(23-24):3198-3211. doi:10.1089/ten.TEA.2013.0659.

72. Erro E, Bundy J, Massie I et al (2013) Bioengineering the liver: scale-up and cool chain delivery of the liver cell biomass for clinical targeting in a bioartificial liver support system. Biores Open Access 2(1):1-11. doi:10.1089/biores.2012.0286.

73. Formo K, Cho CH, Vallier L et al (2015) Culture of hESC-derived pancreatic progenitors in alginate-based scaffolds. J Biomed Mater Res A 103(12):3717-3726. doi:10.1002/jbm.a.35507.

74. de Vos P, Bucko M, Gemeiner P et al (2009) Multiscale requirements for bioencapsulation in medicine and biotechnology. Biomaterials 30(13):2559-2570. doi:10.1016/j.biomaterials.2009.01.014.

75. Zhu J, Marchant RE (2011) Design properties of hydrogel tissue-engineering scaffolds. Expert Rev Med Devices 8(5):607-626. doi:10.1586/erd.11.27.

76. Gauvin R, Parenteau-Bareil R, Dokmeci MR et al (2012) Hydrogels and microtechnologies for engineering the cellular microenvironment. Wiley Interdiscip Rev Nanomed Nanobiotechnol 4(3):235-246. doi:10.1002/wnan.171.

77. Borg DJ, Bonifacio E (2011) The use of biomaterials in islet transplantation. Curr Diab Rep 11(5):434-444. doi:10.1007/s11892-011-0210-2.

78. Barkai U, Rotem A, de Vos P (2016) Survival of encapsulated islets: More than a membrane story. World J Transplant 6(1):69-90. doi:10.5500/wjt.v6.i1.69.

79. Jacobs-Tulleneers-Thevissen D, Chintinne M, Ling Z et al (2013) Sustained function of alginate-encapsulated human islet cell implants in the peritoneal cavity of mice leading to a pilot study in a type 1 diabetic patient. Diabetologia 56(7):1605-1614. doi:10.1007/s00125-013-2906-0.

80. Kerby A, Jones ES, Jones PM et al (2013) Co-transplantation of islets with mesenchymal stem cells in microcapsules demonstrates graft outcome can be improved in an isolated-graft model of islet transplantation in mice. Cytotherapy 15(2):192-200. doi:10.1016/j.jcyt.2012.10.018.

81. Figliuzzi M, Bonandrini B, Silvani S et al (2014) Mesenchymal stem cells help pancreatic islet transplantation to control type 1 diabetes. World J Stem Cells 6(2):163-172. doi:10.4252/wjsc.v6.i2.163.

82. Iacovacci V, Ricotti L, Menciassi A et al (2016) The bioartificial pancreas (BAP): Biological, chemical and engineering challenges. Biochem Pharmacol 100:12-27. doi:10.1016/j.bcp.2015.08.107.

83. Montanucci P, Pennoni I, Pescara T et al (2013) Treatment of diabetes mellitus with microencapsulated fetal human liver (FH-B-TPN) engineered cells. Biomaterials 34(16):4002-4012. doi:10.1016/j.biomaterials.2013.02.026.

84. Tomei AA, Villa C, Ricordi C (2015) Development of an encapsulated stem cell-based therapy for diabetes. Expert Opin Biol Ther 15(9):1321-1336. doi:10.1517/14712598.2015.1055242.

85. Ngoc PK, Phuc PV, Nhung TH et al (2011) Improving the efficacy of type 1 diabetes therapy by transplantation of immunoisolated insulin-producing cells. Hum Cell 24(2):86-95. doi:10.1007/s13577-011-0018-z.

86. Richardson T, Barner S, Candiello J et al (2016) Capsule stiffness regulates the efficiency of pancreatic differentiation of human embryonic stem cells. Acta Biomater 35:153-165. doi:10.1016/j.actbio.2016.02.025.

87. Murua A, Orive G, Hernandez RM et al (2011) Emerging technologies in the delivery of erythropoietin for therapeutics. Med Res Rev 31(2):284-309. doi:10.1002/med.20184.

88. Orive G, De Castro M, Kong HJ et al (2009) Bioactive cell-hydrogel microcapsules for cell-based drug delivery. J Control Release 135(3):203-210. doi:10.1016/j.jconrel.2009.01.005.

89. Orive G, Santos E, Pedraz JL et al (2014) Application of cell encapsulation for controlled delivery of biological therapeutics. Adv Drug Deliv Rev 67-68:3-14. doi:10.1016/j.addr.2013.07.009.

90. Acarregui A, Pedraz JL, Blanco FJ et al (2013) Hydrogel-based scaffolds for enclosing encapsulated therapeutic cells. Biomacromolecules 14(2):322-330. doi:10.1021/bm301690a.

91. Hashemi M, Kalalinia F (2015) Application of encapsulation technology in stem cell therapy. Life Sci 143:139-146. doi:10.1016/j.lfs.2015.11.007.

92. Klinge PM, Harmening K, Miller MC et al (2011) Encapsulated native and glucagon-like peptide-1 transfected human mesenchymal stem cells in a transgenic mouse model of Alzheimer's disease. Neurosci Lett 497(1):6-10. doi:10.1016/j.neulet.2011.03.092.

93. Knippenberg S, Thau N, Dengler R et al (2012) Intracerebroventricular injection of encapsulated human mesenchymal cells producing glucagon-like peptide 1 prolongs survival in a mouse model of ALS. PLoS One 7(6):e36857. doi:10.1371/journal.pone.0036857.

94. Wright EJ, Farrell KA, Malik N et al (2012) Encapsulated glucagon-like peptide-1-producing mesenchymal stem cells have a beneficial effect on failing pig hearts. Stem Cells Transl Med 1(10):759-769. doi:10.5966/sctm.2012-0064.

95. Heile AMB, Wallrapp C, Klinge PM et al (2009) Cerebral transplantation of encapsulated mesenchymal stem cells improves cellular pathology after experimental traumatic brain injury. Neurosci Lett 463(3):176-181. doi:10.1016/j.neulet.2009.07.071.

96. Skinner SJM, Geaney MS, Lin H et al (2009) Encapsulated living choroid plexus cells: potential long-term treatments for central nervous system disease and trauma. - Journal of Neural Engineering(6):065001. doi:10.1088/1741-2560/6/6/065001.

97. Huang SL, Wang J, He XJ et al (2014) Secretion of BDNF and GDNF from free and encapsulated choroid plexus epithelial cells. Neurosci Lett 566:42-45. doi:10.1016/j.neulet.2014.02.017.

98. Aliaghaei A, Digaleh H, Khodagholi F et al (2015) Encapsulated Choroid Plexus Epithelial Cells Actively Protect Against Intrahippocampal Abeta-induced Long-Term Memory Dysfunction; Upregulation of Effective Neurogenesis with the Abrogated Apoptosis and Neuroinflammation. J Mol Neurosci 56(3):708-721. doi:10.1007/s12031-015-0492-y.

99. Pettingill LN, Wise AK, Geaney MS et al (2011) Enhanced auditory neuron survival following cell-based BDNF treatment in the deaf guinea pig. PLoS One 6(4):e18733. doi:10.1371/journal.pone.0018733.

100. Gillespie LN, Zanin MP, Shepherd RK (2015) Cell-based neurotrophin treatment supports long-term auditory neuron survival in the deaf guinea pig. J Control Release 198:26-34. doi:10.1016/j.jconrel.2014.11.026.

101. Lindvall O, Wahlberg LU (2008) Encapsulated cell biodelivery of GDNF: a novel clinical strategy for neuroprotection and neuroregeneration in Parkinson's disease?. Exp Neurol 209(1):82-88. doi:10.1016/j.expneurol.2007.08.019.

102. Grandoso L, Ponce S, Manuel I et al (2007) Long-term survival of encapsulated GDNF secreting cells implanted within the striatum of parkinsonized rats. Int J Pharm 343(1-2):69-78. doi:10.1016/j.ijpharm.2007.05.027.

103. Date I, Ohmoto T, Imaoka T et al (1996) Cografting with polymer-encapsulated human nerve growth factor-secreting cells and chromaffin cell survival and behavioral recovery in hemiparkinsonian rats. J Neurosurg 84(6):1006-1012. doi:10.3171/jns.1996.84.6.1006.

104. Date I, Ohmoto T, Imaoka T et al (1996) Chromaffin cell survival from both young and old donors is enhanced by co-grafts of polymer-encapsulated human NGF-secreting cells. Neuroreport 7(11):1813-1818.

105. Emerich DF, Orive G, Thanos C et al (2014) Encapsulated cell therapy for neurodegenerative diseases: from promise to product. Adv Drug Deliv Rev 67-68:131-141. doi:10.1016/j.addr.2013.07.008.

106. Spuch C, Antequera D, Portero A et al (2010) The effect of encapsulated VEGF-secreting cells on brain amyloid load and behavioral impairment in a mouse model of Alzheimer's disease. Biomaterials 31(21):5608-5618. doi:10.1016/j.biomaterials.2010.03.042.

107. Antequera D, Portero A, Bolos M et al (2012) Encapsulated VEGF-secreting cells enhance proliferation of neuronal progenitors in the hippocampus of AbetaPP/Ps1 mice. J Alzheimers Dis 29(1):187-200. doi:10.3233/JAD-2011-111646.

108. Shen Y, Qiao H, Fan Q et al (2015) Potentiated Osteoinductivity via Cotransfection with BMP-2 and VEGF Genes in Microencapsulated C2C12 Cells. Biomed Res Int 2015:435253. doi:10.1155/2015/435253.

109. Han YF, Han YQ, Pan YG et al (2010) Transplantation of microencapsulated cells expressing VEGF improves angiogenesis in implanted xenogeneic acellular dermis on wound. Transplant Proc 42(5):1935-1943. doi:10.1016/j.transproceed.2009.12.070.

110. Chen W, Yang D, Wang P et al (2011) Microencapsulated myoblasts transduced by the vascular endothelial growth factor (VEGF) gene for the ischemic skin flap. Aesthetic Plast Surg 35(3):326-332. doi:10.1007/s00266-010-9610-y.

111. Kim C, Chung S, Yuchun L et al (2012) In vitro angiogenesis assay for the study of cell-encapsulation therapy. Lab Chip 12(16):2942-2950. doi:10.1039/c2lc40182g.

112. Selimoglu SM, Elibol M (2010) Alginate as an immobilization material for MAb production via encapsulated hybridoma cells. Crit Rev Biotechnol 30(2):145-159. doi:10.3109/07388550903451652.

113. Dubrot J, Portero A, Orive G et al (2010) Delivery of immunostimulatory monoclonal antibodies by encapsulated hybridoma cells. Cancer Immunol Immunother 59(11):1621-1631. doi:10.1007/s00262-010-0888-z.

114. Saenz del Burgo L, Compte M, Aceves M et al (2015) Microencapsulation of therapeutic bispecific antibodies producing cells: immunotherapeutic organoids for cancer management. J Drug Target 23(2):170-179. doi:10.3109/1061186X.2014.971327.

115. Read TA, Sorensen DR, Mahesparan R et al (2001) Local endostatin treatment of gliomas administered by microencapsulated producer cells. Nat Biotechnol 19(1):29-34. doi:10.1038/83471.

116. Kleinschmidt K, Klinge PM, Stopa E et al (2011) Alginate encapsulated human mesenchymal stem cells suppress syngeneic glioma growth in the immunocompetent rat. J Microencapsul 28(7):621-627. doi:10.3109/02652048.2011.599441.

117. Goren A, Dahan N, Goren E et al (2010) Encapsulated human mesenchymal stem cells: a unique hypoimmunogenic platform for long-term cellular therapy. FASEB J 24(1):22-31. doi:10.1096/fj.09-131888.

118. Shah K (2013) Encapsulated stem cells for cancer therapy. Biomatter 3(1):10.4161/biom.24278. Epub 2013 Jan 1. doi:10.4161/biom.24278.

119. Soon-Shiong P, Heintz RE, Merideth N et al (1994) Insulin independence in a type 1 diabetic patient after encapsulated islet transplantation. Lancet 343(8903):950-951.

120. Calafiore R, Basta G, Luca G et al (2006) Microencapsulated pancreatic islet allografts into nonimmunosuppressed patients with type 1 diabetes: first two cases. Diabetes Care 29(1):137-138. doi:10.2337/diacare.29.01.06.dc05-1270.

121. Basta G, Montanucci P, Luca G et al (2011) Long-term metabolic and immunological follow-up of nonimmunosuppressed patients with type 1 diabetes treated with microencapsulated islet allografts: four cases. Diabetes Care 34(11):2406-2409. doi:10.2337/dc11-0731.

122. Tuch BE, Keogh GW, Williams LJ et al (2009) Safety and viability of microencapsulated human islets transplanted into diabetic humans. Diabetes Care 32(10):1887-1889. doi:10.2337/dc09-0744.

123. A Phase I/II Study of the Safety and Efficacy of Sernova's Cell PouchTM for Therapeutic Islet Transplantation. Available via https://clinicaltrials.gov/ct2/show/NCT01652911. Accessed November 2016.

124. Elliott RB, Escobar L, Tan PL et al (2007) Live encapsulated porcine islets from a type 1 diabetic patient 9.5 yr after xenotransplantation. Xenotransplantation 14(2):157-161. doi:10.1111/j.1399-3089.2007.00384.x.

125. Living Cell Technologies: DIABECELL®. Available via http://www.lctglobal.com/products/diabecell/development-to-date. Accessed November 2016.

126. Open-label Investigation of the Safety and Effectiveness of DIABECEL® in Patients With Type I Diabetes Mellitus. Available via https://clinicaltrials.gov/ct2/show/NCT00940173. Accessed November 2016.

127. Open-label Investigation of the Safety and Effectiveness of DIABECELL® in Patients With Type 1 Diabetes Mellitus. Available via https://clinicaltrials.gov/ct2/show/NCT01739829. Accessed November 2016.

128. Open-label Investigation if the Safety and Efficacy of DIABECELL® in Patients With Type 1 Diabetes Mellitus. Available via https://clinicaltrials.gov/ct2/show/NCT01736228. Accessed November 2016.

129. GLP-1 CellBeads® for the Treatment of Stroke Patients With Space-occupying Intrecerebral Hemorrhage. Available via https://clinicaltrials.gov/ct2/show/NCT01298830. Accessed November 2016.

130. Heile A, Brinker T (2011) Clinical translation of stem cell therapy in traumatic brain injury: the potential of encapsulated mesenchymal cell biodelivery of glucagon-like peptide-1. Dialogues Clin Neurosci 13(3):279-286.

131. Luo XM, Lin H, Wang W et al (2013) Recovery of neurological functions in non-human primate model of Parkinson's disease by transplantation of encapsulated neonatal porcine choroid plexus cells. J Parkinsons Dis 3(3):275-291. doi:10.3233/JPD-130214.

132. Open-label Investigation of the Safety and Clinical Effects of NTCELL® in Patients With Parkinson's Disease. Available via https://clinicaltrials.gov/ct2/show/NCT01734733. Accessed November 2016.

133. Investigation of the Safety and Efficacy of NTCELL® [Immunoprotected (Alginate-Encapsulated) Porcine Choroid Plexus Cells for Xenotransplantation] in Patients With Parkinson's Disease. Available via https://clinicaltrials.gov/ct2/show/NCT02683629. Accessed November 2016.

134. Living Cell Technoogies: NTCELL®. Available via http://www.lctglobal.com/products/ntcell/development-to-date. Accessed November 2016.

135. Santos E, Orive G, Hernandez R,M. et al (2011) Cell-Biomaterial Interaction: Strategies To Mimic The Extracellular Matrix. In: Pramatarova L (ed) On Biomimetics. InTech, Rijeka.

136. Cruz-Acuna R, Garcia AJ (2016) Synthetic hydrogels mimicking basement membrane matrices to promote cell-matrix interactions. Matrix Biol. doi:10.1016/j.matbio.2016.06.002.

137. Dalheim MO, Vanacker J, Najmi MA et al (2016) Efficient functionalization of alginate biomaterials. Biomaterials 80:146-156. doi:10.1016/j.biomaterials.2015.11.043.

138. Marturano JE, Schiele NR, Schiller ZA et al (2016) Embryonically inspired scaffolds regulate tenogenically differentiating cells. J Biomech 49(14):3281-3288. doi:10.1016/j.jbiomech.2016.08.011.

139. Garate A, Ciriza J, Casado JG et al (2015) Assessment of the Behavior of Mesenchymal Stem Cells Immobilized in Biomimetic Alginate Microcapsules. Mol Pharm 12(11):3953-3962. doi:10.1021/acs.molpharmaceut.5b0041.

140. Garate A, Santos E, Pedraz JL et al (2015) Evaluation of different RGD ligand densities in the development of cell-based drug delivery systems. J Drug Target 23(9):806-812. doi:10.3109/1061186X.2015.1020428.

141. Santos E, Garate A, Pedraz JL et al (2014) The synergistic effects of the RGD density and the microenvironment on the behavior of encapsulated cells: in vitro and in vivo direct comparative study. J Biomed Mater Res A 102(11):3965-3972. doi:10.1002/jbm.a.35073.

142. Llacua A, de Haan BJ, Smink SA et al (2016) Extracellular matrix components supporting human islet function in alginate-based immunoprotective microcapsules for treatment of diabetes. J Biomed Mater Res A 104(7):1788-1796. doi:10.1002/jbm.a.35706.

143. Mazzitelli S, Luca G, Mancuso F et al (2011) Production and characterization of engineered alginate-based microparticles containing ECM powder for cell/tissue engineering applications. Acta Biomater 7(3):1050-1062. doi:10.1016/j.actbio.2010.10.005.

144. Lou R, Xie H, Zheng H et al (2016) Alginate-based microcapsules with galactosylated chitosan internal for primary hepatocyte applications. Int J Biol Macromol 93(Pt A):1133-1140. doi:10.1016/j.ijbiomac.2016.09.078.

145. Jeon O, Powell C, Solorio LD et al (2011) Affinity-based growth factor delivery using biodegradable, photocrosslinked heparin-alginate hydrogels. J Control Release 154(3):258-266. doi:10.1016/j.jconrel.2011.06.027.

146. Dang TT, Thai AV, Cohen J et al (2013) Enhanced function of immuno-isolated islets in diabetes therapy by co-encapsulation with an anti-inflammatory drug. Biomaterials 34(23):5792-5801. doi:10.1016/j.biomaterials.2013.04.016.

147. Murua A, Herran E, Orive G et al (2011) Design of a composite drug delivery system to prolong functionality of cell-based scaffolds. Int J Pharm 407(1-2):142-150. doi:10.1016/j.ijpharm.2010.11.022.

148. Acarregui A, Herran E, Igartua M et al (2014) Multifunctional hydrogel-based scaffold for improving the functionality of encapsulated therapeutic cells and reducing inflammatory response. Acta Biomater 10(10):4206-4216. doi:10.1016/j.actbio.2014.06.038.

149. Azadi SA, Vasheghani-Farahani E, Hashemi-Najafbabadi S et al (2016) Co-encapsulation of pancreatic islets and pentoxifylline in alginate-based microcapsules with enhanced immunosuppressive effects. Prog Biomater 5:101-109. doi:10.1007/s40204-016-0049-3.

150. Jo EH, Hwang YH, Lee DY (2015) Encapsulation of pancreatic islet with HMGB1 fragment for attenuating inflammation. Biomater Res 19:21-015-0042-2. eCollection 2015. doi:10.1186/s40824-015-0042-2.

151. Arlov O, Skjak-Braek G, Rokstad AM (2016) Sulfated alginate microspheres associate with factor H and dampen the inflammatory cytokine response. Acta Biomater 42:180-188. doi:10.1016/j.actbio.2016.06.015.

152. Zanotti L, Sarukhan A, Dander E et al (2013) Encapsulated mesenchymal stem cells for in vivo immunomodulation. Leukemia 27(2):500-503. doi:10.1038/leu.2012.202.

153. Alunno A, Montanucci P, Bistoni O et al (2015) In vitro immunomodulatory effects of microencapsulated umbilical cord Wharton jelly-derived mesenchymal stem cells in primary Sjogren's syndrome. Rheumatology (Oxford) 54(1):163-168. doi:10.1093/rheumatology/keu292.

154. English K (2013) Mechanisms of mesenchymal stromal cell immunomodulation. Immunol Cell Biol 91(1):19-26. doi:10.1038/icb.2012.56.

155. Vaithilingam V, Evans MD, Rowe A et al (2016) Coencapsulation of Target Effector Cells With Mesenchymal Stem Cells Reduces Pericapsular Fibrosis and Improves Graft Survival in a Xenotransplanted Animal Model. Cell Transplant 25(7):1299-1317. doi:10.3727/096368915X688975.

156. Stucky EC, Schloss RS, Yarmush ML et al (2015) Alginate micro-encapsulation of mesenchymal stromal cells enhances modulation of the neuro-inflammatory response. Cytotherapy 17(10):1353-1364. doi:10.1016/j.jcyt.2015.05.002.

157. Luca G, Mancuso F, Calvitti M et al (2015) Long-term stability, functional competence, and safety of microencapsulated specific pathogen-free neonatal porcine Sertoli cells: a potential product for cell transplant therapy. Xenotransplantation 22(4):273-283. doi:10.1111/xen.12175.

158. Luca G, Bellezza I, Arato I et al (2016) Terapeutic Potential of Microencapsulated Sertoli Cells in Huntington Disease. CNS Neurosci Ther 22(8):686-690. doi:10.1111/cns.12569.

159. Arifin DR, Manek S, Call E et al (2012) Microcapsules with intrinsic barium radiopacity for immunoprotection and X-ray/CT imaging of pancreatic islet cells. Biomaterials 33(18):4681-4689. doi:10.1016/j.biomaterials.2012.03.008.

160. Qie F, Astolfo A, Wickramaratna M et al (2015) Self-assembled gold coating enhances X-ray imaging of alginate microcapsules. Nanoscale 7(6):2480-2488. doi:10.1039/c4nr06692h.

161. Yang F, Zhang X, Maiseyeu A et al (2012) The prolonged survival of fibroblasts with forced lipid catabolism in visceral fat following encapsulation in alginate-poly-L-lysine. Biomaterials 33(22):5638-5649. doi:10.1016/j.biomaterials.2012.04.035.

162. Barnett BP, Arepally A, Stuber M et al (2011) Synthesis of magnetic resonance-, X-ray- and ultrasound-visible alginate microcapsules for immunoisolation and noninvasive imaging of cellular therapeutics. Nat Protoc 6(8):1142-1151. doi:10.1038/nprot.2011.352.

163. Catena R, Santos E, Orive G et al (2010) Improvement of the monitoring and biosafety of encapsulated cells using the SFGNESTGL triple reporter system. J Control Release 146(1):93-98. doi:10.1016/j.jconrel.2010.05.018.

164. Allen AB, Gazit Z, Su S et al (2014) In vivo bioluminescent tracking of mesenchymal stem cells within large hydrogel constructs. Tissue Eng Part C Methods 20(10):806-816. doi:10.1089/ten.TEC.2013.0587.

165. Santos E, Larzabal L, Calvo A et al (2013) Inactivation of encapsulated cells and their therapeutic effects by means of TGL triple-fusion reporter/biosafety gene. Biomaterials 34(4):1442-1451. doi:10.1016/j.biomaterials.2012.10.076.

166. Chan KW, Liu G, van Zijl PC et al (2014) Magnetization transfer contrast MRI for non-invasive assessment of innate and adaptive immune responses against alginate-encapsulated cells. Biomaterials 35(27):7811-7818. doi:10.1016/j.biomaterials.2014.05.057.

167. Krishnan R, Arora RP, Alexander M et al (2014) Noninvasive evaluation of the vascular response to transplantation of alginate encapsulated islets using the dorsal skin-fold model. Biomaterials 35(3):891-898. doi:10.1016/j.biomaterials.2013.10.012.

168. Chang HK, Kim PH, Cho HM et al (2016) Inducible HGF-secreting Human Umbilical Cord Blood-derived MSCs Produced via TALEN-mediated Genome Editing Promoted Angiogenesis. Mol Ther 24(9):1644-1654. doi:10.1038/mt.2016.120.

169. Wang P, Song Y, Weir MD et al (2016) A self-setting iPSMSC-alginate-calcium phosphate paste for bone tissue engineering. Dent Mater 32(2):252-263. doi:10.1016/j.dental.2015.11.019.

170. Zhao L, Weir MD, Xu HH (2010) An injectable calcium phosphate-alginate hydrogel-umbilical cord mesenchymal stem cell paste for bone tissue engineering. Biomaterials 31(25):6502-6510. doi:10.1016/j.biomaterials.2010.05.017.

171. Barkai U, Weir GC, Colton CK et al (2013) Enhanced oxygen supply improves islet viability in a new bioartificial pancreas. Cell Transplant 22(8):1463-1476. doi:10.3727/096368912X657341.

172. Neufeld T, Ludwig B, Barkai U et al (2013) The efficacy of an immunoisolating membrane system for islet xenotransplantation in minipigs. PLoS One 8(8):e70150. doi:10.1371/journal.pone.0070150.

173. Veiseh O, Doloff JC, Ma M et al (2015) Size- and shape-dependent foreign body immune response to materials implanted in rodents and non-human primates. Nat Mater 14(6):643-651. doi:10.1038/nmat4290.