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STUDY OF HUMAN DENTAL PULP STEM CELLS (hDPSCs) COMBINED WITH TITANIUM, DECELLULARIZED ADIPOSE TISSUE AND PLASMA DERIVED PRODUCTS ON OSTEOINDUCTION FOR BONE TISSUE ENGINEERING

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Abstract

Bone tissue engineering is a multidisciplinary and relatively new field that consists on the application of engineering and life science to develop biological substitutes to maintain, restore or improve damaged or lost tissue function. The main three pillars of tissue engineering are scaffolds, stem cells and growth factors. With all the technological advances in the last decades, due to new materials and surface modifications, scaffolds for bone tissue engineering and dental implantology have upgraded a lot. These upgrades have demonstrated a good osteoblastic differentiation induction of different MSCs. Moreover, MSCs showed good features for the use in bone tissue engineering such as high proliferation and differentiation potential. However, the reason which MSC cell type is the best option for bone regeneration therapies is still unclear. The main problem of the use of stem cells in autologous cell therapies is the widespread use of animal origin serums like fetal bovine serum (FBS) for *in vitro* cell cultures. To inactivate the immune response of the cell-grafted patients these serums should be replaced.

In the present work, we studied the human Dental Pulp Stem Cells (hDPSCs) adherence, proliferation, viability and osteo-differentiation potential when cultured on widely used Ti6AL4V titanium surface and a new biomimetic porous surface (BAS^{TM}), in the presence or absence of osteoblastic differentiation media supplemented with plasma rich in growth factors (PRGF) and platelet rich fibrin (PRF).

The results showed that hDPSCs showed a good adherence ability and non affected viability and proliferation when cultured on Ti6AL4V and BAS titanium surfaces. Moreover, both titanium surfaces demonstrated having osteoblastic differentiation induction effect on hDPSCs without using osteoblastic differentiation media. Besides, the two plasma derived products showed interesting results. On one hand, the PRGF induced higher cell proliferation on *in vitro* hDPSC cultures becoming a good FBS substitute for the use on autologous cell therapies. On the other hand, PRF enhanced osteoblastic cell differentiation of hDPSCs with higher calcified bone matrix production. Finally, the combination of PRF with BAS titanium surface maximized hDPSCs osteoblastic differentiation to bone-producing cells. The results obtained on this work provides experimental support to the commonly used fibrin clots on clinical practice to enhance bone production around dental implants.

Once we studied the effects of these two titanium surfaces on hDPSCs, we performed a comparative study of viability, proliferation and osteoblastic differentiation potential between the most promising mesenchymal stem cells for bone regeneration therapies, hDPSCs and hBMSCs. The results suggested a higher proliferation and mineralized bone matrix production of hDPSCs compared to hBMSCs. Even more data is needed to confirm these results, thanks to easier and less invasive isolation, proliferation and differentiation differences mentioned, hDPSCs could be a better option than hBMSCs for bone regeneration therapies.

Lastly, another problem in dental implantology is the loss of periodontal ligament. The function of this tissue is to act like a cushion to absorb part of the masticatory mechanical forces to protect the alveolar bone. Nowadays, the periodontal regenerative therapy is based on the use of barrier membranes in alveolar ridge defects to enhance the bone growth on the surrounding affected area before the placement of dental implants. For this purpose, we studied the porcine decellularized adipose tissue (pDAT). It demonstrated being a good support for the adhesion and viability of hDPSCs. In addition, hDPSCs cultured on pDAT were able to differentiate showing the production of intramembraneous ossification and formation of Sharpey fibre-like attachment structures. Considering the available and abundant human derived adipose tissue material source and that both, DAT and hDPSCs, can be obtained from the patients themselves, their combination for personalized clinical therapies seems to be a great opportunity for bone regeneration therapies and dental implantology.

Keywords: dental pulp stem cells, DPSC, bone marrow stem cells, BMSC, tissue engineering, scaffold, cell differentiation, plasma-derived products, PRGF, PRF, titanium, dezellularized adipose tissue, regenerative medicine.

Abbreviations

ALP= Alkaline phosphatase

αMEM= α minimal essential medium

AMTP= Advanced medical therapy products

APC= Allophycocyanin

ARC= Adventitial reticular cell

ARS= Alizarin red

α-SMA= α-smooth muscle actin

AT-MSC= Adipose tissue MSC

BAS= Biomimetic advanced surface

BDNF= Brain derived neurotrophic factor

BGLAP= osteocalcin

BMPxxx= Bone morphogenetic protein xxx

BMSC= Bone marrow stem cell

BSA= Bovine serum albumin

CDxxx= Cluster differentiation

CFU-F= Colony forming unit fibroblasts

CMAP= The Connectivity Map

DAPI= 4´,6-diamino-2-phenilindol

DAT= Decellularized adipose tissue

DFSC= Dental follicle stem cell

DME= β-mercaptoethanol

DMEM= Dubbelco´s modified eagle´s medium

DPSC= Dental pulp stem cell

DSP= Dentin sialoprotein

DSPP= Dentin sialophosphoprotein

ECC= Embryonal carcinoma cell

EDTA= Ethylenediamine tetraacetic acid

EGF= Epidermal growth factor

EMD= Enamel matrix derivate

ESC= Embryonic stem cell

FBS= Fetal bovine serum

FGF= Fibroblast growth factor

FITC= fluorescein isothiocyanate

GDNF: Glial cell line derived neurotrophic factor

GOBP= Gene Ontology Biological Process

HA= Hydroxyapatite

hBMSC= Human BMSC

HBSS= Hank´s balanced salt solution

hDPSC= Human DPSC

HGF= Hepatocyte growth factor

HSC= Hematopoietic stem cell

IBMX= 3-isobutyl-1-methylxantine

ICM= Inner cell mass of the blastocyst

IL= Interleukins

iPSC= Induced pluripotent stem cell

ISTC= International society for cellular therapy

ITSx= Insulin-transferrin-selenium-x

mESC= Mouse ESC

MNC= Marrow mononuclear cell

MSC= Mesenchymal stem cells

NBT/BCIP= 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium

NeuN= Neuronal nuclei protein

NGF= Nerve growth factor

NSE= Neuron specific enolase

OSTERIX/SP7= Transcription factor 7

PBS= Phosphate buffered saline

pDAT= Porcine DAT

PDGF= Platelet derived growth factor

PDL= Periodontal ligament

PDLSC= Periodontal ligament stem cell

PE= phycoerytrin

PFA= Paraformaldehide

PGA= Poly-glycolic acid

PLA= Poly-lactic acid

PRF= Plasma rich fibrin

PRGF= Plasma rich growth factor

PRP= Plasma rich plasma

RA= Retinoic acid

RUNX2= Runt-related transcriptional factor 2

SCAP= Stem cell from apical papilla

SEM= Scanning electron microscopy

SHED= Exfoliated deciduous teeth stem cell

SHxxx= Src homology xxx

SPARC= osteonectin

SPB= Sorensen phosphate buffer

SSEA-1= Stage specific embryo antigen 1

TEM= Transmission electron microscopy

TCP= Tricalcium phosphate

TGF-β= Transforming growth factor β

V-CAM 1= Vascular cell adhesion protein 1

VEGF= vascular endothelial growth factor

Introduction

Introduction to tissue engineering

The term "Tissue engineering" was created in 1988 by the National Science Foundation workshop as "the application of principles and methods of engineering and life sciences toward the fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain or improve tissue function". Tissue engineering is a multidisciplinary and relatively new field, which interconnects different disciplines as clinical medicine, material science, mechanical engineering and genetics (Berthiaume et al., 2011). Tissue engineering strives for functional restoration of lost or damaged tissues by seeding cells and growth factors on three-dimensional scaffolds (Chaudhari et al., 2016), conforming what is known as "tissue-engineering triad", which can be set up in a bioreactor in a controlled environment (Dlaska et al., 2015; O'Brien, 2011).

The main objective of tissue engineering is to restore, maintain, or improve damaged tissues or whole organs. In the case of bone tissue, the natural remodeling, regeneration and self-repairing that this tissue presents are big advantages. One of the aims of new material investigation is to create biocompatible materials, which can supply regenerative signals to the cells. Moreover, the tissue engineering field is also focused on finding biomimetic materials with similar properties to the natural environment improving proliferation, adhesion and differentiation (Dhandayuthapani et al., 2011).

After blood transfusion, bone transplantation is the most common tissue transplantation type, and due to the aging of the population, the demand is increasing considerably (Kattimani et al., 2016). Bone tissue engineering field has set his eye on stem cells connected with osteoblasts for future therapies (Stevens, 2008).

Taking into account bone tissue engineering criteria, craniofacial tissue engineering goes forward by trying to develop biomaterials for the regeneration of dental and oral tissues, like bone, salivary glands, periodontal ligaments, mucosa, cementum and dentin (Rahman et al., 2018).

Particularly, periodontal regenerative therapy has a well-reported guided bone/tissue regeneration technique, also named as "membrane protected bone

regeneration". This technique is based on the use of barrier membranes in alveolar ridge defects to enhance the bone growth on the surrounding affected area of dental implants. The alveolar bone growth had to be enhanced first to give local mechanical stability for the correct bone-implant interface creation (Pilipchuk et al., 2015).

Summarizing, the three pillars on which tissue engineering is sustained are stem cells, scaffolds and growth factors (figure 1).

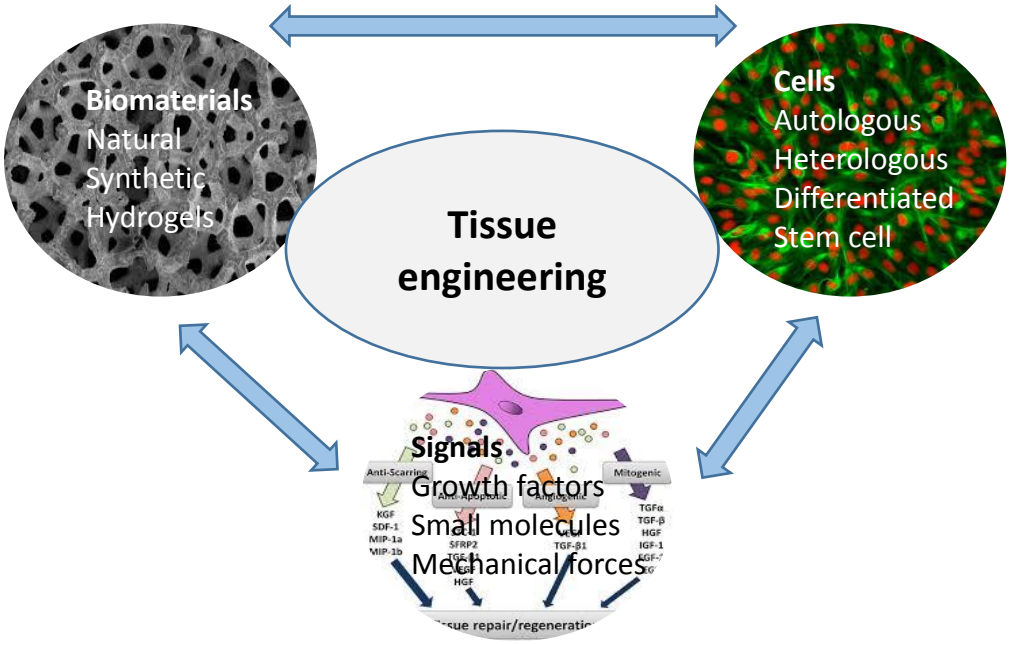


Figure 1. The tissue engineering triad. A combination of cells cultured on a biomaterial scaffold with appropriate biophysical and chemical signals coordinate to regenerate the desired tissue.

1. Stem cells

Stem cells must meet, to be defined as such, three main characteristics. Clonality, the ability to self-renew, and the potential to differentiate into mature cells of different tissues and organs (Potten and Loeffler, 1990).

The first described stem cells were obtained by the isolation of the embryonal carcinoma cells (ECCs) from teratocarcinomas in 1954 (Stevens and Little, 1954). Ten years later, the two main characteristics of these cells were described: their self-renewal ability and their capacity to differentiate to the three different germ layers *in vitro*, describing them as pluripotent stem cells (Kleinsmith and Pierce, 1964). In 1970, they

were also described on *in vivo* models (Kahan and Ephrussi, 1970). Few years later, in 1981, they isolated the mouse embryonic stem cells (mESCs) from the inner cell mass of the blastocyst (ICM) and in 1998, the human ESCs (Martin, 1980; Thomson et al., 1998). In recent years, a new concept of stem cells was born, induced pluripotent stem cells (iPSCs). In 2006, it was firstly described on mouse cells and in 2007 on human cells (Takahashi et al., 2007; Takahashi and Yamanaka, 2006).

Depending on the developmental stage, the stem cells show different differentiation potentials. According to this, they can be categorized into five groups:

Totipotent: These types of stem cells have the potential to create an entire individual. They are able to form an entire embryo and the temporary support tissues (umbilical cord and placenta). This potential lasts until the blastomeric stage (Posfai et al., 2021).

Pluripotent: After the blastocyst is formed, the pluripotent stem cells forming the IMC are able to differentiate into the three germ layers. These stem cells are capable to generate all the adult tissues and organs but not the temporary support tissues (Smith, 2001).

Multipotent: The differentiation potential of these stem cells is restricted to their original organ or tissue, being their main function the reparation and maintenance of adult tissues (Slack, 2000).

Oligopotent: These term refers to the stem cells which can only differentiate to very few and restricted types of cells like the myeloid and lymphoid cells (Majo et al., 2008).

Unipotent: The unipotent stem cells are able only to produce one type of cell. They are considered stem cells because of their self-renewal capacity, like muscular stem cells (Seale et al., 2001).

1.1. Adult stem cells

When the blastocyst is developed, the ESCs from the ICM create the three germ layers: endoderm, mesoderm and ectoderm. After the tissue and organ formation, some stem cells are retained without a terminal differentiation as in the bone marrow, blood, liver, skin, brain, teeth, bone or muscle (Denham et al., 2005; Vats et al., 2005).

The plasticity of these stem cells can vary from the potential to differentiate to many different cell types (multipotent) to being able only to differentiate to a single type of cell (unipotent) (Almeida-Porada et al., 2001; Wagers and Weissman, 2004). Besides, the proliferation rate of these cells varies from tissue to tissue. The highest proliferative stem cells can be found in high cell turnover tissues as bone marrow, skin or intestine (Baulies et al., 2020; Lenkiewicz, 2019; Yadav et al., 2020). On the other hand, other stem cells will only proliferate to replace damaged cells or in a response to an injury such as in the liver, heart or the nervous system (Angelini et al., 2004; Duncan et al., 2009; Mansergh et al., 2000) (figure 2).

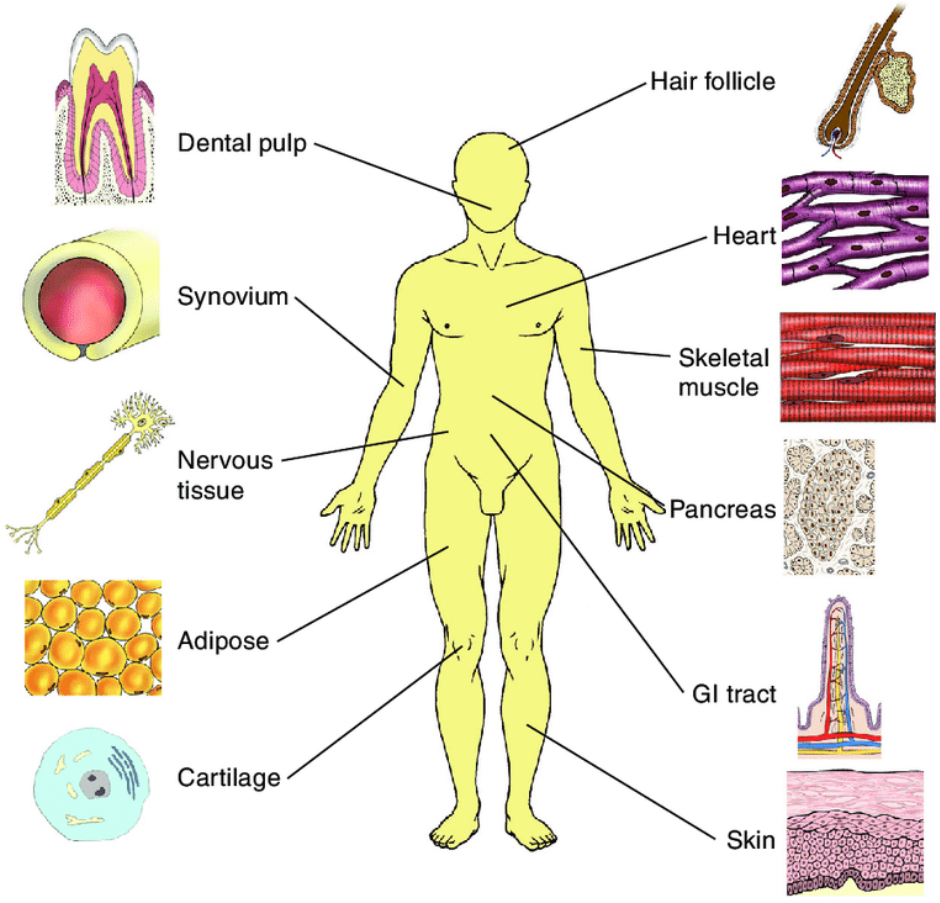


Figure 2. Human adult stem cell locations. (Hodgkinson et al., 2009)

1.1.1. Mesenchymal stem cells (MSCs)

It was 1990 when the term “mesenchymal stem cell “ started to be popular thanks to its use by Caplan but it took until 2000 to be accepted by the scientific community (Caplan, 1991; Horwitz and Keating, 2000). They are named MSCs the non-hematopoietic stem cells with the capacity to differentiate to different mesenchymal tissue cells. They can be isolated from various tissues and organs such as muscles, cartilage, bone, tendons, adipose tissue and perivascular area (Chamberlain et al., 2007; Crisan et al., 2008), and from umbilical cord, menstrual blood, placenta, large intestine and dental pulp (Du et al., 2016; Ma et al., 2014; Portmann-Lanz et al., 2006; Tirino et al., 2011).

Mesenchymal stem cells must fulfill certain requirements to be considered as such. The Committee of Mesenchymal Stem Cells and Tissues of the international Society for Cellular Therapy (ISCT) established these requirements in 2005: They must be plastic adherent cells when cultured *in vitro* and must retain the potential for chondrogenic, adipogenic and osteogenic differentiation. Moreover, their surface marker expression must be negative to hematopoietic linage markers as CD14, CD34 and CD45, but positive for CD13, CD44, CD73, CD90 and CD105 (Dominici et al., 2006).

Different MSC populations have been described in the adult body. The first MSC population to be described were the bone-marrow mesenchymal stem cells (BMSCs) (Anjos-Afonso and Bonnet, 2007; Friedenstein et al., 1976). Then they followed different MSC populations such as umbilical cord mesenchymal stem cells, discovered in 1991 and confirmed as MSCs in 2004 (McElreavey et al., 1991; Wang et al., 2004), dental pulp mesenchymal stem cells (Gronthos et al., 2000), cardiac mesenchymal stem cells (Beltrami et al., 2003), pulmonary mesenchymal stem cells (Griffiths et al., 2005), peripheral blood mesenchymal stem cells (Cao et al., 2005) or adipose tissue mesenchymal stem cells (AT-MSCs) (Fraser et al., 2006). Despite most of the cell types mentioned before come from the mesenchymal germ layer, the origin of oral cavity mesenchymal stem cells, such as DPSCs, is in the neural crest, as DPSCs. Even been widely classified as mesenchymal stem cells, the ectodermal germ layer origin gives these cells have a higher ability to differentiate to neuron-like cells compared to MSCs, being possible to call them ectomesenchymal stem cells (Ibarretxe et al., 2012a).

1.1.1.1. Dental pulp stem cells (DPSCs)

a. General characteristics

As mentioned before, DPSCs have a neural crest origin. During the embryogenesis, ectodermal cells from the neural crest migrate to the oral region to create different craniofacial structures as the dental pulp, the tongue, craniofacial nerves, periodontal ligament, bones and muscles (Ibarretxe et al., 2012a; Shyamala et al., 2015; Vega-Lopez et al., 2017). Due to this characteristic, DPSCs have the potential to differentiate not only to mesenchymal-linage cells but also to neural-lineage cells (Ibarretxe et al., 2012a).

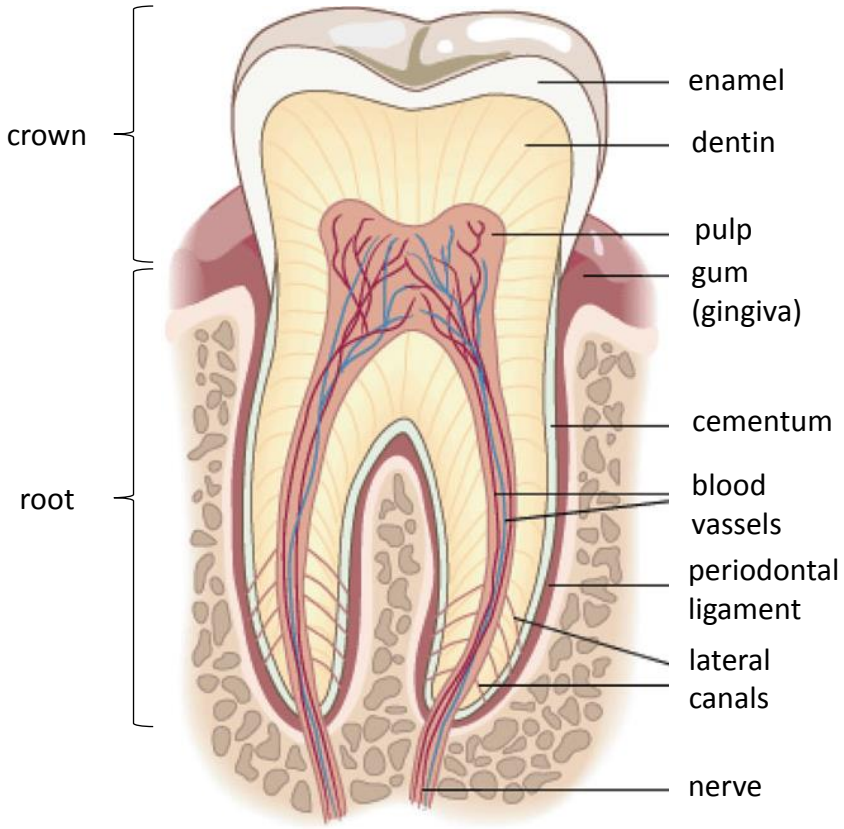


Figure 3. The main structures of the tooth. (Yildirim, 2013)

DPSCs were described for the first time in 2000 as mesenchymal stem cells from the dental pulp similar to BMSC (Gronthos et al., 2000). It was two years later when they were named as dental pulp stem cells and they were characterized as highly clonogenic

and proliferative cells (Gronthos et al., 2002). These cells are located in the "pulp chamber" inside the dental crown (Figure 3). Dental pulp is a connective tissue with a heterogeneous composition, including different cell types such as odonto/osteoprogenitor cell populations, neural cells, vascular cells, fibroblasts (known as pulpoblasts) and immune cells as granulocyte and macrophages (Goldberg and Smith, 2004). It is also important to mention that some studies located the DPSCs in the vascular pericyte compartment (Shi and Gronthos, 2003).

Hard tissues as cementum, dentin and enamel compose the adult tooth as well as the soft tissues, like the dental pulp. Dental formation starts with the interaction between the MSCs and the oral ectodermal epithelial cells. Dentin is the first tissue being formed, followed by enamel and the dental follicle. The epithelial cells form ameloblasts, which create the enamel. On the other hand, MSCs are the responsible for generating the dentin, cementum, periodontal ligament and pulp (Sedgley and Botero, 2012; Thesleff and Aberg, 1999) (Figure 3).

One of the main functions of the dental pulp in the adult body is the repair and maintenance of the dentin. In cases with severe damage as in deep caries, the DPSCs migrate to the injury zone and they differentiate to odontoblasts to create reparative dentin (Dimitrova-Nakov et al., 2014; Tziafas et al., 2000).

Even if the DPSCs are the most known and more commonly used dental stem cells in research, we can find different stem cell population with different properties. The dental follicle stem cells (DFSCs) can be isolated during tooth development from the ectomesenchymal embryonic tissue where they are covering the tooth germ (Zhang et al., 2019). They can differentiate *in vitro* to other cell linages apart from periodontal cells (Honda et al., 2010). Moreover, stem cells from apical papilla (SCAP) are localized in the apices of the root of developing tooth as in pre-erupting wisdom teeth with high proliferative ability (Bakopoulou et al., 2011). As the other stem cell subpopulation of this group, because of the ectomesenchymal origin, they are also able to differentiate to angiogenic and neurogenic cells (Dagnino et al., 2020; Nada and El Backly, 2018). Furthermore, periodontal ligament stem cells (PDLSCs) were found to have very similar characteristics to DPSCs as pluripotency markers and differentiation potential (Liu et al., 2018; Seo et al., 2004). Finally, primary exfoliated deciduous teeth stem cells (SHED) also

show pluripotency markers, neural markers and high proliferation rate (Kerkis et al., 2006; Miura et al., 2003). Like the rest of the dental stem cells, SHED have demonstrated the ability to differentiate into mesenchymal linage cells at the same time as to neural cells (Karbalaie et al., 2021; Sordi et al., 2021; Zhang et al., 2016) (Figure 4).

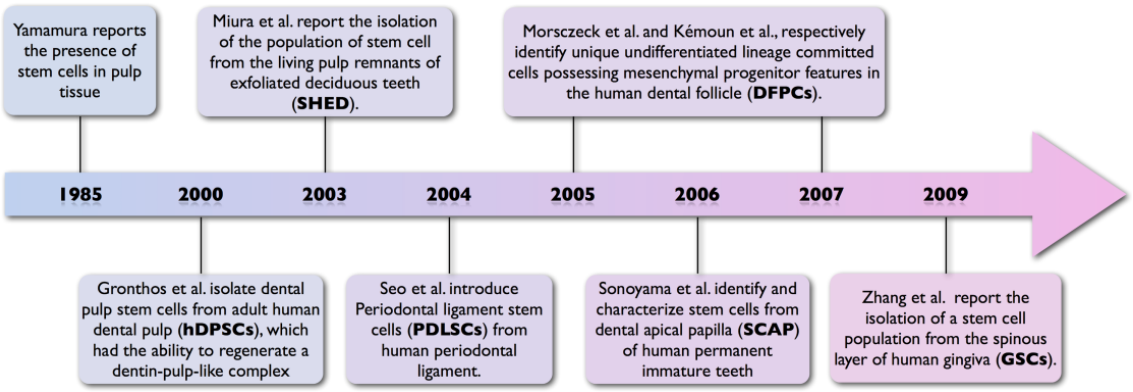


Figure 4. Timeline about the highlights in the history of the isolation of dental-related stem cells. (Karamzadeh and Eslaminejad, 2013).

b. DPSC surface markers

DPSCs demonstrated having cell marker differences as a consequence of the heterogeneity of stem cells subpopulation within the dental pulp (Alraies et al., 2020; Kawashima, 2012; Simonović et al., 2019).

Due to their neural crest origin, it is important to mention the expression of mesenchymal (Vimentin, Collagen I), neural (Nestin, GFAP) and stem pluripotency markers (OCT4, Nanog, Sox2) (Bae et al., 2021; Ibarretxe et al., 2012a; Wang et al., 2020).

Moreover, because of the variety of DPSC subpopulations, the expression of surface markers is not well established. In general, these cells are considered a subtype of MSCs because of the positive expression of the CD73 (5´-ectonucleatidase), CD90 (glycosylphosphatidylinositol-anchored glycoprotein) and CD105 (endoglin) markers. Moreover, DPSCs also have a positive expression for CD27, CD29, CD44, CD146, CD166 CD271, V-CAM-1, SSEA4 and STRO-1. On the other hand, DPSCs are negative for CD14 (monocyte or macrophage marker), CD19 (B cell marker) and hematopoietic markers CD34 and CD45 (Gang et al., 2007; Gronthos et al., 2003). Despite this being the most

commonly reported marker expression pattern for DPSCs, some studies showed expression of CD34 in certain DPSC subpopulations (Carnevale et al., 2018) (table 1).

	DPSCS	**SHED**	**PDLSCS**	**DFPCS**	**SCAPS**	**GSCS**	**BMSCS**
CD (+)	STRO-1	STRO-1	STRO-1	STRO-1	STRO-1	STRO-1	STRO-1
	CD29	CD29	CD29	CD29	CD29	CD29	CD29
	CD44	CD44	CD44	CD44	CD44	CD44	
	CD73	CD73	CD73	CD73	CD73	CD73	CD73
	CD105	CD105	CD105	CD105	CD105	CD105	CD105
	CD146	CD146		CD146	CD146	CD146	
		CD166			CD166	CD166	CD166
CD (-)	CD14	CD14					CD14
	CD19						
	CD34*	CD34	CD34	CD34	CD34	CD34	CD34
	CD45	CD45	CD45	CD45	CD45	CD45	CD45

Table 1. Cell surface marker profiles of dental-related stem cells and bone marrow stem cells. DPSCs: Dental pulp stem cell, SHED: Stem cells from human exfoliated deciduous teeth, PDLSC: Periodontal ligament stem cells, DFPC: Dental follicle precursor cells, SCAP: Stem cells from dental apical papilla, GSC: Gingival stem cells and BMSC: Bone marrow stem cells. CD34*: certain DPSC subpopulation expressed CD34 (Carnevale et al., 2018) (Own creation).

c. Cell Differentiation of DPSCs

As previously said, DPSCs have an ectomesenchymal origin. Due to this characteristic they have the ability to differentiate to different connective tissues as osteo/odontoblasts, adipocytes, chondroblasts and muscle cells (Gronthos et al., 2002; Kawashima, 2012). Moreover, because of the neural crest origin, these cells have a higher ability than other MSCs to differentiate to neuron-like cells (Luzuriaga et al., 2021). In addition, some studies suggested the capacity to differentiate to endoderm-lineage cells, suggesting that DPSCs could have pluripotency-like features (Atari et al., 2011). However, even obtaining endodermal-like cells, the pluripotency of DPSCs has not been conclusively demonstrated yet.

The osteogenic differentiation is one of the most well-known cell differentiation fates of DPSCs. Even if in different studies the osteodifferentiation induction cocktail can

vary, dexamethasone, β-glycerol phosphate and L-ascorbic acid are constantly repeated, as they are the most important components. These molecules are essential to activate the expression of collagen type I, alkaline phosphatase, Run-related transcription factor 2 (RUNX2), osterix, osteopontin, osteocalcin and osteonectin, necessary for the osteodifferentiation of the DPSCs (Ajlan et al., 2015; Atari et al., 2012a; Bhuptani and Patravale, 2016; Goto et al., 2016; Riccio et al., 2010). Among all these genes, *RUNX2* is one of the first being expressed. *RUNX2* is an early stage osteodifferentiation gene whose activation provokes the expression of both dentin sialoprotein (DSP) and dentin sialophosphoprotein (DSPP) (Han et al., 2014; Vimalraj et al., 2015; Xu et al., 2015).

For chondroblastic differentiation, the most used culture media contain L-proline, L-ascorbic acid, dexamethasone, insulin-transferrin-selenium (ITSx), sodium pyrubate and TGF-β3 (Hilkens et al., 2013; Jang et al., 2016; Nemeth et al., 2014). In the chondroblastic differentiation TGF-β is an important protein superfamily, responsible for cartilage differentiation/de-differentiation processes (Dexheimer et al., 2016). Although they can be differentiated to chondrocytes, DPSCs had less chondroblastic differentiation potential compares to other MSCs due to their population heterogeneity and *in vitro* high oxygen levels (Pisciotta et al., 2020).

In the case of the adipogenic differentiation media, apart from dexamethasone, there is need of insulin and 3-isobutyl-1-methylxantine (IBMX). The specific markers for adipogenic differentiation are fatty acid binding protein 4, glucose transporter type 4, lipoprotein lipase adipogenic markers and peroxisome proliferator-activated receptor ϒ. As in chondroblastic differentiation, DPSCs had less adipogenic differentiation potential than other MSCs (Grottkau et al., 2010; Lee et al., 2015a; Zhang et al., 2016).

DPSCs are also capable to differentiate to other cells types such as endothelial cells (Luzuriaga et al., 2019a; Luzuriaga et al., 2020; Marchionni et al., 2009), cardiomyocytes (Ferro et al., 2012) and smooth muscle (Song et al., 2016; Zhao et al., 2012). In addition, recent studies obtained endothelial cell differentiation using serum-free media, an interesting approach for autologous cell therapies (Luzuriaga et al., 2019a; Luzuriaga et al., 2020).

Apart from the differentiation to mesenchymal germ layer cells one of the most interesting characteristic of DPSCs is their neural crest origin. Due to this origin, DPSCs are able to differentiate to neuron-like cells. Many neuron differentiation inductive media used in the last decade are based on neurotrophins and other small molecules like NGF, GDNF, BDNF, epidermal growth factor (EGF), fibroblast growth factor (FGF), sonic hedgehog, forskolin, heparin, NT-3 and retinoic acid (RA). It is also remarkable the use of ITSx, N2, B27 and non-essential amino acids (Arthur et al., 2008; Chang et al., 2014; Gervois et al., 2015; Kanafi et al., 2014; Király et al., 2011; Luzuriaga et al., 2019b; Osathanon et al., 2014; Xiao and Tsutsui, 2013; Zhang et al., 2017) (figure 5).

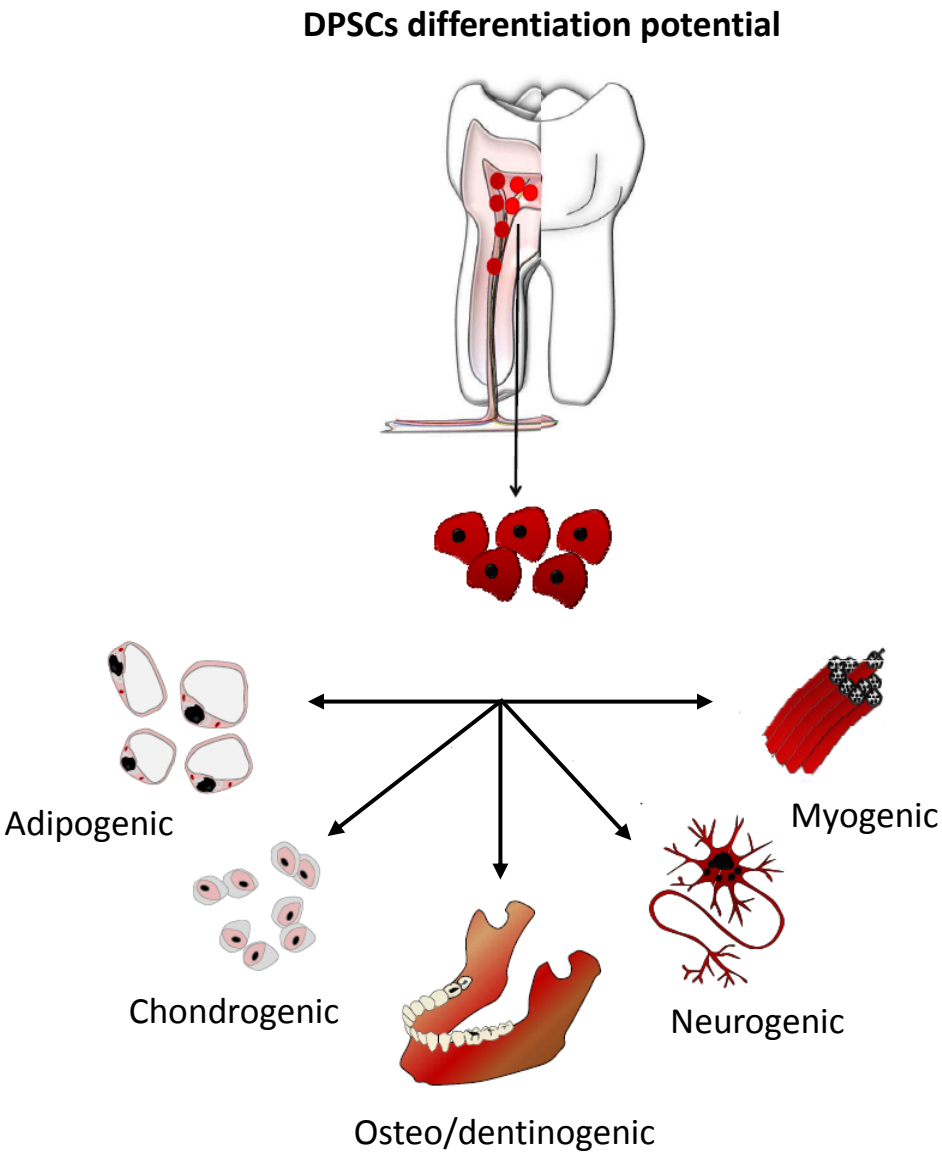


Figure 5. DPSCs differentiation potential. (Aurrekoetxea et al., 2015).

DPSCs showed the potential to generate endodermal lineage cells (Atari et al., 2012b). The ability to differentiate to endoderm germ-line cells as pancreatic cells was demonstrated by obtaining cells capable to produce glucagon, somatostatin, insulin and pancreatic polypeptide being the CD117+ cells the subpopulation with the best differentiation potential. The WNT and PI3K/AKT pathways showed to have a key role on this differentiation (Ishkitiev et al., 2013; Yagi Mendoza et al., 2018). Moreover, hepatic-like differentiation had been carried with the use of dexamethasone, ITSx, HGF and oncostatin M cultured in serum-free or low serum (1 %-2 %) media (Chen et al., 2016; Han et al., 2017; Ishkitiev et al., 2012).

1.1.1.2. Bone marrow stem cells (BMSCs)

a. General characteristics

Friedenstein and colleagues discovered bone marrow mesenchymal stem cells in 1970 with the isolation method based on the adherence in plastic of the cells derived from the bone marrow (Friedenstein et al., 1970). When these cells are cultured in plastic, they form high proliferative compact colonies with fibroblast-like spindle-shaped cells, termed colony forming unit fibroblasts (CFU-F) (Castro-Malaspina et al., 1980; Gothard et al., 2013).

The CFU-Fs showed to have a low frequency, ranging from 1/10.000 to 1/100.000 of bone marrow mononuclear cells (MNCs) (Castro-Malaspina et al., 1980). The frequency of BMSCs is much lower than hematopoietic stem cells (HSCs) also clustered in the bone marrow, being 1% of the MNC fraction (Civin et al., 1996).

Moreover, the BMSCs cell cycle investigations showed that a small fraction of these cells are proliferative (10 % at S + G2 + M), and all the rest at G0/G1 phase (Conget and Minguell, 1999). Depending on patients variability, the proliferation potential of BMSCs can vary from four doublings to going over 15 doublings (Digirolamo et al., 1999; Phinney et al., 1999). Even if the proliferative cells are a small fraction, they have high proliferation rate *ex vivo*. This proliferation potential can vary depending on the procedure to harvest the cells, the frequency of BMSCs and the age of the donor (Blazsek et al., 1999; Koç et al., 1999). Recent studies have improved *in vitro* proliferation of

BMSCs using bioreactors (Bhat et al., 2021; Santos et al., 2011). Despite telomerase activity being lost in somatic cell proliferation and differentiation causing cell death by senescence, BMSCs maintain the telomerase activity (Pittenger et al., 1999). However, after extensive passages, they show apoptosis and senescence signs (Dhanasekaran et al., 2013).

It is being suggested that the main function of BMSCs is to contribute to the formation and maintenance of the stromal microenvironment. The modulation of the microenvironment, via inductive regulatory signals, is not only for other BMSCs but also for other non-mesenchymal stromal and hematopoietic progenitor cells (Cheng et al., 2000). This theory is strengthened by the data showing that the BMSCs produce different matrix molecules, such as collagen, laminin, fibronectin and proteoglycans (Chichester et al., 1993; Connelly et al., 2008; Pittenger et al., 1999). They also express cell-to-cell adhesive interaction and matrix associated counter-receptor molecules.

Some investigations suggested that BMSCs, as DPSCs, are located in the vascular pericyte, the cell lining of the outer surface of blood vessels (Shi and Gronthos, 2003; Short et al., 2003; da Silva Meirelles et al., 2006). Besides, other studies indicates that the BMSCs could be identical to bone marrow stromal supportive cells termed adventitial reticular cells (ARCs). As a result, BMSCs are also referred as multipotent mesenchymal stromal cells or marrow stromal stem cells (Gronthos et al., 2003; Horwitz et al., 2005).

b. BMSC surface markers

BMSCs are characterized by the negative expression of markers as CD14 (monocyte or macrophage marker), CD31 (PECAM) and hematopoietic markers CD34 and CD45 (Conget and Minguell, 1999; Jones et al., 2002; Pittenger et al., 1999). On the contrary, these cells shows positive expression for specific antigens like α-smooth muscle actin (α-sma), STRO-1, SH2, SH3 and SH4 (Haynesworth et al., 1992; Simmons and Torok-Storb, 1991). Furthermore, they are also positive to adherence molecules as, CD54 (ICAM-1), CD102 (ICAM-2), CD106 (VCAM-1) and CD166 (ALCAM-1) (Chichester et al., 1993; Conget and Minguell, 1999; Prockop, 1997). They also have a positive expression for CD13 (ANPEP), CD29 (β1 integrin), CD73 (NTSE), CD90 (THY1), CD105

(Endoglin) and SSEA-4 (stage-specific embryonic antigen) (Boiret et al., 2005; Delorme and Charbord, 2007; Gang et al., 2007; Jones et al., 2006). Finally, it is important to point CD271 (low-affinity nerve growth factor receptor), as one of the best bone marrow subpopulation markers for BMSCs. This expression difference between subpopulations is based on the high expression on these cells and low expression in all other marrow cell populations (Bühring et al., 2007; Cuthbert et al., 2015) (Table 1).

c. Differentiation

The MSCs from the bone marrow are non-hematopoietic cells that exhibit multilineage differentiation capacity. Many studies demonstrated the ability of these cells to give rise to diverse mesenchymal derivatives like cartilage, cardiac muscle, skeletal muscle, bone and adipose tissue (Arthur et al., 2009; Bianco et al., 2001; Conget and Minguell, 1999; Matsuda et al., 2005; Prockop, 1997; Zheng et al., 2013). Contrary to their original germ line, they have also the potential to differentiate into neuroectodermal cells as neuron-like cells (Krampera et al., 2007; Sanchez-Ramos et al., 2000; Zhang et al., 2012). Even so, this neuron-like cell differentiation potential is lower than DPSCs differentiation ability (Isobe et al., 2016; Pagella et al., 2020).

The osteogenic differentiation of BMSCs is based on the supplementation of the media with dexamethasone, L-ascorbic acid and β-glycerol phosphate (or other phosphate-containing molecule to work as phosphate source) like DPSCs. These molecules enhances the transcription of the gene RUNX2, one of the first genes involved in osteodifferentiation process (Langenbach and Handschel, 2013).

Otherwise, for the adipogenic differentiation, the most used media cocktails are based on dexamethasone, indomethacin and IBMX (Zheng et al., 2013).

Moreover, for the chondrogenic differentiation the essential molecules are dexamethasone, sodium pyrubate, L-ascorbic acid, proline, (ITS) and TGF-β1 (Solchaga et al., 2011). For these two, adipogenic and chondrogenic, differentiations, BMSCs demonstrated higher differentiation potential that DPSCs (Isobe et al., 2016).

In addition, BMSCs are also capable to differentiate to other mesenchymal cell such as, cadiomyocytes, skeletal muscle, tendon and hematopoietic-supporting stroma (Guo et al., 2018; Matsuda et al., 2005; Tian et al., 2010; Wu et al., 2018) (figure 6).

Since the BMSCs are mesenchymal origin cells, the aforementioned differentiation outcomes can be considered as “natural”. Even so, these cells have the potential to differentiate to neuron-like cells (cells expressing some neuron markers) from neuroectodermal germ line. For this differentiation, there are two most used media cocktails. On the one hand is the one using basic fibroblast growth factor (bFGF). In various cell types and tissues four FGF receptors can be found with different expression levels (Eswarakumar et al., 2005). This receptors can activate signaling pathways as Src, Crk, phospholipase C-ϒ (PLC-ϒ) and SNT-1/FRS2, some of them involved in neurogenic differentiation (Powers et al., 2000; Reuss and von Bohlen und Halbach, 2003). Some investigations conclude that the SNT-1/FRS2 signaling pathway activates MAPK/ERK cascade, determinant for the secretion of neurotrophic factors (Abe and Saito, 2000). On the other hand we have β-mercaptoethanol (BME) being able to induce the expression of neuron specific enolase (NSE), NeuN and neurofilament-M after 7 days of culture. (Khanabdali et al., 2016; Khang et al., 2012; Scintu et al., 2006).

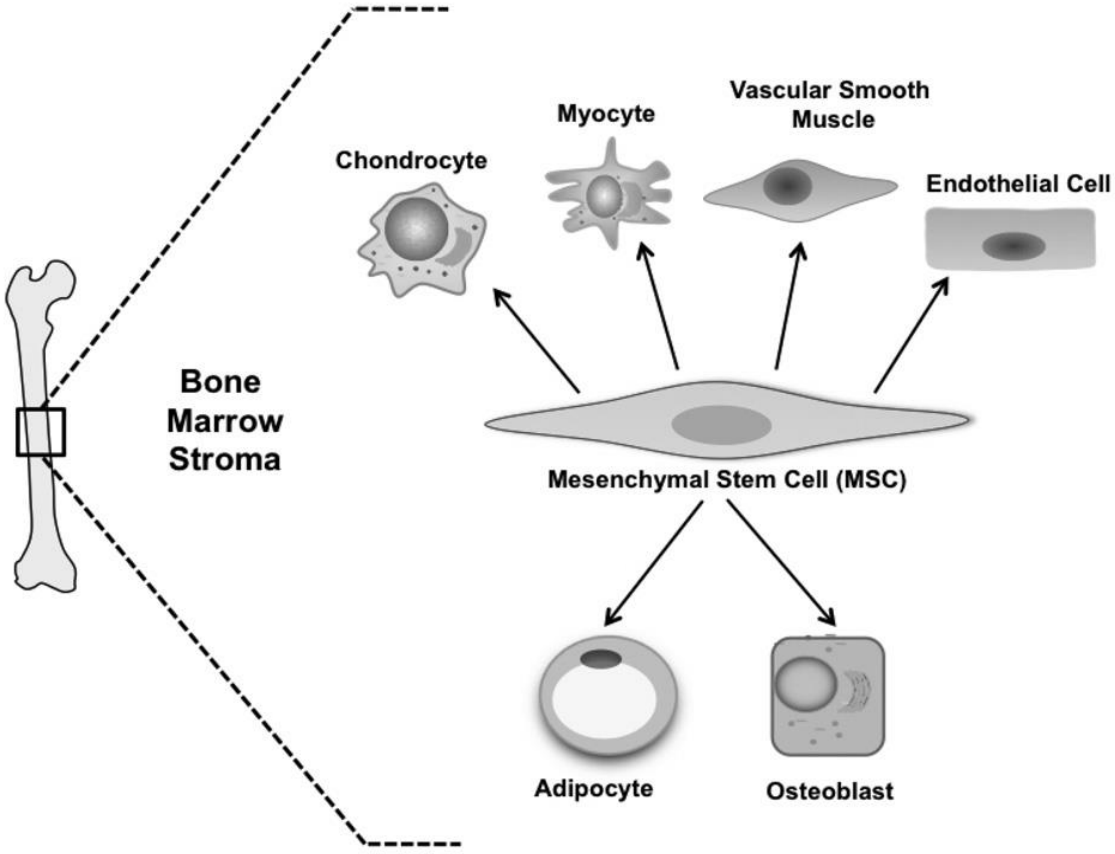


Figure 6. Differentiation potential of bone marrow mesenchymal stem cells (BMSCs). (Muruganandan et al., 2009).

2. Scaffolds for bone tissue engineering

The term scaffold refers to three-dimensional (3D) biomaterials that provide an adequate environment for regeneration of tissues and organs. The purpose of creating new biomaterials is to develop new scaffolds with similar properties to the natural environment and multi-functional features (Dhandayuthapani et al., 2011) (Figure 7).

In bone tissue engineering, scaffolds must meet three characteristics to be used as bone grafts: osteoconductivity, osteoinductivity and osteointegration (Albrektsson and Johansson, 2001). Osteoconductivity indicates that the bone could grow in the graft, while osteointegration is referred to the direct contact between the implant surface and the bone. This could vary depending on the type of material, site of implantation, etc. (Khan et al., 2005). Osteoinductivity is the property to induce the differentiation of multipotent stem cells to bone-forming cells mediated by growth factors (Roberts and Rosenbaum, 2012).

Historically, the main characteristic of the first-generation of biomaterials was the biocompatibility. The purpose of these materials was to keep similar physical properties of the replaced tissue while being inert. Ceramics (zirconia and alumina), metals (titanium, stainless steel and alloys) and polymers (rubber, silicone, polypropylene and polymethylmethacrylate) compose these first-generation biomaterials for bone tissue engineering. Some of these scaffolds lead to a non-specific immune response, ending with the encapsulation of the material by fibrotic connective tissue, isolating it from the surrounding tissues (Anderson, 2001).

Moreover, trying to avoid those non-specific immune response, biointeractivity was the goal for the second-generation. This was performed by coating first generation materials surfaces with hydroxyapatite (HA) or β-tricalcium phosphate, allowing mineralization. Other bone grafts used in this generation were biodegradable scaffolds. With a rate of degradation matching bone-healing rate, the aim of this material is to provide support to the bone generating cells while the body reabsorbs it. Natural

polymers (hyaluronic acid and chitosan) and synthetic ones (polyglicolide and polylactide) are common examples (Navarro et al., 2008).

The third-generation of scaffolds, in which we are nowadays, are bioresponsive materials. Those are biomaterials capable of activating specific genes to promote proliferation and differentiation of cells. This generation is based on tissue engineering, promoting bone regeneration and vascularization using natural or synthetic scaffolds coated with stem cells and growth factors (Amini et al., 2012; Hench and Polak, 2002; Rahman et al., 2018).

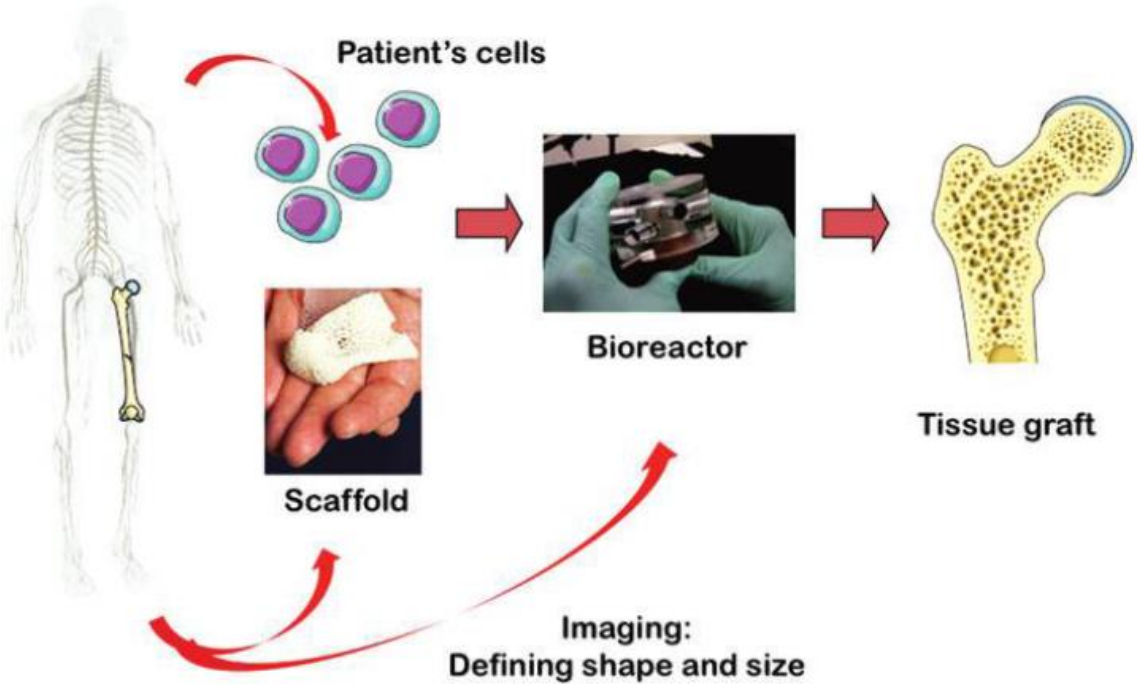


Figure 7. Bone tissue engineering. Bone scaffold with autologous stem cells put in a bioreactor with growth factors enable the growth of autologous bone grafts *in vitro* for bone repair applications *in vivo*. (Ng et al., 2017).

Nowadays, natural or synthetic polymers and inorganic materials compose the scaffolds for bone tissue engineering.

The most commonly used synthetic polymers are poly-glycolic acid (PGA), poly-lactic acid (PLA) and their copolymers. Their degradation rate control and excellent mechanical properties make them a good choice for cell transplantation for tissue engineering (Dorati et al., 2017).

Moreover, natural biological components are proteins (collagen, fibrin gels, soy and silk) and polysaccharides (alginate, chitin/chitosan, starch, and hyaluronic acid derivates). They are a good option for enhancing cell adhesion, however, they allow less control on the biodegradability and mechanical characteristics. Besides, they could produce some immunogenicity (Dorati et al., 2017).

Finally, we can find inorganic materials such as, metals, tricalcium phosphate (TCP), bioactive glasses, hydroxyapatite (HA), wollastonite and several combination of them as HA-TCP biphasic ceramics. The best feature they have is the similarity to the bone mineral phase (Kokubo and Takadama, 2006; Lakshmi and Sasikumar, 2015; Stevens, 2008). The main disadvantage of these materials is the low or lack of degradation so they cannot be reabsorbed, thus hindering the creation of new tissue.

One of the features that seems to be crucial for cell proliferation and differentiation in recent investigations is the porosity of the scaffolds. Some studies showed that pore sizes between 100 and 400 μm were the perfect size for the formation of mature bone by enhancing vascularization and cell infiltration (Boyan et al., 2016a; Murphy et al., 2010), while other studies suggested that 800 μm pore size was more appropriate for the cell growth (Roosa et al., 2010). In contrast, small pore sizes (<100 μm) are related with fibrous tissue and non-mineralized osteoid formation (Iviglia et al., 2019; Liu et al., 2018). At the same time, micropores have more surface area, promoting ion exchange and cell and bone protein adhesion (Abbasi et al., 2019; Diaz-Rodriguez et al., 2018; Morejón et al., 2019).

Another relevant characteristic of scaffolds to take account of is the form of the material. Concave surfaces exhibit better tissue formation comparing to flat and convex surfaces. In concave surfaces cell alignment is greater due to more density of actin and myosin fibers while in convex surface the tissue growth is delayed (Bianchi et al., 2014; Knychala et al., 2013).

2.1. Titanium

In the last half a century, titanium implants have been established as the best option for teeth replacement due to their resistance, durability and their high

osteointegration capacity (Albrektsson et al., 1981; Breine and Brånemark, 1980). However, the lack of sufficient alveolar bone, bacterial infections (e.g. Perimplantitis), implant surface microporosity or the lack of activation of surrounding mesenchymal stem cells compromise the efficacy of these implants (Boyan et al., 2016a; Graziano et al., 2008). Hence, new scaffolds, surface topography and stem cells based research for the regeneration of maxillary and mandibular bone can lead to a substantial improvement for clinical implantology.

Despite titanium proved to be the best material for dental implants, in the last decades it has been demonstrated the importance of the design and manufacturing different implant surfaces in order to enhance their osteointegraton (Gasik et al., 2015; Rani et al., 2012; Rupp et al., 2018; Salou et al., 2015). Apart from the composition of the titanium, it is important to consider the topography of its surface for a durable anchorage of the titanium implant (Annunziata and Guida, 2015; Jemat et al., 2015; Le Guéhennec et al., 2007; Naves et al., 2015).

Rough titanium surfaces, maximizing the contact surface with the surrounding alveolar bone, has proven to have a better osteointegration compared to smooth titanium ones (Coelho et al., 2009). The better integration in the bone could be attributed to the microporosity of the titanium surface due to the promotion of osteoblastic differentiation of surrounding mesenchymal cells. Lastly, these mesenchymal cells are responsible for consolidation and bone healing after the placement of the implant (Boyan et al., 2016a; Graziano et al., 2008).

As mentioned before, different investigations concluded that the small pores (<180 μm) promoted cell differentiation at the beginning of implant healing, while large pores (>300 μm) enhanced cell proliferation and bone growth (Coelho et al., 2015). However, the porosity affects directly to the mechanical properties of the material so it is essential a correct balance between porosity and toughness depending on the type of the implant. Furthermore, other investigations also showed that porous titanium scaffolds have weaker corrosion resistance due to dynamic flow of the body fluid through. Porosity allows higher body fluid flow through the scaffold accelerating its degradation (Chen et al., 2017).

Another important reason in the development of porous titanium is also to decrease implant stiffness. It has seen that hard titanium surfaces decrease bone creation and remodeling because of the negative implications of stress shielding, causing bone reabsorption and finally, implant loosening (Ma et al., 2006; Świeczko-Żurek, 2009) (figure 8).

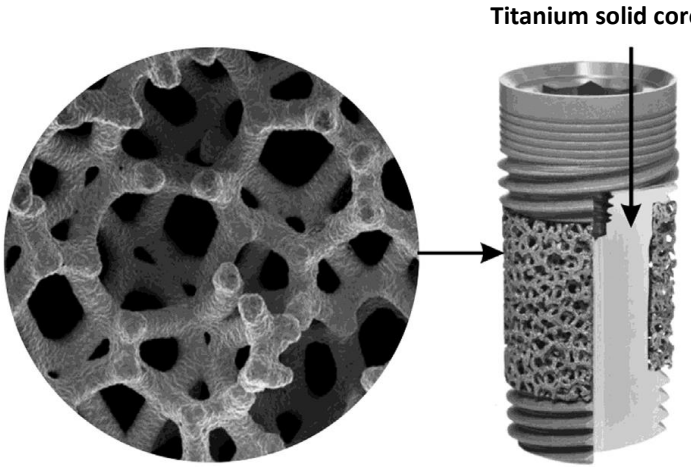


Figure 8. Porous surface made on titanium dental implant. (Pałka and Pokrowiecki, 2018)**.**

The titanium alloys can be classified into three groups:

1- A phase stabilizers: carbon (C), oxygen (O), aluminium (Al) and nitrogen (N).
2- B phase stabilizers:
    a. Isomorphous: tantalum (Ta), molybdenum (Mo), vanadium (V) and niobum (Nb).
    b. Eutectoid: silicon (Si), chromium (Cr), copper (Cu), hydrogen (H), manganese (Mn), nickel (Ni) and iron (Fe).
3- Neutral additions: tin (Sn) and zirconium (Zr).

The activation of surrounding mesenchymal cells of the affected alveolar bone area is essential to enhance the implant integration to the bone to improve durability, resistance and avoid implant failure due to bacterial infections (*e.g.* Perimplantitis). For this reasons, the combination of titanium implants with cellular allografts/autografts promises to offer considerable advantages for clinical implantology.

2.2. Decellularized Adipose Tissue (DAT)

Most of the actual dental implant roots are inert surface-micropatterned metal alloys. The use of these implants alone or in combination with decellularized bone matrix and plasma derived growth factors promotes the osteodifferentiation of mesenchymal stem cells, generating bone directly in the implant surface (Anitua et al., 2016; Boyan et al., 2016a; Mayer et al., 2018; Zanicotti et al., 2018). The main problem of actual dental implants is developing periimplantitis. This is caused by the continuous mechanical pressure transferred from the implant to the bone tissue during natural mastication. In the worst scenario, periimplantitis leads to the loss of the dental implant (Bertolini et al., 2019). In natural teeth, this problem is solved thanks to the periodontal ligament (PDL), a highly innervated and vascularized connective tissue composed by strong collagen bundles. These collagen fiber bundles are anchored to the dental root cement and the alveolar bone by lightly calcified edges, also named as Sharpey´s perforating fibers (Aaron, 2012). The principal PDL function is acting as a cushion to absorb the masticatory forces to protect alveolar bone.

Decellularized Adipose Tissue (DAT) seems to be a promising biomaterial for tissue engineering due to great characteristic such as malleability, accessibility and bioactive properties (Yang et al., 2020). It had already showed *in vivo* biocompatibility (Wang et al., 2013). One of the most interesting features of DAT is the softness of this material, which may help in the creation of connective tissue interface between dental implant root and the alveolar bone. DAT is obtained from white adipose tissue, a highly vascularised tissue. It also helps the cell adhesion and integration because of the high concentration of basement membrane proteins it contains such as collagen (type I, III and IV), perlecan (sulphatated proteoglycan) and laminin (Yang et al., 2020). The decellularization process has also been improved (Madarieta Pardo et al., 2017). It is also important to mention that the DAT can be processed in different ways depending on the application. In the case of bone tissue regeneration, the most promising

formulation is the solid foam due to the porosity and the high volume per weight ratio, allowing nutrients, signals and cell mobility through the scaffold matrix (figure 9).

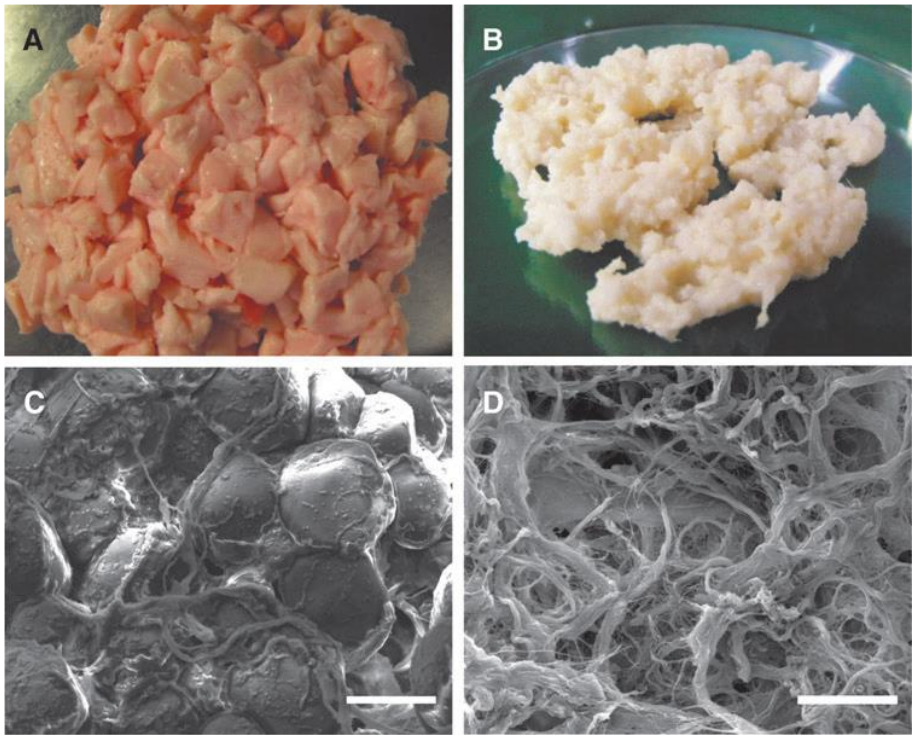


Figure 9. Macroscopic (A, B) and scanning electron microscopic (SEM) (C, D), images of native porcine adipose tissue (A, C) and decellularized extracellular matrix (ECM) (B, D). Scale bar: 50 µm. (Choi et al., 2012)**.**

3. Growth factors

In the last decades, molecules as bone morphogenetic proteins (BMPs) and enamel matrix derivate (EMD) have been using to promote the regeneration of damaged or lost tissues (Dumic-Cule et al., 2018; Miron et al., 2016; Rao et al., 2013). However, in recent years, platelet derived products have become a very promising tool for enhancing cell regeneration and differentiation in tissue engineering.

3.1. Plasma derived products

With the purpose of enhancing local healing in the damaged zone, the concept of concentrated platelets and autologous growth factors was born by collecting plasma solutions (Anfossi et al., 1989; Fijnheer et al., 1990). The name given in the late 1990s was platelet rich plasma (PRP) (Jameson, 2007; Marx et al., 1998; Whitman et al., 1997).

PRP is composed of platelets that when activated releases growth factors enhancing cell proliferation, adhesion and migration of the cells in the damaged zone (Jameson, 2007; Marx, 2004). After PRP, a second plasma derived product was formulated using anticoagulants, which was termed platelet-rich growth factor (PRGF) (Anitua, 1999). Moreover, to avoid the biggest limitation of these two products, being difficult to handle, a second generation of plasma derived product was created by Choukoun et al. in 2001 to enhance hard and soft tissue regeneration in maxillofacial and oral surgery, platelet rich fibrin (PRF) (Kumar and Shubhashini, 2013).

Among the growth factors and cytokines being released by activated platelets the following can be included: transforming growth factor β1 (TGF-β1), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), epidermal growth factor (EGF) and interleukins (IL) as IL-1β, IL-4 and IL-6 (Dohan et al., 2006).

Despite the preparation of PRP and PRGF is almost the same, PRGF contains more plasma proteins and coagulation factors, enhancing the effect comparing to PRP (Anitua, 1999).

The mixture of factors of both plasma-derived products, PRGF and PRF, induces the proliferation and differentiation of the surrounding mesenchymal cells to osteoblastic cells. One of the best characteristics of this product is the possibility to be used as autologous personalized therapy (Anitua et al., 2013). The benefits of the use of this product are widely exploited in different experimental and clinical tissue regeneration therapies and especially in implant dentistry therapies (Anitua et al., 2016; Masuki et al., 2016) (figure 10).

Figure 10. Images showing PRGF (left) and PRF (right).

PRGF is composed of a platelet-enriched plasma fraction. It is obtained by centrifugation of patient´s blood sample and afterwards by activation with calcium chloride. The calcium treatment triggers platelet degranulation, obtaining a polymerized fibrin clot and a complex mixture of growth factors (Anitua et al., 2009; Jovani-Sancho et al., 2016; Paknejad et al., 2012). The following centrifugation separates the fibrin clot from the growth factors rich soluble fraction (Anitua et al., 2012).

To obtain PRF it is necessary a fast centrifugation after the blood extraction since it is extracted in absence of anticoagulants. After the separation of blood fractions, the clot containing platelet and leukocytes, can be easily manipulated and flattened into a membrane. The most interesting feature of it is the progressive release of the growth factors due to the degranulation of the platelet over time. After surgery, these growth factors stimulate tissue healing upon enhancing cell proliferation and chemotaxis. The fibrin clot is easy to manipulate, resistant, strong and flexible, giving them great adaptability characteristics to be used in varied anatomical surfaces (Khurana et al., 2017; Kumar et al., 2016).

Nowadays, the use of plasma rich in growth factors (PRGF) and platelet rich fibrin (PRF) in the dental clinic to promote bone healing for the placement of the titanium implant after the tooth extraction is widely extended. It is known as plasma-derived advanced medical therapy products (AMTP) (Giannini et al., 2015; Kobayashi et al., 2016; Nishiyama et al., 2016).

Materials and Methods

hBMSC isolation and culture

The hBMSCs were obtained in the Laboratory for Cell and Tissue Engineering (Ehrbarlab) of the University of Zurich (Zurich, Switzerland) with the following procedure: the hBMSCs were obtained from aspirates of the bone marrow during orthopedic surgical procedures of human healthy donors (average age 45 years old). The aspirate was obtained after informed consent established by the local ethical committee (University Hospital Basel; Prof. Kummer; approval date 26/03/2007 Ref. Number 78/07). For the isolation of nucleated cells, the red blood cells from the aspirate were lysed using a buffer containing 0.15 M NH_4Cl (Sigma, Switzerland), 1 mM $KHCO_3$ (Sigma, Switzerland) and 0.1 mM Na_2EDTA (Fluka, Switzerland). hBMSC cells were cultured in minimal essential medium (αMEM, Gibco, NY, USA) supplemented with 10 % FBS (Gibco, NY, USA), 1 % penicillin-streptomycin (Gibco, NY, USA) and 5 ng/ml fibroblast growth factor 2 (FGF2) (PeproTech, NJ, USA) at 37 °C in 5 % CO_2. Passages between 3 and 8 maximum culture age were used on experimentation.

hDPSC isolation and culture

Human dental pulp stem cells (hDPSCs) were isolated from the third molar teeth of young healthy patients (age 18-30) in dental clinics. All the patients signed an informed consent. The competent authority (Ethics committee of the University of the Basque Country) under the M10_2016_088 protocol had previously approved the procedures officially.

Dental pulp extraction was performed by fracturing the molar. The obtained pulp was digested with an enzymatic solution containing 3 mg/mL collagenase type I (Gibco, NY, USA) and 4 mg/mL dispase (Sigma, MO, USA) in Hank´s balanced salt solution (HBSS, Gibco, NY, USA), and then incubated at 37 °C for 1 hour. The enzymatic digestion solution was neutralized with Dulbecco´s modified eagle medium (DMEM, Lonza, Switzerland) supplemented with 10 % fetal bovine serum (FBS, HyClone, UT, USA). Then, the cells were centrifuged at 1500 rpm for 5 minutes. The pellet was mechanically disrupted by 18G needles (BD Microlance, Fisher Scientific, UK). After the disassociation,

the cells were seeded in 25 cm^2 flask (Sarstedt, Nümbrecht, Germany) with DMEM supplemented with 10 % FBS, 1 % penicillin-streptomycin (Gibco, NY, USA) and 1 % L-glutamine (Sigma, MO, USA) at 37 °C in 5 % CO_2. After reaching 80 % confluence, the cells were subcultured to 75 cm^2 flasks (Sarstedt, Nümbrecht, Germany). Passages between 4 and 10 and maximum culture age were used on experimentation.

Osteogenic differentiation media

For hDPSC cultured on titanium surfaces and pDAT solid foams experimentation the osteogenic media was composed by DMEM (10 % FBS, 1 % penicillin/streptomycin and 1 % L-glutamine) supplemented with 50 µM ascorbic acid (Sigma, MO, USA), 20 mM β-glycerolphosphate (Sigma, MO, USA) and 10 nM dexamethasone (Sigma, MO, USA). For the comparative study of hDPSCs and hBMSCs the osteogenic media were composed by DMEM (10 % FBS, 1 % penicillin/streptomycin and 1 % L-glutamine) supplemented with 50 µM ascorbic acid, 10 mM β-glycerolphosphate and 100 nM dexamethasone. For these comparative experiments, both control and osteogenic media were supplemented with 5 ng/ml EGF (AF-100-15-500UG, PeproTech, NJ, USA) .

Titanium disc manufacture

Ti6Al4V 2 mm thick discs were obtained cutting a 5.5 mm diameter bar, provided by Avinent Implant System SLU (Barcelona, Spain). For the manufacture of BAS™ (Biomimetic Advanced Surface) titanium surface, one face of the titanium disc was treated by shot blasting using white corundum (Al_2O_3) F60 with projection particle sizes of 212-300 µm. Next, the discs were washed in an ultrasonic bath for 10 min and rinsed with deionized water. Afterwards, they were anodized by connecting them to a DC power source anode and using calcium and phosphorus as an electrolyte in an aqueous solution. A current was applied to this solution with a density of 0.75 mA/mm^2 and the potential was freely allowed to increase until reaching 130 V. Subsequently, the titanium discs were rinsed by deionized water for 10 minutes in an ultrasonic bath. Ti6Al4V titanium discs did not undergo any surface treatment or polishing.

pDAT decellularization and processing as a solid foam

Porcine adipose tissue was harvested in a local food company (Jaucha SL, Navarra, Spain). The tissue was defrosted at room temperature, cleaned and creamed by a beater. Then, tissue was homogenized on ice by Polyton (PT3100) with two different rods at 1200 rpm for 5 minutes. To produce phase separation of lipids, homogenized tissue was centrifuged at 900 g with ultrapure water for 5 minutes. Lipids were discarded manually and proteins conserved. The protein pellets were treated overnight under orbital shaking with isopropanol (Merk Life Science, Spain) at room temperature. Afterwards, it was cleaned with PBS and treated using 1 % Triton X-100 and 0.1 % ammonium hydroxide (Merk Life Science, Spain) in an orbital shaker for 36 hours at room temperature. After cleaning the material again with PBS it was lyophilized to completely dry up. Then, the tissue was milled by a mixer mill (Retsch MM400) to obtain fine-grained powder suitable for processing. The resulting powder was frozen in liquid nitrogen and storage at 4℃ in a vacuum desiccator.

Solid foams were prepared by pDAT freeze-drying method. 0.5 M acetic acid was added to 0.5 % pDAT, the solution was homogenized by magnetic stirring for 48 hours at room temperature. Teflon moulds of 20 mm diameter and 3 mm thickness were used for Scanning Electron Microscopy (SEM) with 1 ml solution. In the case of *in vitro* cell culture assays, solid foams were formed in Millicell EZ-slide 8 well glass slides (Merk Millipore) using 120 µl solution per well. The resulting slides were compatible for optic and fluorescence microscopy. To obtain solid foams the samples were frozen at -20 ℃ and freeze-dried (0.63 mbar and -10 ℃). Solid foams for cell culture were sterilized by ethylene oxide (Esterilizacion SL, Barcelona, Spain).

PRGF and PRF preparation

For the preparation of PRGF, blood from human healthy young donors was collected in 5 ml blood collection tubes with 3.8 % sodium citrate anticoagulant (BD Vacutainer, Plymouth, UK). After centrifugation at 580 g for 8 minutes, platelet-rich fraction was transferred to 15 ml falcon tubes. Then, the platelets were activated with

10 % calcium chloride (Braun medical, Melsungen, Germany) for 1 hour at 37 ℃. Following the incubation, the plasma was centrifuged at 3000 g for 15 minutes at 4 ℃ and filtered by 0.2 µm pore-size filters (Sarstedt, Nümbrecht, Germany). Then, the plasma was aliquoted and stored at -20 ℃ until use. The PRGF was added in media changes every two days (figure 11).

For obtaining PRF, blood was collected in tubes without anticoagulant (BD Vacutainer, Plymouth, UK). The samples were immediately centrifuged at 580 g for 8 minutes to avoid the blood coagulation. After the coagulation and separation of blood fraction and fibrin clot, the platelet-containing fibrin clot was separated from the hematocrit fraction by scalpel and forceps. Then it was compressed between two glasses by weight for 1 hour at room temperature. The fibrin clots were cut to obtain smaller sections of around 1 cm^2 (figure 11).

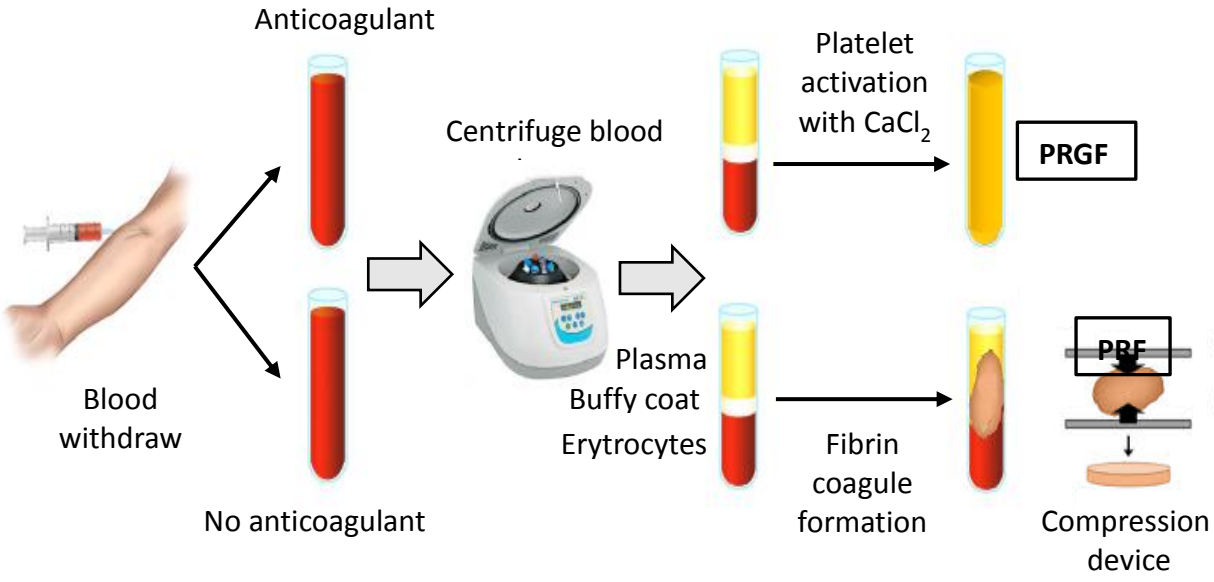


Figure 11. Illustration of plasma-derived PRGF and PRF preparation. (Own creation).

Culture of hDPSCs and hBMSCs on titanium discs

When the cells reached 80 % confluence, they were detached from the bottom of the flask with 0.05 % trypsin-EDTA, centrifuged and counted. Different densities of cells were seeded on Ti6Al4V and BAS™ titanium discs, depending on the assay. To

improve attachment, 15.000 cells were seeded in a 70 µl drop on the discs, and incubated for 3 hours at 37 °C. Afterwards, 700 µl of DMEM medium was added for 24 well plates and 4 ml for 6 well plates.

Culture of hDPSCs on porcine Decellularised Adipose Tissue (pDAT)

When the cells reached 80 % confluence, they were detached from the bottom of the flask with 0.05 % trypsin-EDTA, centrifuged and counted. pDAT (Tecnalia, Donostia, Spain) was placed in 24 well plates (Sarstedt, Nümbrecht, Germany) and 8 well Ibidi plates (Ibidi, Munich, Germany). After hydrating the pDAT with DMEM medium, the hDPSCs were seeded in a concentration of 10.000-20.000 cells/well (depending on the assay). The medium was changed every 2-3 days.

Calcein/ propidium iodide assay

After 4 days of culture, the cells were washed with PBS. This step was followed by an incubation of the cells with 3 µM calcein-AM (Molecular probes, OR, USA) and 2.5 µM propidium iodide (Sigma, MO, USA) for 30 minutes at 37 ℃. Afterwards, the cells were washed with PBS three times and photographed by using an Axioskop fluorescence microscope (Zeiss, Overkochen, Germany) and a Nikon DS-Qi1 camera (Nikon, Tokyo, Japan).

Immunocytochemistry

After culture, the cells were washed with PBS and fixed in 4 % paraformaldehyde (PFA) at room temperature for 10 minutes. Then, the cells were incubated for 10 minutes with 10 % goat serum (Invitrogen, CA, USA) at room temperature. The blocking step was followed by overnight incubation at 4 °C in 0.1 % Triton X-100/ 1 % BSA/ PBS with the following antibodies: BGLAP (ab93876, Rabbit polyclonal, Abcam, Cambridge, UK), Caspase-3 (ab32351, Rabbit monoclonal, Abcam, Cambridge, UK), Ki67 (ab15580, Rabbit polyclonal, Abcam, Cambridge, UK), Osterix (ab22552, Rabbit polyclonal, Abcam, Cambridge, UK) and SPARC (ab14174, Rabbit polyclonal, Abcam, Cambridge, UK). Alexa

488 conjugated rabbit IgG (A11008, Abcam, Cambridge, UK) was used as secondary antibody for primary antibody localization in 1 % BSA/ PBS for 1 hour at room temperature. The nuclei were stained with 4',6-diamino-2-phenilindol (DAPI, Invitrogen, CA, USA). The images were taken using an Axioskop fluorescence microscope (Zeiss, Overkochen, Germany) and Nikon DS-Qi1 camera (Nikon, Tokyo, Japan) and by Leica DM6000 B microscope (Leica, Wetzlar, Germany), Leica DFC420 C camera with 3.3.3.16958 Leica application suite X (Leica, Wetzlar, Germany).

Flow cytometry

The phenotype of hDPSCs and hBMSCs was confirmed by flow cytometry analysis. For each antibody, 500.000 cells were enzymatically detached and incubated in PBS 0.15 % bovine serum-albumin (BSA, Sigma, MO, USA) with CD45-APC 1:50 (304011, Biolegend, CA, USA), CD73-APC 1:50 (17-0739-41, eBioscience, MA, USA), CD90-FITC 1:50 (328107, Biolegend, CA, USA) and CD105-PE 1:50 (12-1057, eBioscience, MA, USA) for 40 minutes on ice. The cells were washed by PBS 0.15 % BSA and finally resuspended in 500 µl of PBS. The analysis was performed using a Gallios flow cytometer (Beckman Coulter, CA, USA) and data were analyzed using Kaluza 1.1 software (Beckman Coulter, CA, USA).

Scanning electron microscopy (SEM)

The cells cultured on both titanium surfaces were fixed with 2 % glutaraldehyde (Sigma, MO, USA) in 0.1 M Sorensen phosphate buffer (SPB, Thermo Fisher Scientific, MA, USA) for 1 hour at 4 °C. The samples were washed 3 times with 4-8 % sucrose in 0.1 M SPB and followed by other three washes in 0.1 M SPB. Then, the cells were dehydrated with 15 minutes series of ethanol (30 %, 50 %, 70 %, 90 % and 100 %) and a final wash by hexamethyldisilazane (Sigma, MO, USA). After dehydration, the samples were air-dried and sputten coated by 15 nm gold. The images were taken using a scanning electron microscope (SEM) (S4800, Hitachi High Technologies, Tokyo, Japan). Moreover, images of titanium discs without cells were also obtained by SEM.

Transmission electron microscopy (TEM)

The samples were fixed with 2 % glutaraldehyde for 15 minutes and embedded in Epon Polarbed resin (Electron Microscopy Science, Hatfield, PA, USA). 70 nm ultrathin sections were deposited onto 150 mesh copper grids (150 Square Mesh xopper 3,05 mm, AGAR Scientific, UK). Post-staining was performed with 2 % uranyl acetate in distilled water (AGAR Scientific, UK) and 0.2 % lead in citrate in distilled water (AGR1210, AGAR Scientific, UK). The images were taken in a Philips EM208S transmission electron microscope with an integrated Jeol JEM 1400 Plus camera.

RNA extraction and retrotranscription

Cell pellets were stored frozen at -80 °C until use. The RNA extraction was performed using RNeasy mini kit (Qiagen, Hilden, Germany). RNA concentration and purity was measured by 260/280 nm absorbance in Nanodrop Synergy HT device (Biotek, VT, USA). cDNA synthesis was made by retrotranscription was made starting from 1000 ng of RNA using iScript cDNA Kit (Biorad, CA, USA). The retrotranscription cycle started with 5 minutes at 25 °C, followed by 20 minutes at 46 °C and finished by 1 minute at 95 °C. Once the retrotranscription finished the lid stayed at 4 °C until the samples were frozen at -20 °C.

RNA sequencing

hDPSCs and hBMSCs were cultured on plastic, Ti6AL4V and BAS titanium surfaces in presence or absence of osteoblastic differentiation media for 14 days. 24 seeded discs were used for each condition, after tripsinization the cells were put together and the RNA was extracted using Qiagen RNA extraction kit. The RNA extracted from hDPSCs and hBMSCs were sequenced by the Genomic center of the University of Zurich using Illumina (Illunmina, CA, USA) obtaining 25.000 RNA lectures per sample. The gene expression were analyzed by presence/absence (ON/OFF) term of different condition

comparison and the threshold was set at limit of 10 raw reads to categorize a gene as effectively expressed (ON). Statistical significantly activated pathways were analyzed by "Pathway Enrichment Analysis" using The Connectivity Map (CMAP) and Gene Ontology enrichment Biological Process (GOBP) by the Genome Analysis Platform of the CIC bioGUNE (CIC bioGUNE, Derio, Spain).

Quantitative real-time PCR (qPCR)

Power SYBR Green PCR Master Mix (Applied biosystems, CA, USA) was used to perform the Q-PCR. The initial step for the amplification program was at 95 °C for 10 minutes, followed by 40 cycles of 95°C for 20 seconds, 59 °C for 1 minute. The housekeeping genes used were β-actin 5´-GTTGTCGACGACGAGCG-3´ and 5´-GCACAGAGCCTCGCCTT-3´ and Gapdh 5´ CTTTTGCGTCGCCAG -3´ and 5´-TTGATGGCAACAATATCCAC -3´. The target genes forward and reverse sequences were as follows: collagen I (*COL I*) 5´ -GGCCCCCTGGTATGACTGGCT-3´ and 5´-CGCCACGGGGACCACGAATC-3´, osteonectin (*SPARC*) 5´-GAAAGAAGATCCAGGCCCTC-3´ and 5´-CTTCAGACTGCCCGGAGA-3´, osteocalcin (*BGLAP*) 5´-CGCCTGGGTCTCTTCACTAC-3´ and 5´-CTCACACTCCTCGCCCTATT-3´, dentin sialophosphoprotein (*DSPP*) 5´-TGCCCAAATGCAAAAATATG-3´ and 5´-GTGGGCCACTTTCAGTCTTC-3´, osterix (*OSX*) 5´-TGAGGAGGAAGTTCACTATG-3´ and 5´-CATTAGTGCTTGTAAAGGGG-3´and runx2 (*RUNX2*) 5´-CACTCACTACCACACCTACC-3´ and 5´-TTCCATCAGCGTCAACAC-3´. The target gene expression was normalized against the housekeeping genes.

Alkaline phosphatase (ALP) assay

Briefly, the cells were fixed with 4 % PFA for 1 minute and washed 3 times by 0.05 % Tween 20-PBS. For the ALP staining, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (NBT/BCIP; Sigma, MO, USA) was used as a substrate and the staining was checked every 3 minutes. After the staining was concluded, cells were washed three times for 5 minutes with PBS. The images were taken using a Zeiss Stemi 2000-C stereoscopic microscope (Zeiss, Germany) and Canon PowerShot A80 camera (Canon, Tokyo, Japan). For ALP enzymatic activity quantification, cells were detached by 0.05 %

Trypsin-ethylenediaminetetraacetic acid (EDTA) (Trypsin, Gibco, NY, USA). The enzymatic activity absorbance was measured at 420 nm using a Synergy HT microplate reader (BioTek, VT, USA) equipped with the Gen5 1.11 program.

Alizarin Red (ARS) assay

To prove the osteogenic potential of hDPSCs and hBMSCs, Alizarin Red was used to stain extracellular calcium deposits. After 21 days of culture, the cells were fixed for 10 minutes with 4% paraformaldehyde. The samples were washed with distilled water and then stained in dark using 2 % ARS (Acros organics, Sigma, MO, USA), pH 4.2 at room temperature for 45 minutes. The staining was then washed with distilled water, followed by 3 rinses of 5 minutes each until no ARS remaining was observed. The images were taken by a Zeiss Stemi 2000-C stereoscopic microscope (Zeiss, Germany) and a Canon PowerShot A80 camera (Canon, Tokyo, Japan). Afterwards, for quantification, the Alizarin Red stained calcium-containing deposits were dissolved in acetic acid (Sigma, MO, USA) and the absorbance of solubilized ARS was measured at 405 nm using a Synergy HT microplate reader (BioTek, VT, USA).

Statistical analysis

Statistical analyses were carried out using the IBM SPSS Statistical software (v. 26.0), calculating the mean and standard error for each condition. The data from the different experimental conditions were compared using a Mann Whitney test or ANOVA followed by the Bonferroni or Games-Howell test depending on the homogeneity of the variances. The confidence intervals were fixed at 95 % ($p < 0.05$), 99 % ($p < 0.01$) and 99.9 % ($p < 0.001$).

Hypothesis

In the last decades, dental implants and scaffolds for bone tissue engineering and dental implantology have improved a lot. New materials, surface topography and roughness changes showed to have a direct impact on mesenchymal stem cell differentiation to osteoblasts. With all these new scaffolds that had shown a good induction of osteoblastic differentiation potential due to upgrades in porosity, roughness or shape, the best option for clinical use is still unclear. Thanks to their high proliferation and differentiation potential, MSCs seemed to be the future of bone tissue engineering; however, the debate about the best MSCs for bone regeneration therapies is still open. Finally, tissue engineering is facing the challenge of a widespread use of animal-derived sera like fetal bovine serum (FBS), which must be replaced in order to inactivate the immune response of the cell grafted patients.

With the aim to create a substitute for crucial tissue as periodontal ligament to avoid implant loose due to periimplantitis, we hypothesized that biomimetic BAS^{TM} titanium surfaces and Decellularized Adipose Tissue (DAT) would provide a more effective implant material and scaffold for stem cell osteogenic differentiation for bone tissue engineering. Furthermore, the easily extracted, highly proliferative and osteodifferentiable human dental pulp stem cells (hDPSCs) would be a very good option for autologous regenerative cell therapies. Lastly, plasma-derived products like Plasma rich in growth factors (PRGF) and Platelet rich fibrin (PRF) could provide an excellent FBS substitute for *in vitro* cell cultures. Since it is possible to obtain these plasma products from the patients themselves, this would avoid non-desirable immune responses on regenerative cell therapies.

General Objectives

The main objective of this work was the study of hDPSCs osteoblastic differentiation potential and interactions with biomaterials for bone tissue engineering. To this end, we assessed the effect of Ti6AL4V and BAS^{TM} titanium surfaces and porcine Decellularized Adipose Tissue (pDAT), in combination with plasma-derived products and osteoblastic differentiation media.

Specific objectives

1. Evaluation of cell viability and proliferation of hDPSCs when cultured on Ti6AL4V and BAS titanium surfaces.
2. Assessment of the effect of plasma-derived products PRGF and PRF in hDPSCs proliferation and osteoblastic differentiation.
3. Study of the synergistic effects of combining plasma-derived products in cultures of hDPSCs with Ti6AL4V and BAS titanium growth surfaces in the presence or absence of osteoblastic differentiation media, in terms of cell proliferation and osteoblastic differentiation.
4. Comparison of the proliferation and osteoblastic differentiation potential of hDPSCs and hBMSCs when cultured on Ti6AL4V and BAS titanium surfaces with the presence or absence of osteoblastic induction media.
5. Evaluation of hDPSCs adherence and viability when cultured in Decellularized Adipose Tissue (DAT) scaffolds.
6. Study of DAT scaffold effect on hDPSCs osteoblastic differentiation on *in vitro* cultures in combination with osteoblastic differentiation media.

Results

Isolation, culture and characterization by flow cytometry of the hDPSCs

Human dental pulp stem cells (hDPSCs) were isolated from third molars and cultured in DMEM supplemented with fetal bovine serum (10 %), penicillin/ streptomycin (1 %) and L-Glutamine (1 %). After 2-3 weeks, the cells formed multiple adherent colonies reaching subconfluence. On culture, the hDPSCs showed spindle-like fibroblast shape morphology; the cells maintained this feature as well as being highly proliferative for several passages (Figure 12).

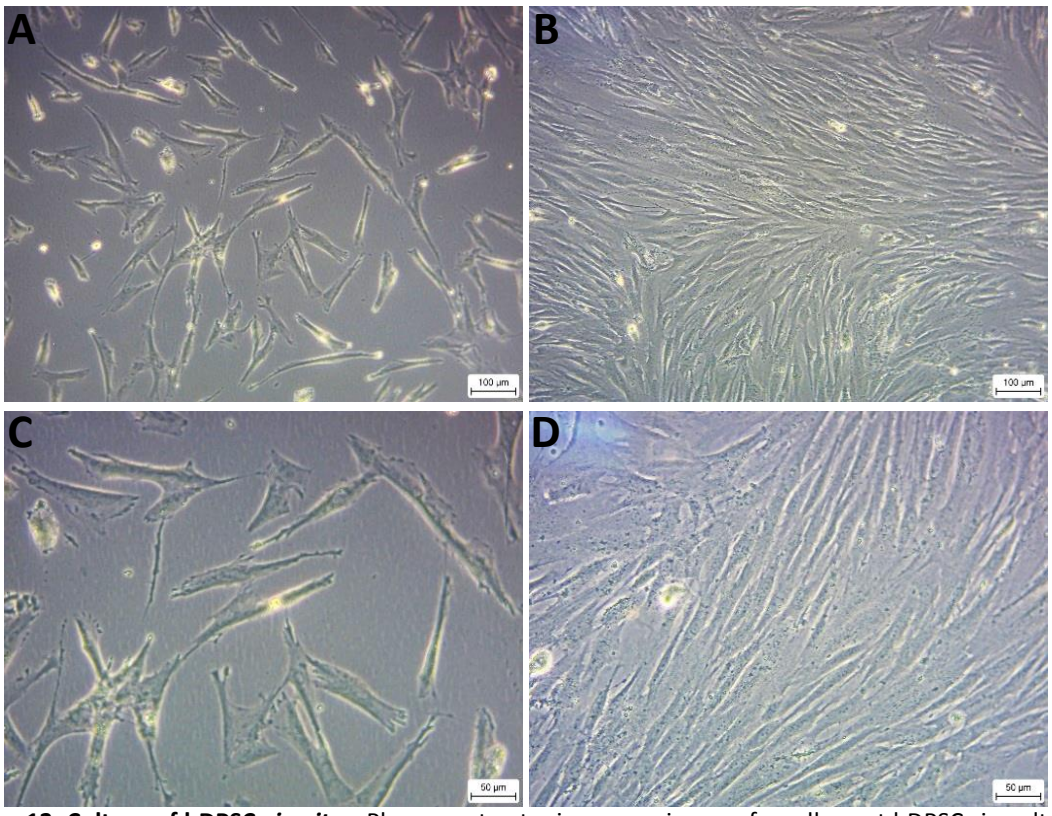


Figure 12. Culture of hDPSCs *in vitro*. Phase-contrast microscopy images for adherent hDPSCs in culture under subconfluence (**A**, **C**) and confluence (**B**, **D**) at low and high magnification. Adapted from (Irastorza et al., 2019).

For the evaluation of the cellular markers of the hDPSCs, we analyzed the cell markers by flow cytometry using antibodies against the following CD (clusters of differentiation) antigens: CD45, CD73, CD90 and CD105. The hDPSCs were negative for CD45, the hematopoietic marker. On the other hand, they were positive for mesenchymal stem cell markers CD73 (100 %), CD90 (100 %) and CD105 (100 %). With these results, we can conclude the mesenchymal stem cell phenotype of the dental pulp stem cells on culture (Gronthos et al., 2003) (Figure 13).

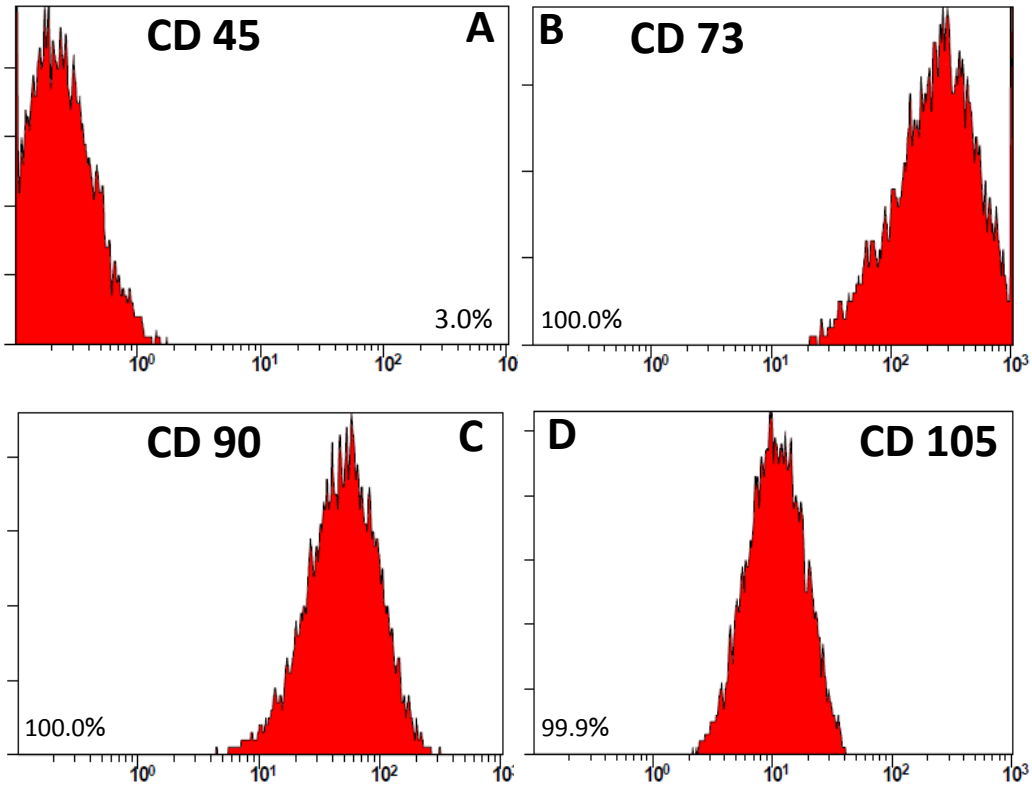


Figure 13. Stem cell markers of the hDPSCs by flow cytometry. Flow cytometry revealed that hDPSCs were all negative for the hematopoietic marker CD45 (**A**) and all positive for the mesenchymal stem cell markers CD73, CD90 and CD105 (**B, C, D**, respectively). Adapted from (Irastorza et al., 2019).

Scanning electron microscopy of Ti6AL4V and BAS™ titanium surfaces for microtopographical analysis

Cell-free raw titanium surfaces SEM images (Ti6AL4V) displaying concentrically parallel grooves (Figure 14A, B), on the other hand, the rough titanium surface (BAS™) showed a surface with numerous pits and bumps and uniformly dispersed small micro pores (< 10 µm) through the whole surface (Figure 14C, D).

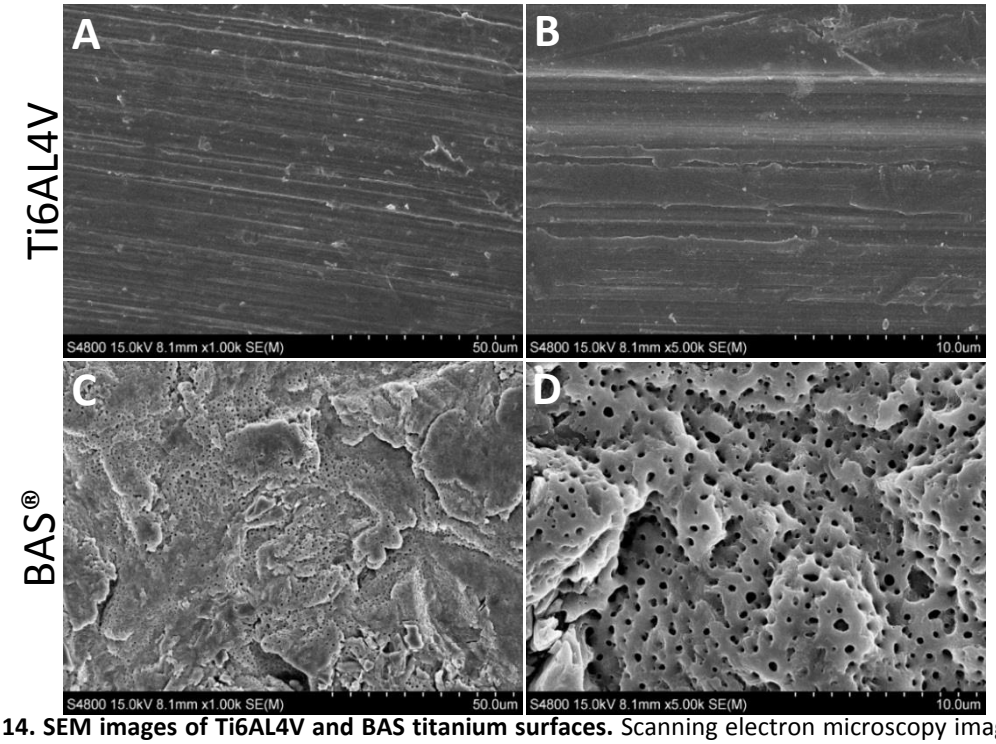


Figure 14. SEM images of Ti6AL4V and BAS titanium surfaces. Scanning electron microscopy images of titanium surfaces, Ti6AL4V (**A, B**) and BAS (**C, D**) with small and high magnifications. Adapted from (Irastorza et al., 2019).

Cell adhesion, migration and viability of hDPSCs on Ti6AL4V and BAS™ titanium surfaces

hDPSCs were seeded on Ti6A14V and BAS™ titanium surfaces, 20,000 cells/disc, for 4 days. The hDPSCs strongly adhered and spread all over the smooth titanium surface Ti6AL4V. The cells grew following the orientation of the disc´s groove pattern and showed several cell elongations similar to lamellipodia (Figure 15 A). On the other hand, the hDPSCs seeded on BAS titanium surface adhered by adapting their morphology to the porous surface, penetrating deeper into the porous structure (Figure 15 B). In both titanium surfaces the cell adhesion showed to be consistent and maintained throughout the entire culture time.

We performed a cell viability and death assay of the hDPSCs seeded on both titanium surfaces by culturing them in the presence of Calcein-AM (green fluorescence) and propidium iodide (red fluorescence). The fluorescence images showed that the hDPSCs were 100 % calcein positive on both titanium discs, demonstrating the high

viability of the hDPSCs grown on the smooth and rough titanium surfaces (Figure 15C, D). Moreover, none of the hDPSCs showed PI red fluorescence, demonstrating that cell death was negligible in these cultures.

In addition, where the nuclei of the hDPSCs had been stained in blue by DAPI, revealed that the cells followed the groove directions on the Ti6A14V surface orientating themselves in a spiral form (Figure 15 E), whereas the hDPSCs had no preferential orientation while been seeded in the BAS titanium (Figure 15 F).

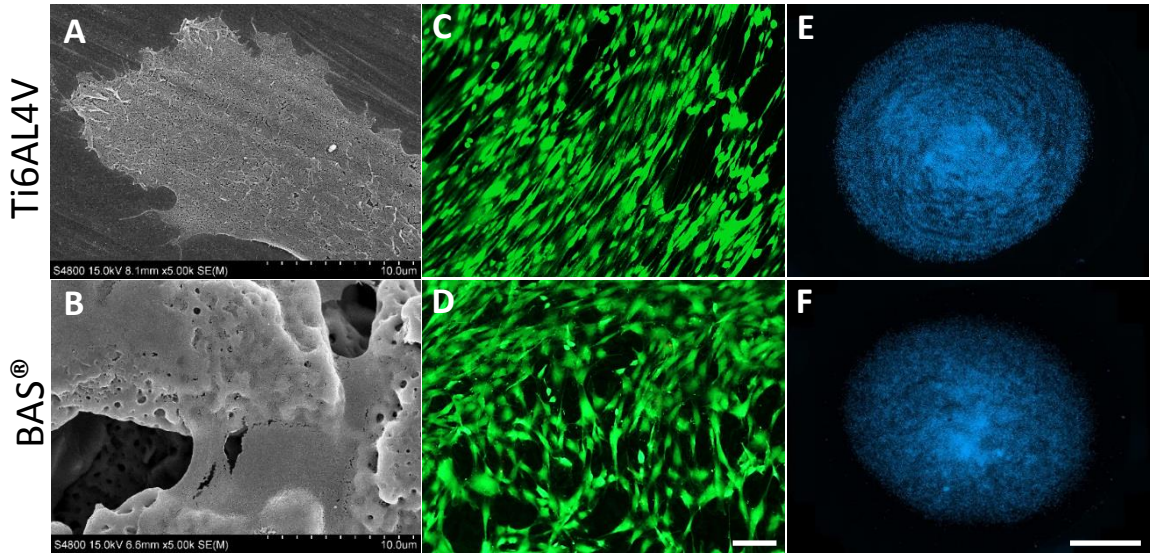


Figure 15. SEM images, viability, orientation and mobility of hDPSCs on titanium surfaces. Scanning electron microscopy images of both titanium surfaces with hDPSC cells seeded on them (**A**, **B**, respectively). Fluorescent microscopy images of the hDPSCs cultured for 4 days on both titanium discs to test viability by calcein (green) and cell death by propidium iodide (red) (**C**, **D**). Scale bar: 100 µm. Fluorescent microscopy image mosaic showing the entire titanium discs with hDPSCs nuclei stained in blue with DAPI on Ti6A14V (**E**) and on BAS (**F**). Scale bar: 2 mm. Adapted from (Irastorza et al., 2019).

Cell proliferation assay of hDPSCs combined with PRGF grown on Ti6AL4V and BAS™ titanium surfaces

The photometric/fluorometric quantification of the viable proliferating cells was precluded due to the opacity of the titanium surfaces. Because of this, the total number of viable cells (interphase or mitosis) were counted assessing their nuclear morphology by using the DNA fluorescent stain DAPI. The images revealed that the nuclear morphology was regular for interphase cells whereas condensed chromosomes could

be seen in mitotic cells. As presumed, most hDPSCs appeared to be in interphase, nevertheless, some cells were at different stages of mitosis, being most of them in metaphase and early and late anaphase. These results indicated the normal growth and proliferation of the hPDSCs. Even so, when the hDPSCs were grown in the presence of soluble PRGF, the number of proliferative cells was significantly higher (20 to 50 %; $p < 0.05$) after 4 days of culture with respect to control conditions (Figure 16F). These results indicated that culturing the hDPSCs in presence of plasma-derived supplements induces an increase of the cell proliferation on titanium surfaces. To corroborate these results, an immunoassay of Ki67 was performed. The Ki67 positive hDPSCs, cultured in presence of PRGF and grown on both titanium surfaces, were counted showing again a significant increase of the proliferative cells (Figure 16A-E).

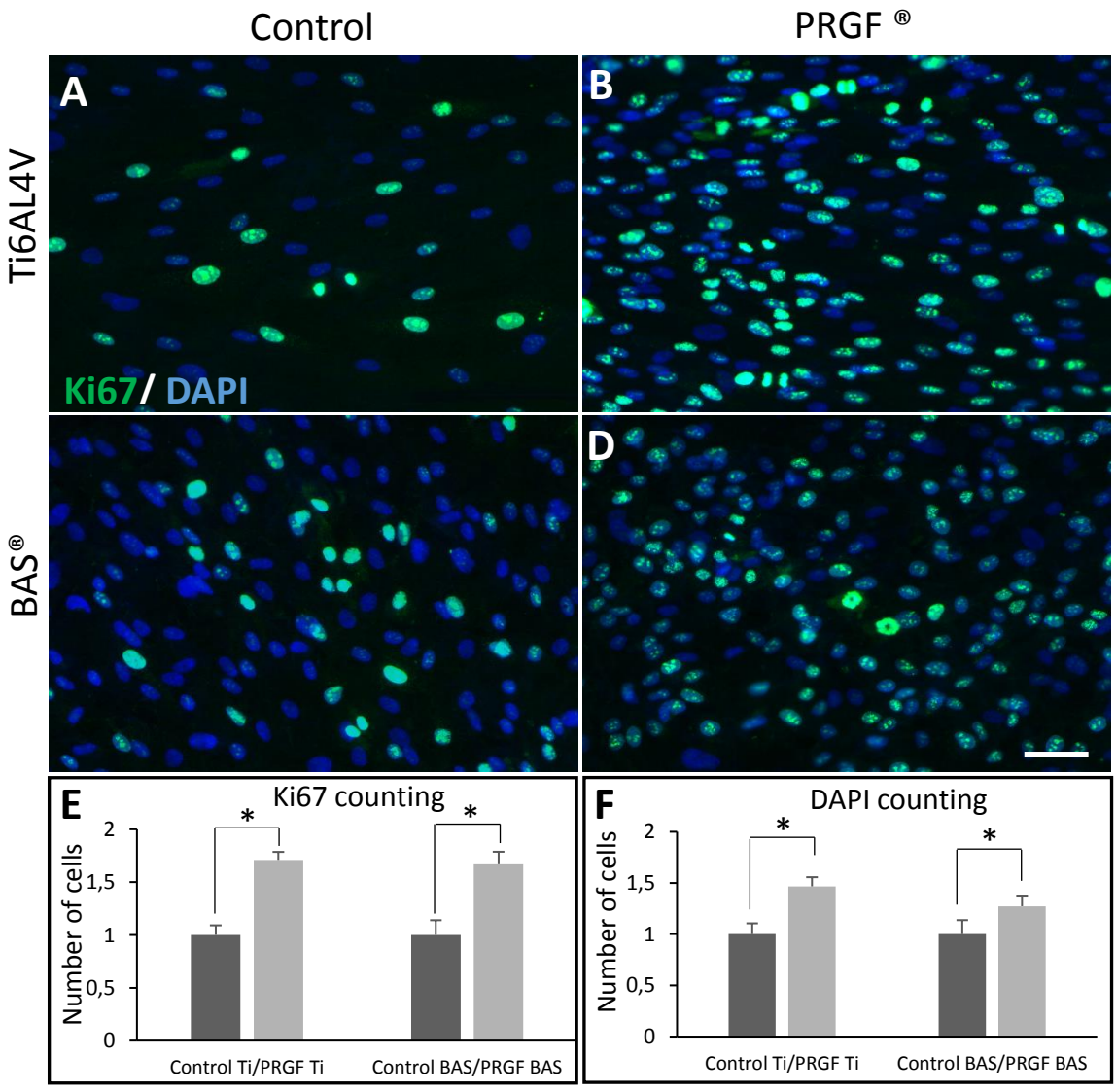
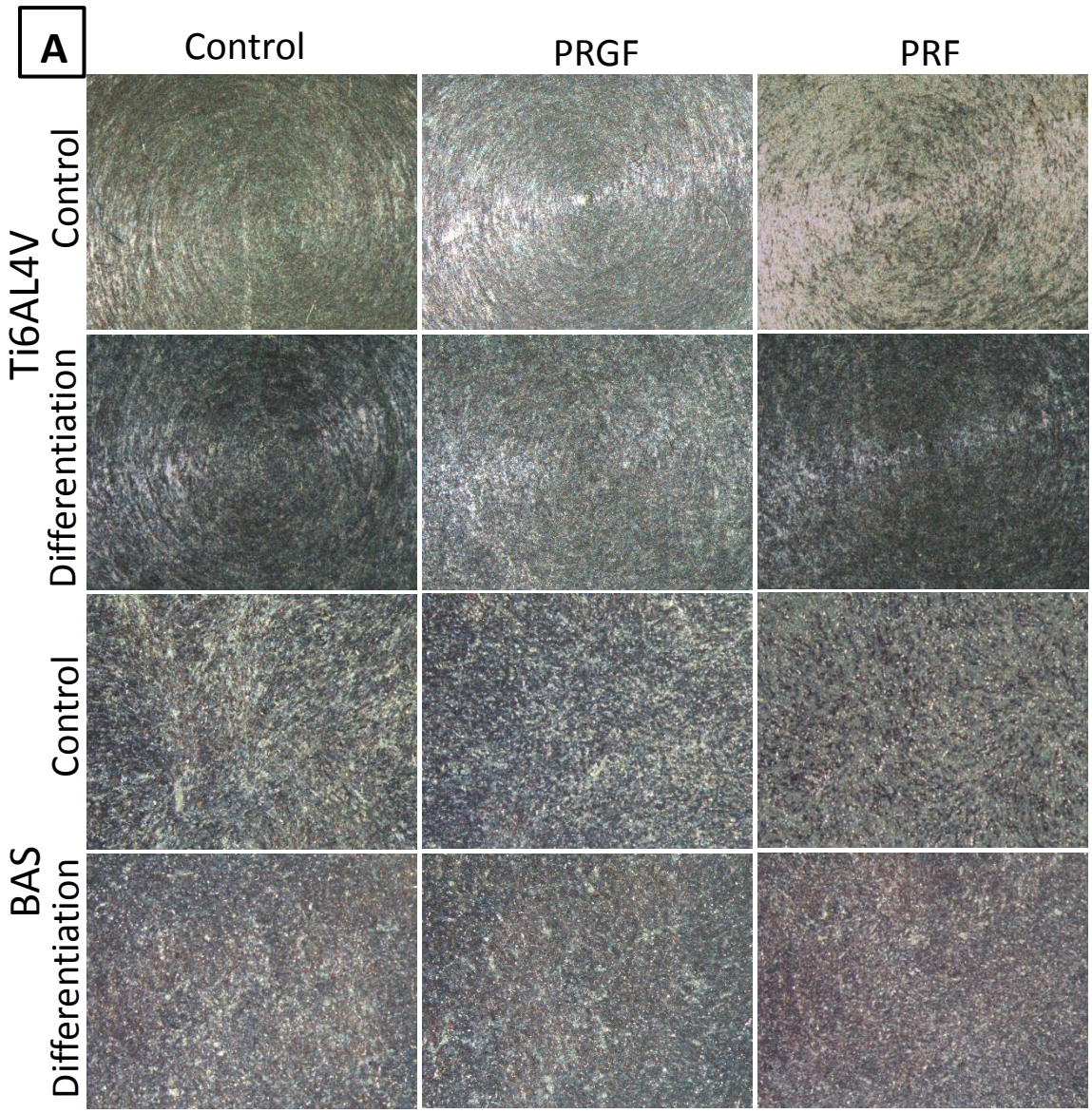


Figure 16. Proliferation assay of the hDPSCs cultured on presence of PRGF on titanium surfaces. Immunofluorescence images of hDPSCs for Ki67 proliferation marker (green) and the nuclei stained with DAPI (blue) in control conditions on Ti6AL4V (**A**) and BAS (**C**) titanium discs, and cultured in presence of 20 % PRGF on Ti6AL4V (**B**) and BAS (**D**) discs for 4 days. PRGF treatment increased the number of Ki67+ cells. Scale bar: 50 μm. Graphs illustrating comparison of total amount of cycling cells (KI67+) between control (normalized number of cells) and PRGF conditions (**E**) and total number of nuclei (DAPI; **F**) of hDPSCs grown on both titanium surfaces. Statistical significance was set at $p \leq 0.05$. Adapted from (Irastorza et al., 2019).

Alkaline phosphatase activity of hDPSCs cultured on Ti6AL4V and BAS™ titanium surfaces

Examination of ALP activity level is a common marker of stem cells early osteoblast differentiation. Alkaline phosphatase enzyme is essential for extracellular matrix mineralization (Orimo, 2010). To promote osteoblastic differentiation, the hDPSCs seeded on the Ti6AL4V and BAS titanium discs were cultured for 7 days in presence of a widely used osteoblastic differentiation induction media consisting of the supplementation of control media with dexamethasone, ascorbic acid and β-glycerol phosphate (Winning et al., 2019). Moreover, two different plasma derived products, PRGF and PRF, were also added to the culture media. ALP activity level rised markedly on the hDPSCs cultured with osteoblastic differentiation medium in both titanium surfaces. However, even in presence of the differentiation medium, the hDPSCs grown with PRGF did not increase their alkaline phosphatase activity (Figure 17A, B). This result is consistent with the high proliferation rates that hDPSCs showed when being cultured with PRGF (Figure 16). However, the hDPSCs seeded on smooth titanium discs and grown in the presence of PRF increased their ALP activity in comparison to the control conditions. Besides, even without the differentiation treatment, the hDPSCs grown on BAS titanium with PRGF also increased their ALP activity compared to the control. This result is possibly due to the high cell density because of the cell proliferation increase, induced by PRGF, as seen before (Figure 16).


A
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PRGF
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Control
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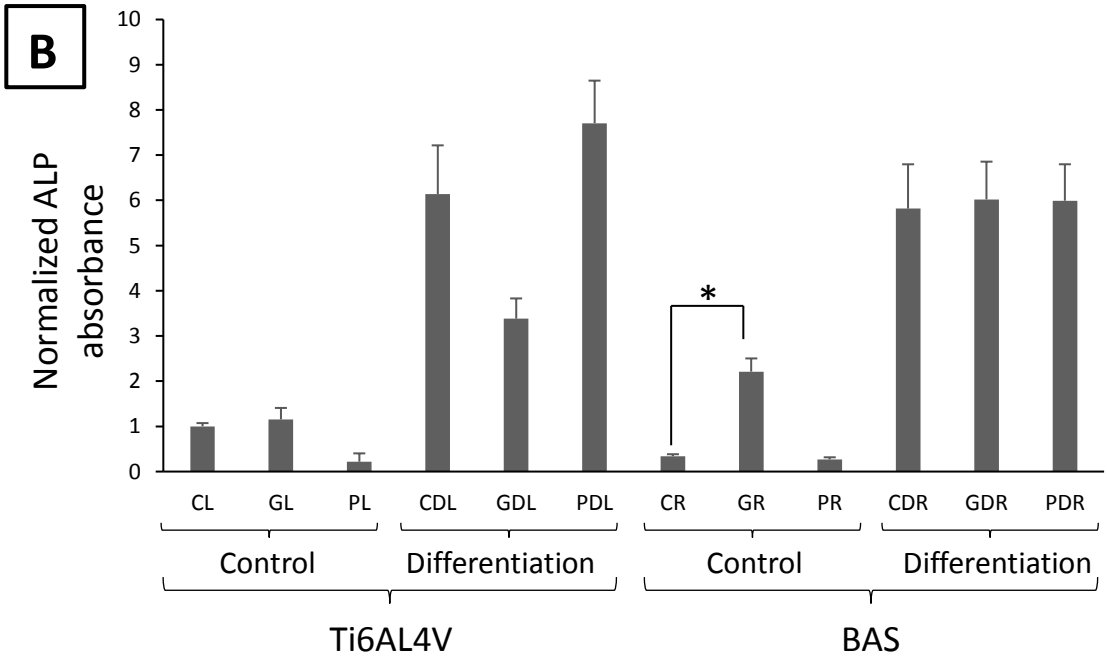
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Control
Differentiation
Control
Differentiation
Ti6AL4V
BAS

Figure 17. Alkaline phosphatase assay of hDPSCs cultured on both titanium surfaces, in presence/absence of osteoblastic differentiation media and plasma derived products (PRGF and PRF). Images of alkaline phosphatase staining of the hDPSCs cultured on Ti6AL4V and BAS titanium discs for 14 days with osteoblastic differentiation media, PRF and PRGF (**A**). Graph illustrating the normalized quantification of the ALP activity (**B**). Graph acronym meaning: C (control), G (PRGF), P (PRF), D (differentiation medium), L (Ti6AL4V titanium) and R (BAS titanium). Statistical significance was set at (*) $p \leq 0.05$. Scale bar: 1 mm. Adapted from (Irastorza et al., 2019).

Alizarin red staining of hDPSCs grown on Ti6AL4V and BAS^{TM} titanium surfaces on terminal osteogenic differentiation

The main evidence of terminal osteogenic differentiation of the stem cells is the formation of calcified bone matrix nodules. This staining was performed by Alizarin red, which stains the calcified extracellular bone matrix nodules in red. The hDPSCs were seeded on plastic, Ti6AL4V and BAS titanium surfaces, grown with the osteoblastic differentiation media for 14 days before alizarin red staining. Images taken by the stereoscopic microscope demonstrated that there was no alizarin red staining on the hDPSCs cultured on plastic in control condition. On the contrary, the hDPSCs grown on both titanium surfaces, showed alizarin stained red spots corresponding to calcified bone matrix depositions. These bone matrix depositions could be identified at the macroscopic level and even in control conditions, being more consistent on the BAS titanium surface (Figure 18C-J). Therefore, as assessed by alizarin red staining, the effect of titanium surfaces demonstrated by itself to be enough to induce the hDPSCs osteoblastic differentiation. Comparing to controls, the effect of the pharmacological osteoblastic induction media seemed not to produce an additional increase of the total number of stained matrix depositions spots when the hDPSCs were grown on the titanium discs. The calcium depositions of the HDPSCs treated with the osteoblastic induction cocktail give the impression of being more intensely stained with alizarin red, particularly on the cells seeded on BAS titanium (Figure 18. I, J). The biggest evidence of the effect of osteoblastic differentiation induction media could be found on the hDPSCs cultured on plastic. On this condition, the differentiation cocktail composed by β-glycerophosphate, dexamethasone and ascorbic acid, lead to a high number of

mineralized bone matrix depositions with respect to the control conditions (Figure 18A, B).

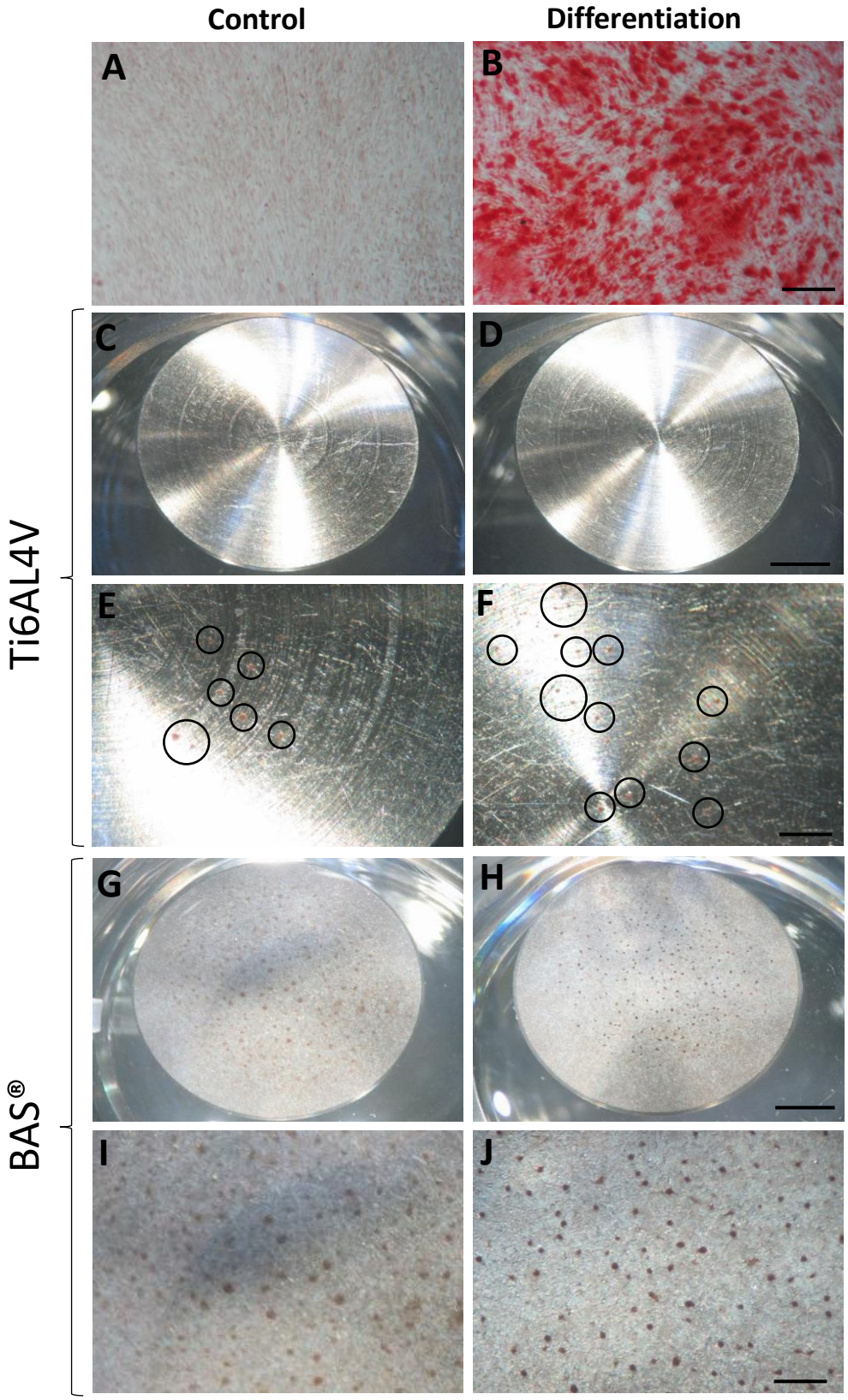
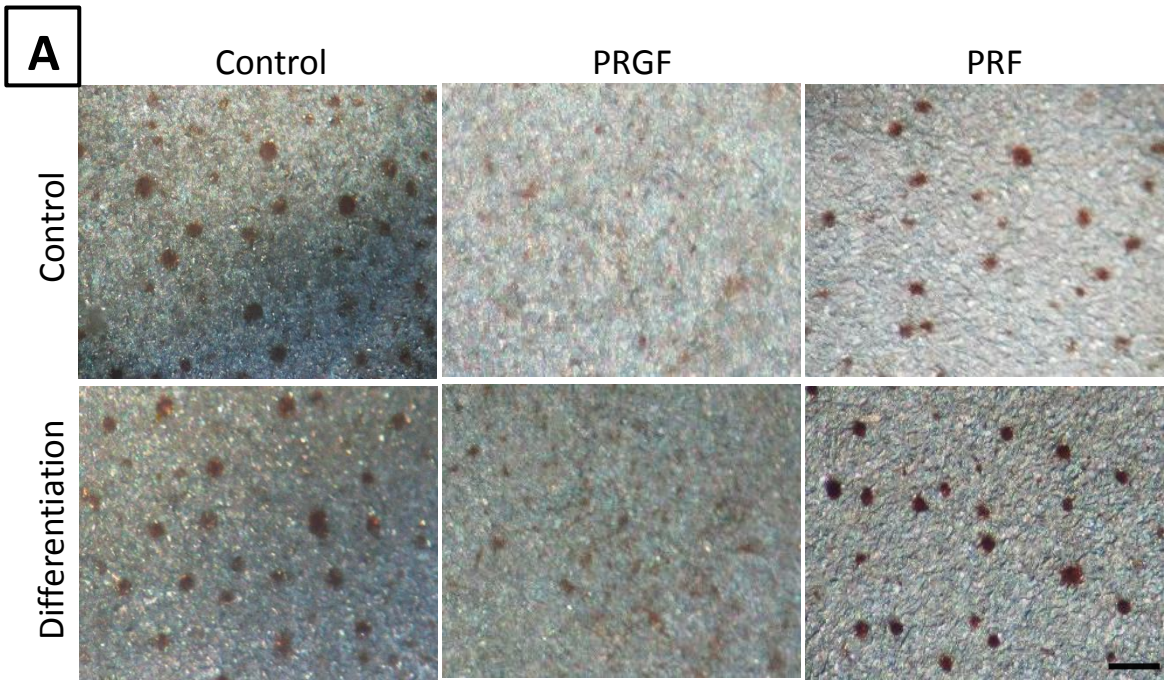


Figure 18. Alizarin red staining assay of hDPSCs grown on Ti6AL4V and BAS titanium surfaces. Alizarin red staining of deposited bone matrix spots in control conditions on plastic (**A**), Ti6AL4V (**C, E**) and BAS (**H, E**) in comparison with the cells treated with osteoblastic differentiation media for 14 days on plastic (**B**), Ti6AL4V (**D, F**) and BAS (**I, J**). Scale bar (**A, B**): 100 µm; scale bar (**C**, **D**, **G**, and **H**): 2mm; scale bar (**E**, **F**, **I** and **J**): 0.5 mm. Adapted from (Irastorza et al., 2019).

Effect of PRGF and PRF supplementation on hDPSCs osteoblastic differentiation cultured on BAS™ titanium surface

After showing the hDPSCs potential to differentiate to bone-producing cells on both titanium surfaces, we investigated the effect of PRGF and PRF on hDPSCs osteodifferentiation. To evaluate the effect of the two plasma derived products, PRGF and PRF, they were added to the hDPSCs seeded on BAS titanium surface in control and differentiation conditions for 21 days. The bone matrix depositions produced by hDPSCs were found in all conditions in presence or absence of PRGF and PRF. However, the decrease of stained mineralized nodules was evident in both hDPSCs conditions grown with the supplementation of PRGF (Figure 19 A). On the contrary, even not showing an increase of the total number of mineralized nodules, the hDPSCs cultured in presence of PRF produced a more intense staining of the deposited bone matrix spots. This result was assessed by photometric quantification after the dissolution of the alizarin red precipitates (Figure 19 B).



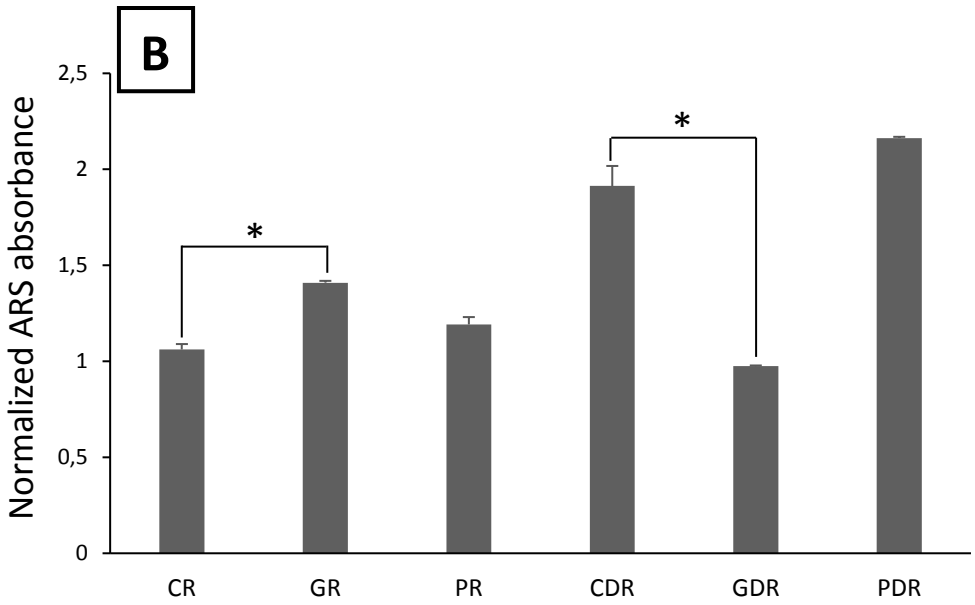


Figure 19. Alizarin red staining and photometric quantification of hDPSCs grown in presence of PRGF and PRF on BAS titanium surface. Alizarin red staining of hDPSCs cultures on BAS titanium surface in control and osteoblastic differentiation conditions and in presence or absence of PRGF and PRF for 21 days (**A**). Normalized photometric quantification of Alizarin red staining (**B**). Graph acronym meaning: C (control), G (PRGF), P (PRF) and D (differentiation medium). Statistical significance was set at (*) $p \leq 0.05$. Scale bar: 250 µm. Adapted from (Irastorza et al., 2019).

RT-QPCR of osteoblastic differentiation markers of the hDPSCs cultured on Ti6AL4V and BAS^{TM} titanium surfaces in presence or absence of plasma-derived products, PRGF and PRF

The staining of the mineralized bone matrix deposits by alizarin red showed the osteoblastic differentiation of the hDPSCs cultured in the presence of PRGF and PRF. However, how the osteoblastic differentiation molecular signaling pathways were affected by the plasma derived products remained unclear. To shed light to this question, we chose representative gene markers involved on the different stages of the osteoblastic differentiation. This battery of gene markers was composed by Collagen I (main organic compound of the bone extracellular matrix), RUNX2 (immature osteoblast marker), SPARC (osteonectin, intermediate secretory osteoblast marker) and OSTERIX/SP7 (fully mature secretory osteoblast marker).

The RT-QPCR results showed that even the hDPSCs cultured without osteoblastic differentiation media expressed Col-I, RUNX2, SPARC and OSTERIX when they were

seeded on both titanium surfaces (Figure 20). Besides, most of the conditions where the hDPSCs were grown in presence of PRGF or PRF, revealed an increase of the expression of these markers. The expression of Collagen 1 between control and plasma derived products containing conditions showed certain variability. Nevertheless, the conditions where the hDPSCs were cultured in presence of PRGF and PRF demonstrated a remarkable increase of the expression of pre-osteoblastic markers RUNX2 and SPARC. Between these two markers, we have to mention especially SPARC marker, whose expression levels in the conditions containing PRGF and PRF raised 6-11 fold with respect to the controls, either in presence or absence of the osteoblastic differentiation treatment (Figure 20 C). Even when the PRGF and PRF demonstrated having an enhancing effect in the osteoblastic differentiation process of the hDPSCs as demonstrated by the raise of expression of the pre-osteoblastic markers RUNX2 and SPARC, we found other interesting differences between these two plasma-derived products. On contrary to RUNX2 and SPARC expression increase can make us think, the expression levels of fully mature osteoblast marker OSTERIX was only increased on the conditions containing PRF. It is important to mention that the hDPSCs in control conditions expresses Collagen 1, RUNX2 and SPARC, being naturally on an osteoblastic pre-differentiated stage. It is noticeable the significant augment (10-30 fold) of OSTERIX gene expression in all conditions containing PRF, either in presence or absence of the osteoblastic differentiation media (Figure 20 D). Nevertheless, it is also remarkable that the conditions where the hDPSCs were cultured in presence of PRGF did not have an increase of the expression of this marker but the expression of OSTERIX was even lower than in control conditions.

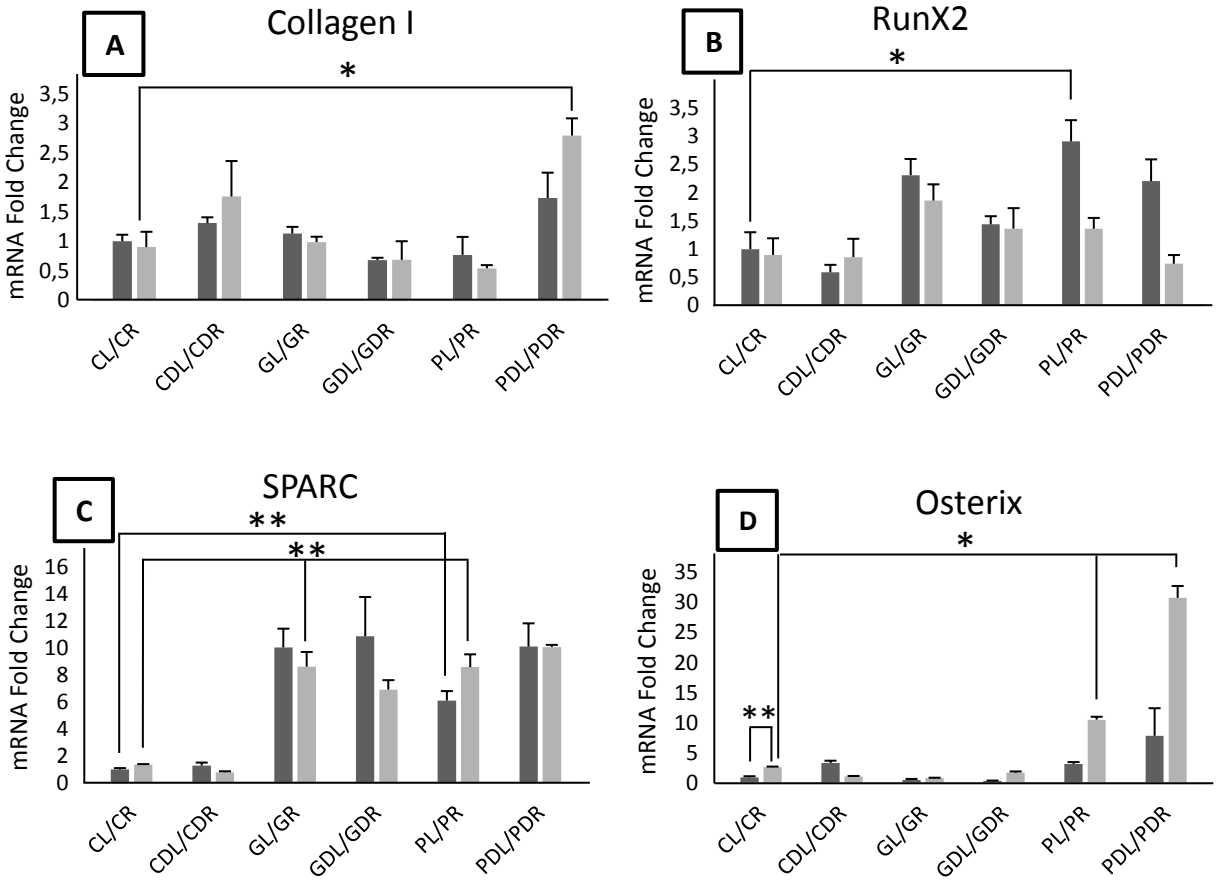


Figure 20. QPCR of osteoblastic differentiation gene expression of hDPSCs cultured on Ti6AL4V and BAS™ titanium surfaces in presence or absence of PRGF and PRF. Normalized mRNA expression of hDPSCs after 14 days of culture for Collagen I, RUNX2, SPARC and OSTERIX. Statistical significance was set at (*$p \leq 0.05$) and (**$p \leq 0.01$). CL: control Ti6aI4V; CR: control BAS; CDL: control/ Ti6aI4V/differentiation treatment; CDR: control/BAS/differentiation treatment; GL: Ti6aI4V/PRGF; GR: BAS/PRGF; GDL: Ti6aI4V/ PRGF/differentiation treatment; GDR: BAS/PRGF/differentiation treatment; PL: TI6AI4V/PRF; PR: BAS/PRF; PDL: TI6AI4V/PRF/differentiation treatment; PDR: BAS/PRF/differentiation treatment. Adapted from (Irastorza et al., 2019).

Comparative study of cellular death by Caspase 3 between hDPSCs and hBMSCs cultured on Ti6AL4V and BAS™ titanium surfaces at 24 and 48 hours

BMSCs are other MSCs with extensively investigated osteoblastic differentiation potential. Nowadays, the election of the best stem cell choice for bone regeneration therapies is still unclear. Because of this, we performed a comparative study of viability, proliferation and osteoblastic differentiation between these two cell types. Caspase 3 is

a widely used marker of cell death by apoptosis. To assess the differences in cellular death between hDPSCs and hBMSCs, influenced by titanium surfaces, both types of cells were seeded on Ti6AL4V and BAS titanium discs for 24 and 48 hours. After that period we performed an immunofluorescence assay to detect apoptotic cells (caspase 3 +). The results demonstrated that none of the two titanium surfaces had any cytotoxic effect on hDPSCs and hBMSCs at 24 and 48 hours. Both types of cells showed having only one or two apoptotic cell in the whole disc, being dying cell proportion less than 0.1 %. The cell nuclei were stained in blue with DAPI (Figure 21).

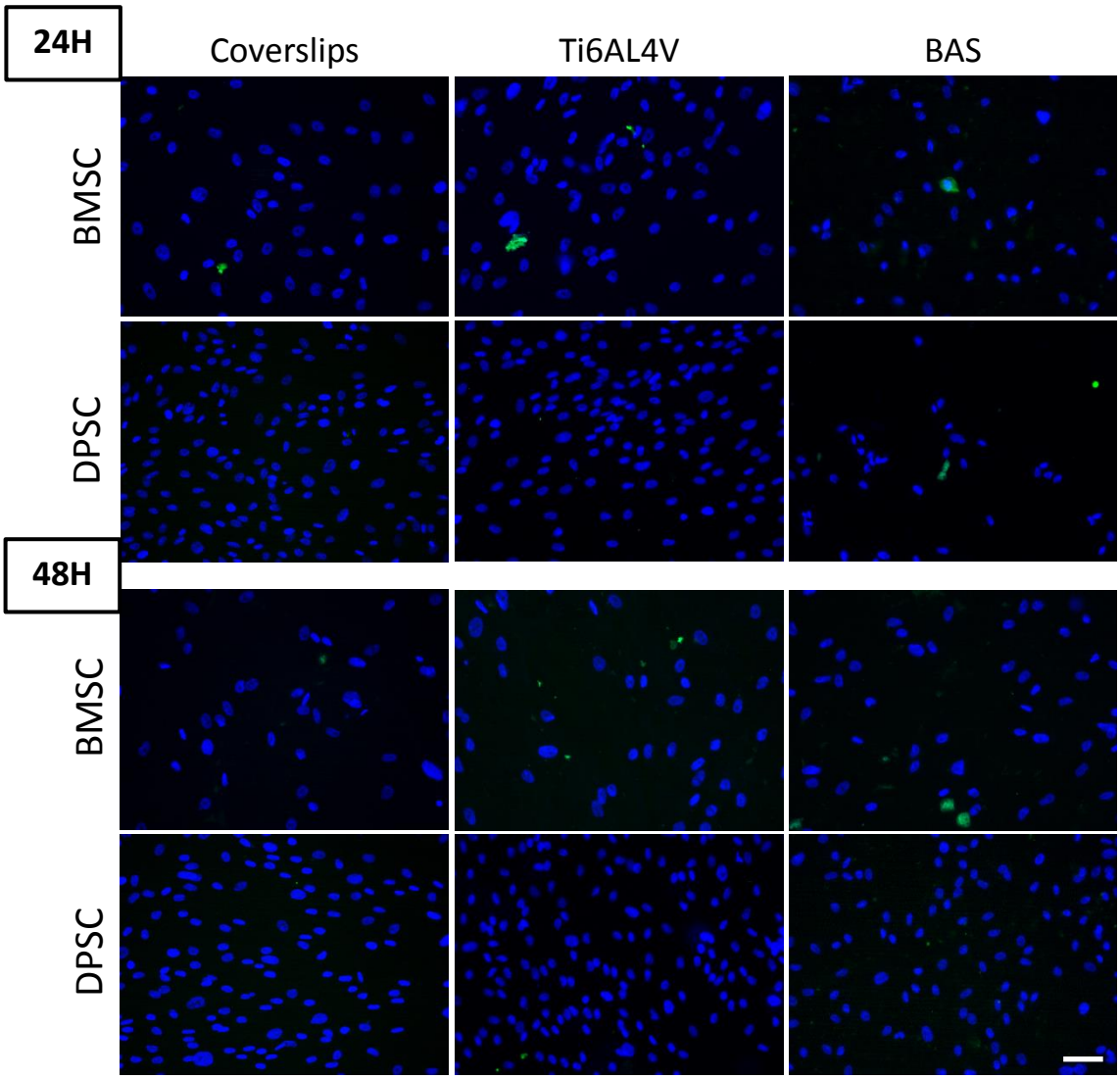
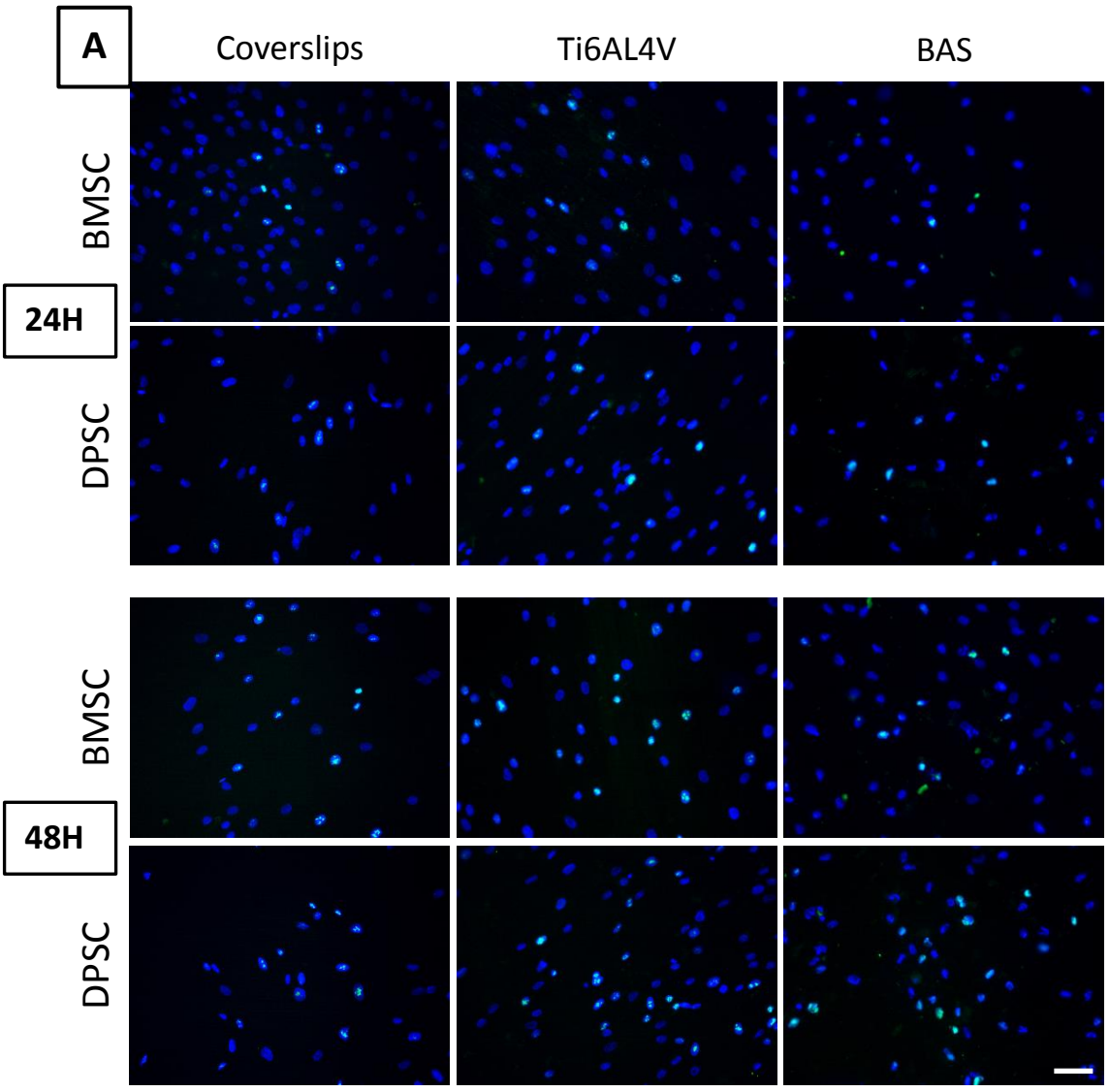


Figure 21. Immunofluorescence images of cell death marker Caspase 3 in hDPSCs and hBMSCs cultured for 24 and 48 hours on coverslips, Ti6AL4V and BAS titanium surfaces. Immunofluorescence images of hDPSCs and hBMSCs stained with apoptotic cell marker Caspase 3 (green) and nuclei stained with DAPI

(blue) seeded on coverslips, Ti6AL4V and BAS titanium discs for 24 and 48 hours. The images demonstrated that both titanium surfaces had no cytotoxic effect on hDPSCs and hBMSCs finding only one or two positive cells for Caspase 3 in the whole disc. Scale bar: 100 µm.

Comparative study of cell proliferation between hDPSCs and hBMSCs cultured on Ti6AL4V and BAS^{TM} titanium surfaces at 24 and 48 hours

To assess cell proliferation difference between hDPSCs and hBMSCs when cultured on coverslips, Ti6AL4V and BAS titanium discs for 24 and 48 hours, we performed an immunofluorescence assay for the detection of proliferative cell marker Ki67 (green) and nuclei stained with DAPI (blue). Due to the opacity of the titanium discs, a manual count of total number of cells (DAPI +) and total proliferative cells (ki67 +) was required. As expected, the majority of both types of cells were found in interphase, however, we found some interesting differences between hDPSCs and hBMSCs and also because of the influence of the titanium surfaces (Figure 22 A). As we can see in the graphs of normalized Ki67/DAPI, after the first 24 hours, there is a significant difference between hDPSCs and hBMSCs proliferation proportion in coverslips, having the first ones bigger proliferation rate. This proportion is also bigger in hDPSCs when cultured on Ti6AL4V and BAS titanium discs, being slightly higher in Ti6AL4V titanium surface in both types of cells than in BAS. After 48 hour of culture, hDPSCs and hBMSCs proliferation rate on cristal seemed to equalize, not having significant differences. However, in Ti6AL4V titanium, hDPSCs demonstrated a significant higher proliferation rate than the hBMSCs. Finally, BAS titanium surface showed interesting results where the proliferation rate of hDPSCs is also significantly higher respect to hBMSC. In addition, hDPSCs proliferation rate increased almost reaching control conditions. On the other hand, hBMSCs exhibit lower proliferation than even after 24 hour of culture, being the lowest proliferation rate of the experiment (Figure 22 B).


A
Coverslips
Ti6AL4V
BAS
BMSC
24H
DPSC
BMSC
48H
DPSC

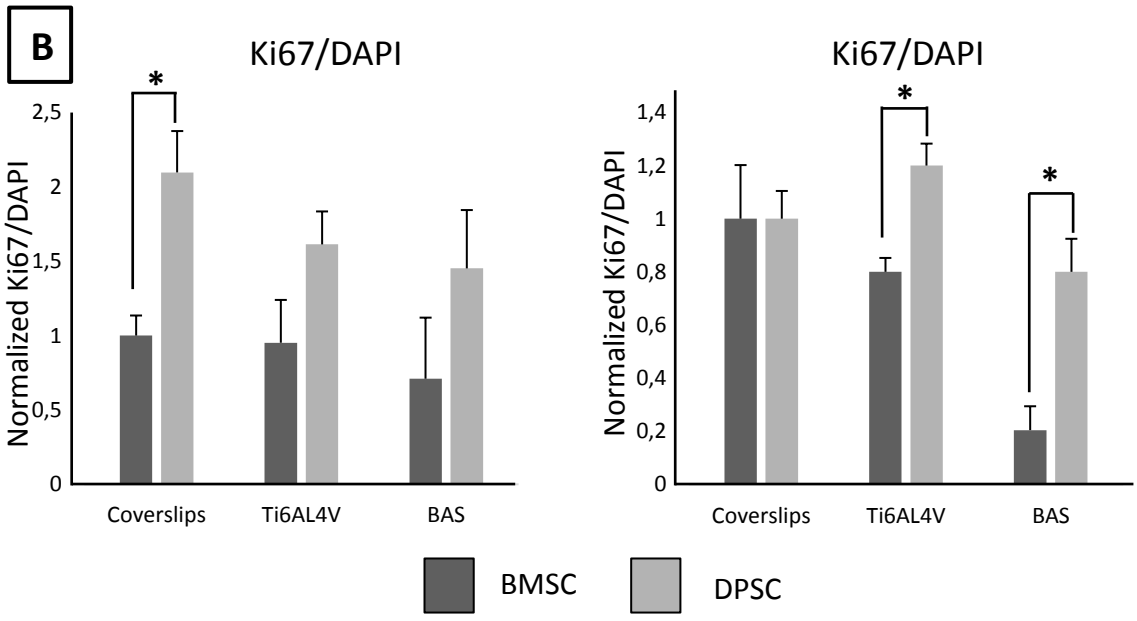

B
Ki67/DAPI
Normalized Ki67/DAPI
2,5
2
1,5
1
0,5
0
*
Coverslips
Ti6AL4V
BAS
Ki67/DAPI
Normalized Ki67/DAPI
1,4
1,2
1
0,8
0,6
0,4
0,2
0
*
*
Coverslips
Ti6AL4V
BAS
BMSC
DPSC

Figure 22. Proliferation assay between hDPSCs and hBMSCs in coverslips, Ti6AL4V and BAS titanium surfaces at 24 and 48 hour of culture. Images taken by immunofluorescence microscope of hDPSCs and hBMSCs stained with proliferation marker Ki67 (green) and the nuclei counterstained with DAPI (blue) at 24 and 48 hours of culture (**A**). Graphs showing the proliferation rate difference between both types of cells while being cultured on different surfaces (**B**). Scale bar: 100 µm. Statistical significance was set at (*$p \leq 0.05$).

Immunofluorescence images of osteoblastic differentiation markers SPARC and Osterix of hDPSCs and hBMSCs cultured on Ti6AL4V and BAS^{TM} titanium surfaces in presence or absence of osteoblastic differentiation media.

With the aim of studying the expression of osteoblastic differentiation markers hDPSCs and hBMSCs were seeded on both titanium discs and were grown with control and osteoblastic induction media for 14 days. After fixation, we performed an immunofluorescence assay for the detection of Osteonectin (SPARC) and Osterix. The nuclei of both cells were counterstained with DAPI. We observed that after 14 days of culture both cells expressed SPARC independently of the culture medium (Figure 23). SPARC seemed to localize in the cytosol with higher congregations around the nuclei. Moreover, only the hBMSCs seeded on Ti6AL4V titanium and treated with differentiation media showed a somewhat enhance of SPARC expression. On the other hand, Osterix showed higher expression in hBMSCs than in hDPSCs in all conditions (Figure 24). Contrary to SPARC, Osterix (transcription factor) was located in the nuclei. Even been more expressed on hBMSCs, this cells did not have big differences between control and osteoblastic treatment in contrast to hDPSC, were we saw an enhance expression of Osterix after growing in presence of osteoblastic differentiation media.

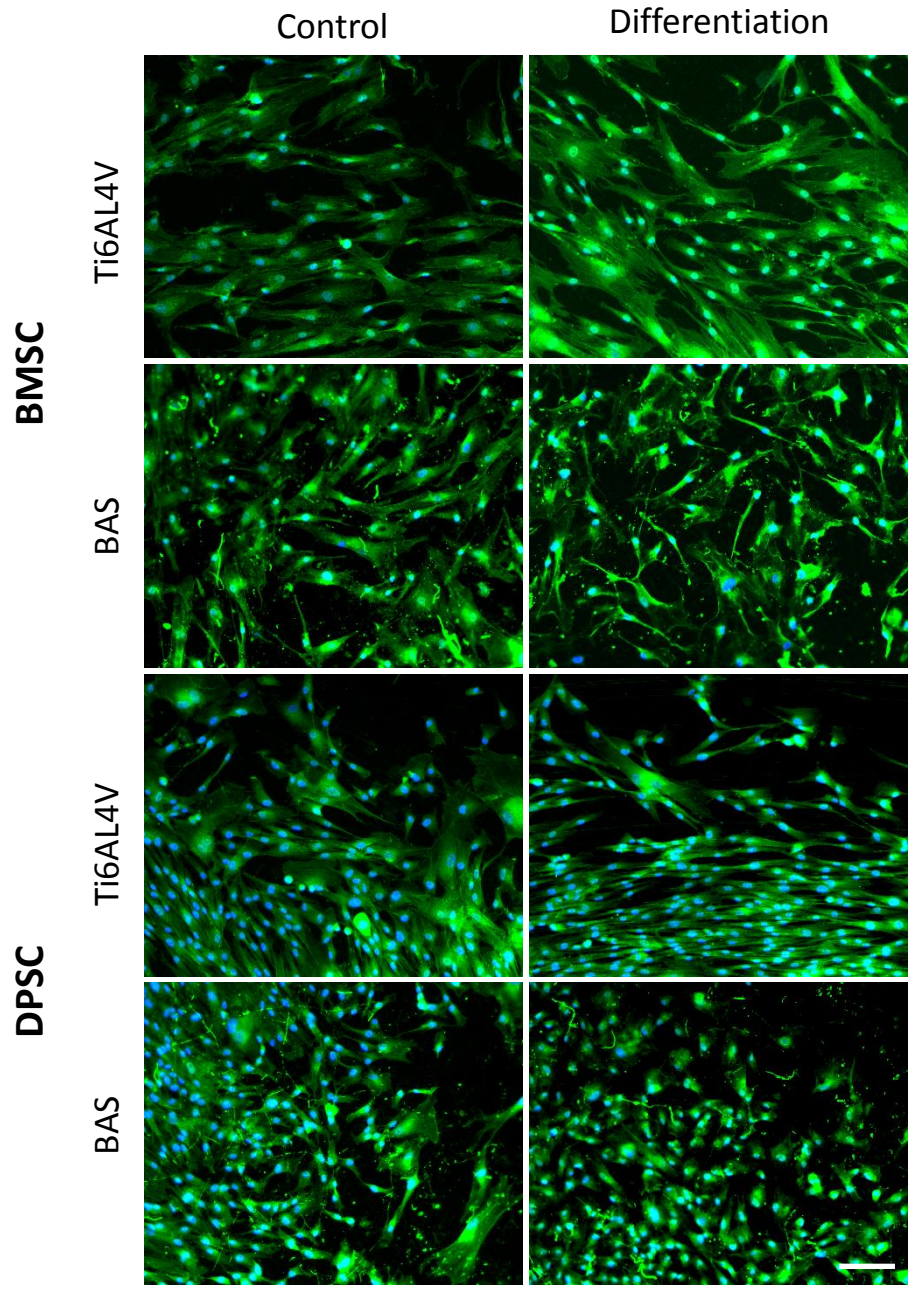


Figure 23. Immunofluorescence images of osteoblastic differentiation marker SPARC in hDPSCs and hBMSCs seeded on Ti6AL4V and BAS titanium discs and grown in presence or absence of osteoblastic differentiation media. hDPSCs and hBMSCs were cultured on both titanium discs and grown with control and osteoblastic induction media for 14 days. After fixation, they were stained for osteoblastic differentiation marker SPARC (green) and counterstained with DAPI (nuclei). Scale bar: 100 µm.

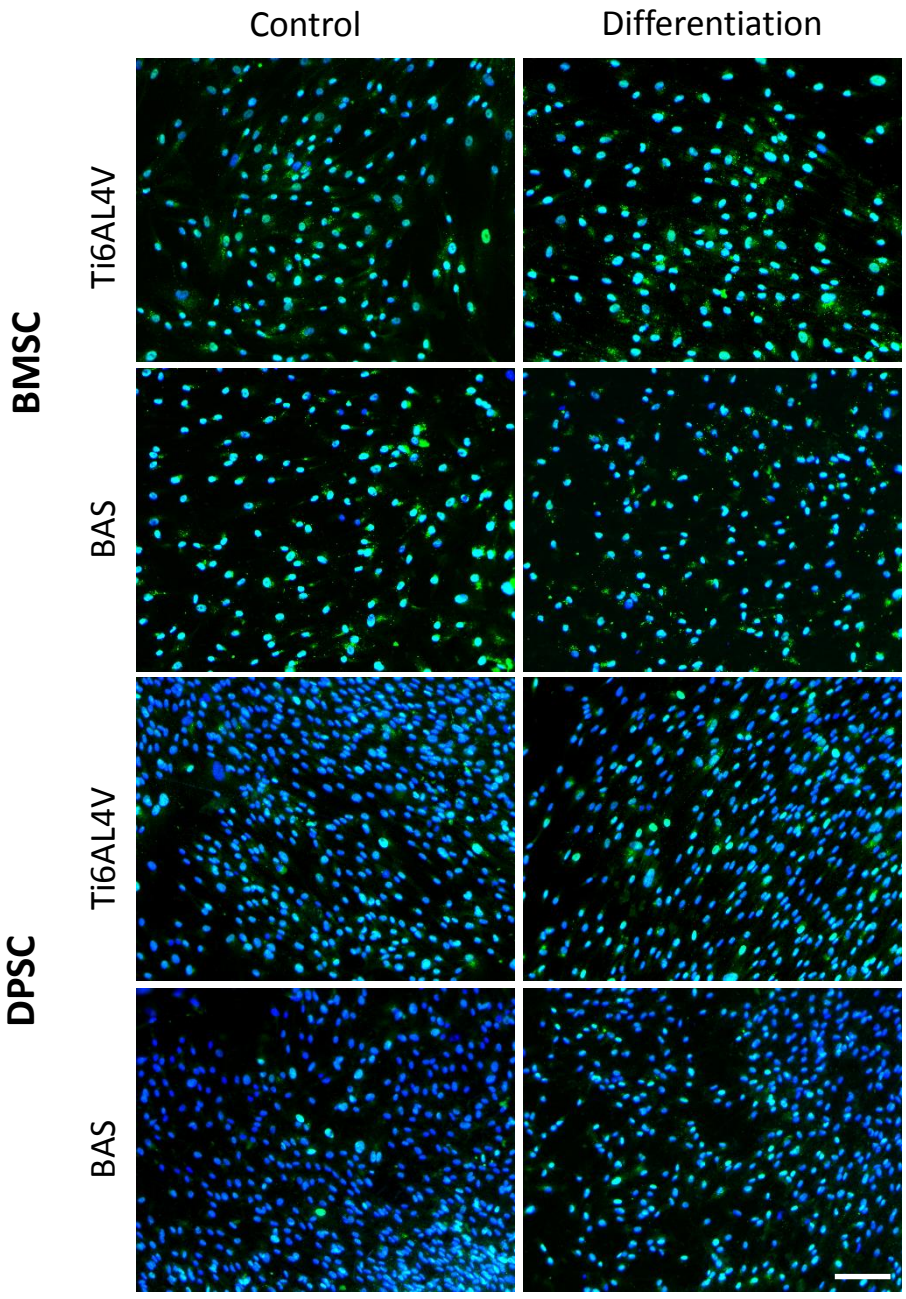


Figure 24. Immunofluorescence images of osteoblastic differentiation marker Osterix in hDPSCs and hBMSCs seeded on Ti6AL4V and BAS titanium discs and grown in presence or absence of osteoblastic differentiation media. hDPSCs and hBMSCs were cultured on both titanium discs and grown with control and osteoblastic induction media for 14 days. After fixation, they were stained for osteoblastic differentiation marker Osterix (green) and counterstained with DAPI (nuclei). Scale bar: 100 µm.

Comparison of mineralized bone matrix deposits by Alizarin red of hDPSCs and hBMSCs cultured on Ti6AL4V and BAS^{TM} titanium surfaces

Extracellular calcified bone matrix nodules are the confirmation of fully differentiated osteoblastic cells. These deposits were stained by Alizarin red obtaining an orange/red color. The hDPSCs and the hBMSCs were seeded on plastic, Ti6AL4V and BAS titanium surfaces for 21 days in presence or absence of osteoblastic differentiation media. In general, the hDPSCs seemed to have slightly more mineralization than the hBMSCs in all conditions but it was not enough to reach statistical significance. However, the hDPSCs seeded on both titanium discs and cultured with osteoblastic differentiation media demonstrated having more mineralization in comparison with the hBMSCs cultured in the same condition (Figure 25), but it still needs to be repeated to make firm conclusions. In addition, this hDPSCs cultured on titanium discs and treated with osteoblastic differentiation media are the only conditions where the mineralization augmented respect to the control conditions. These results corroborated the osteoblastic differentiation induction effect of titanium surfaces to hDPSCs. After the staining of the calcified nodules with Alizarin red, the samples were treated with Acetic acid to dissolve alizarin red so it can be measured in a microplate reader.

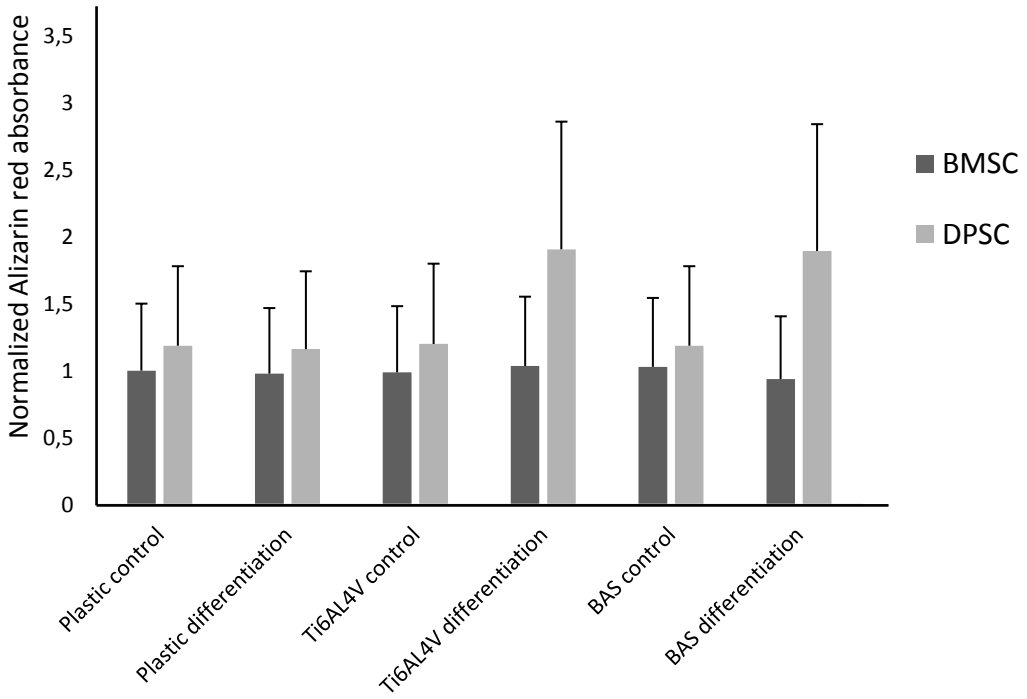


Figure 25. Graph illustrating Alizarin red absorbance of hDPSCs and hBMSCs cultured on plastic, Ti6AL4V and BAS titanium surfaces grown in presence or absence of osteoblastic differentiation media. After culturing the cells for 21 days and dying the extracellular bone matrix deposits, the Alizarin red was dissolved in Acetic acid. A microplate reader measured the absorbance.

RNA sequencing of hDPSCs and hBMSCs cultured on plastic, Ti6AL4V and BAS™ titanium surfaces in presence or absence of osteoblastic differentiation media

To investigate the up and downregulation of different genes and pathways involved in the osteoblastic differentiation process, the hDPSCs and the hBMSCs were seeded on plastic, Ti6AL4V and BAS titanium discs and cultured for 14 days in presence or absence of osteoblastic differentiation media. After the culture, the cells were detached and RNA was extracted using a Qiagen RNA extraction kit and sequenced by Illumina in the Genomic center of the University of Zurich obtaining 25.000 RNA lectures per sample. The data was analyzed by "Pathway Enrichment Analysis" with The Connectivity Map (CMAP) and Biological Process Gene Ontology (GOBP) by the Genome Analysis Platform of the CIC bioGUNE.

We focused on the comparative study of genes that were differentially expressed, in terms of presence/absence (ON/OFF) between the different experimental conditions. We set the threshold limit of 10 raw reads to categorize a gene as effectively expressed (ON) in each condition. The quantity of differently expressed genes were found in the comparison of different conditions (Table 2).

Comparative conditions	Genes
DPSC-C-FLASK VS BMSC-C-FLASK	69
DPSC-C-FLASK VS DPSC-C-TI	7
BMSC-C-FLASK VS BMSC-C-TI	16
BMSC-C-TI VS BMSC-C-BAS	23
DPSC-C-FLASK VS DPSC-T-FLASK	34
BMSC-C-FLASK VS BMSC-T-FLASK	43
DPSC-C-FLASK VS DPSC-T-TI	17
DPSC-T-TI VS DPSC-T-BAS	1
BMSC-C-FLASK VS BMSC-T-TI	64
BMSC-T-TI VS BMSC-T-BAS	35

Table 2. Table illustrating the quantity of genes expressed in one condition and not in the other. Different quantity of genes expression patterns were found when comparing different conditions. Abbreviations: DPSC: dental pulp stem cells; BMSC: bone marrow stem cells; C: control media; T: osteoblastic differentiation media; Flask: plastic culture surface; TI: Ti6AL4V titanium and BAS: Biomimetic Advanced Surface titanium.

The comparative analysis of the ON/OFF genes between different conditions showed some interesting results. hDPSCs and hBMSCs grown with control media over plastic demonstrated the differential expression of many HOX genes between hBMSCs and hDPSCs (Table 3). In addition, the expression of genes specifically involved in osteoblastic differentiation such as SPARC, OSTERIX/SP7 and ZBTB16 showed to be increased when both cells types were cultured with osteoblastic differentiation media and/or seeded on titanium surfaces (Table 3). The case of ZBTB16 was worth mentioning as this was the only gene whose expression was triggered in both cell types (hDPSCs and hBMSCs) under osteoblastic differentiation (pharmacological treatment and/or titanium) conditions, suggesting its possible role as a master gene for osteogenesis. Finally, the neurotrophin receptor and hDPSCs stemness marker NTRK3 and a related gene GFRA2, were expressed in control conditions but we found a shutdown of expression in hDPSCs cultured on titanium with osteoblastic differentiation media (Table 3).

	Identifier	Gene-name	Description	DPSC-C-FLASK	BMSC-C-FLASK
DPSC-C-FLASK VS BMSC-C-FLASK	ENSG00000037965	HOXC8	homeobox C8	0	76
	ENSG00000078399	HOXA9	homeobox A9	0	66
	ENSG00000105991	HOXA1	homeobox A1	0	12
	ENSG00000105997	HOXA3	homeobox A3	0	38
	ENSG00000106004	HOXA5	homeobox A5	0	48
	ENSG00000106511	MEOX2	mesenchyme homeobox 2	0	185
	ENSG00000108511	HOXB6	homeobox B6	0	19
	ENSG00000120093	HOXB3	homeobox B3	0	129
	ENSG00000122592	HOXA7	homeobox A7	0	41
	ENSG00000123388	HOXC11	homeobox C11	0	36
	ENSG00000170370	EMX2	empty spiracles homeobox 2	0	19

	ENSG00000175879	HOXD8	homeobox D8	0	21
	ENSG00000180806	HOXC9	homeobox C9	0	22
	ENSG00000180818	HOXC10	homeobox C10	0	231
	ENSG00000197757	HOXC6	homeobox C6	0	85
	ENSG00000198353	HOXC4	homeobox C4	0	27
	ENSG00000253293	HOXA10	homeobox A10	0	116
	ENSG00000260027	HOXB7	homeobox B7	0	37
				DPSC-C-Flask	**DPSC-C-Ti**
DPSC-C-FLASK VS DPSC-C-TI	ENSG00000170374	SP7	Sp7 transcription factor	0	71
				DPSC-C-Flask	**DPSC-T-Flask**
DPSC-C-FLASK VS DPSC-T-FLASK	ENSG00000109906	ZBTB16	zinc finger and BTB domain containing 16	0	182
	ENSG00000140538	NTRK3	neurotrophic receptor tyrosine kinase 3	12	0
	ENSG00000168546	GFRA2	GDNF family receptor alpha 2	107	0
				BMSC-C-Flask	**BMSC-T-Flask**
BMSC-C-FLASK VS BMSC-T-FLASK	ENSG00000109906	ZBTB16	zinc finger and BTB domain containing 16	0	673
				DPSC-C-Flask	**DPSC-T-Ti**
DPSC-C-FLASK VS DPSC-T-TI	ENSG00000109906	ZBTB16	zinc finger and BTB domain containing 16	0	358
	ENSG00000140538	NTRK3	neurotrophic receptor tyrosine kinase 3	12	0
	ENSG00000170374	SP7	Sp7 transcription factor	0	51
				BMSC-C-Flask	**BMSC-T-Ti**
BMSC-C-FLASK VS BMSC-T-TI	ENSG00000107742	SPOCK2	SPARC (osteonectin), cwcv and kazal like domains proteoglycan 2	0	10
	ENSG00000123364	HOXC13	homeobox C13	0	11
	ENSG00000152583	SPARCL1	SPARC like 1	0	45

Table 3. Gene expression differences between the comparisons of different conditions. Abbreviations: DPSC: dental pulp stem cells; BMSC: bone marrow stem cells; C: control media; T: osteoblastic differentiation media; Flask: plastic culture surface; TI: Ti6AL4V titanium and BAS: Biomimetic Advanced Surface titanium.

Apart from individual gene expression comparisons, we performed a pathway enrichment analysis with The Connectivity Map (CMAP) and Gene Ontology enrichment (GO). On one hand, CMAP is a collaborative effort that utilizes transcriptional expression data to evidence relationships between cell physiology, diseases and therapeutics. On the other hand, GO is a resource to develop and use ontologies to support biologically meaningful annotation of genes and their products. The analyses are divided in three categories: Cellular component (CC), Biological process (BP) and Molecular function (MF). In the present work, we used GOBP for the pathways analysis (Tables 4, 5). Both CMAP and GOBP analysis pipelines highlight the signaling pathways and processes that are predicted to be affected between different experimental conditions, providing an associated probability (p) value and a list of the differential genes (Count) within the total amount of genes (Size) for each described pathway. We set a threshold of a minimum of two affected genes and a significant associated p value ($p < 0.05$) for a pathway to be included in the output data.

CMAP	ID	Size	Count	pvalueadj	Genes	Description
DPSC-C-FLASK VS BMSC-C-FLASK	ALCALAY_AML_NPMC_UP	133	8	6.98724e-09	COCH, HOXA1, HOXA10, HOXA5, HOXA7, HOXB3, HOXB6, HOXB7	Genes up-regulated in acute myeloid leukemia (AML)
	VERHAAK_AML_NPM1_MUT_VS_WT_UP	173	7	1.16885e-06	EREG, HOXA10, HOXA5, HOXA7, HOXB3, HOXB6, TNFSF10	Genes up-regulated in acute myeloid leukemia (AML)
	TAKEDA_NUP8_HOXA9_16D_UP	125	6	3.89713e-06	EREG, HOXA3, HOXA5, HOXA7, HOXB3, TNFSF10	Hematopoietic disorder
	BOQUEST_CD31PLUS_VS_CD31MINUS_UP	552	8	0.000117134	BST2, CHI3L1, COCH, CSF2RB, DOK5, HOXB7, STEAP4, TNFSF10	
	TAKEDA_NUP8_HOXA9_3D_UP	143	5	0.000184436	BST2, HOXA3, HOXA5, HOXA7, HOXB3	effects of NUP98-HOXA9 on gene transcription at 3 days after transduction UP
	TAKEDA_NUP8_HOXA9_6H_UP	66	4	0.000205999	HOXA3, HOXA5, HOXB3, HOXC6	effects of NUP98-HOXA9 on gene

						transcription at 6 hours after transduction UP
	TAKEDA_NUP8_HOXA9_8D_UP	118	4	0.00178462	HOXA3, HOXA5, HOXA7, HOXB3	effects of NUP98-HOXA9 on gene transcription at 8 days after transduction UP
	TAKEDA_NUP8_HOXA9_10D_UP	147	4	0.00369089	HOXA3, HOXA5, HOXA7, HOXB3	effects of NUP98-HOXA9 on gene transcription at 10 days after transduction UP
DPSC-C-FLASK VS DPSC-T-FLASK	BASSO_GERMINAL_CENTER_CD40_UP	93	3	0.0191607	BATF, BCL2A1, HLA-DQB1	Gene up-regulated by CD40 signaling in Ramos cells
	CMV_HCMV_TIMECOURSE_12HRS_UP	25	2	0.0377068	RSAD2, TNFSF10	Genes up-regulated after infection with HCMV at 12 h
	VERHAAK_AML_NPM1_MUT_VS_WT_UP	173	3	0.040305	BCL2A1, SERPINA1, TNFSF10	Genes up-regulated in acute myeloid leukemia (AML)
	CARIES_PULP_UP	200	3	0.0462674	BCL2A1, HLA-DQB1, SERPINA1	Immune/cytokine response

Table 4. Pathway enrichment analysis performed with The Connectivity map (CMAP). Pathway enrichment comparative analysis between hDPSCs and hBMSCs grown on plastic and titanium surfaces, in the presence or absence of osteoblastic differentiation media. The absent comparisons are not in the table because they did not meet the established n= 2 and p< 0.05 requirements. Abbreviations: DPSC: dental pulp stem cells; BMSC: bone marrow stem cells; C: control media; T: osteoblastic differentiation media; Flask: plastic culture surface; TI: Ti6AL4V titanium and BAS: Biomimetic Advanced Surface titanium.

GOBP	ID	Size	Count	pvalueadj	Genes	Description
DPSC-C-FLASK VS BMSC-C-FLASK	GO:0009952	104	12	1,32926E-12	HOXA1, HOXA5, HOXA7, HOXA10, HOXB3, HOXB6, HOXB7, HOXC6, HOXC8, HOXC9, HOXC10, HOXC11	anterior/posterior pattern specification
	GO:0048704	80	7	8,00122E-06	HOXA1, HOXA5, HOXA7, HOXB3, HOXB6, HOXB7, HOXC9	embryonic skeletal system morphogenesis
	GO:0009792	607	13	9,5092E-05	HOXA1, HOXA5, HOXA7, HOXB3, HOXB6, HOXB7, HOXC6, HOXC9, HOXC11, MEOX2, PITX2, TBX18, HEY2	embryo development ending in birth or egg hatching
	GO:0001568	531	10	0,006077272	CHI3L1, EREG, HOXA1, HOXA3, HOXA5, HOXA7, HOXB3, MEOX2, PITX2, HEY2	blood vessel development
	GO:0065007	7094	38	0,009102425	BST2, CHI3L1, CSF2RB, CSTA, EREG, HAS1, HOXA1, HOXA3, HOXA7, HOXA9, HOXA10, HOXB3, HOXB7, HOXC4, HOXC6, HOXC8, HOXC9, HOXC10, HOXC11, HOXD8, LSP1, MEOX2, OPCML, PITX2, SIM1, ZIC1, TNFSF10, WISP3, TBX18, RASSF9, ABCC9, CNKSR2, DOK5, SUCNR1, HHIP, EBF2, NDNF, FOXP2	biological regulation
	GO:0032774	3408	26	0,01596934	EREG, HOXA1, HOXA3, HOXA5, HOXA7, HOXA9, HOXA10, HOXB3, HOXB6, HOXB7, HOXC4, HOXC6, HOXC8, HOXC9, HOXC10, HOXC11, HOXD8, MEOX2, PITX2, SIM1, ZIC1, TBX18, HEY2, EBF2, SPX, FOXP2	RNA biosynthetic process
	GO:0072358	414	8	0,016913155	CHI3L1, EREG, HOXA1, HOXA3, HOXA5, HOXA7, HOXB3, MEOX2	cardiovascular system development
	GO:0009954	30	3	0,022750337	HOXA10, HOXC10, HOXC11	proximal/distal pattern formation
DPSC-C-FLASK VS DPSC-C-TI	GO:0042755	26	2	0,023359184	LEP, TACR1	eating behavior

	GO:0046887	93	2	0,031493385	LEP, TACR1	positive regulation of hormone secretion
	GO:0050880	127	2	0,031493385	LEP, TACR1	regulation of blood vessel size
	GO:0002520	737	3	0,040817267	LEP, RSAD2, SP7	immune system development
	GO:0050867	258	2	0,04102342	HLA-DQB1, TACR1	positive regulation of cell activation
	GO:0009914	281	2	0,048691666	LEP, TACR1	hormone transport
BMSC-C-FLASK VS BMSC-C TI	GO:0010719	22	2	0,022505055	LDLRAD4, TBX5	negative regulation of epithelial to mesenchymal transition
BMSC-C-FLASK VS BMSC-T-FLASK	GO:0035912	5	2	0,042243127	HEY2, DLL4	dorsal aorta morphgenesis
DPSC-C-FLASK VS DPSC-T-TI	GO:0060218	13	2	0,027739491	BATF, SP7	hematopoietic stem cell differentiation

Table 5. Pathway enrichment analysis performed with Gene Ontology Biological Process enrichment (GOBP). Pathway enrichment comparative analysis between hDPSCs and hBMSCs grown on plastic and titanium surfaces, in the presence or absence of osteoblastic differentiation media. The absent comparisons are not in the table because they did not meet the established n= 2 and p< 0.05 requirements. Abbreviations: DPSC: dental pulp stem cells; BMSC: bone marrow stem cells; C: control media; T: osteoblastic differentiation media; Flask: plastic culture surface; TI: Ti6AL4V titanium and BAS: Biomimetic Advanced Surface titanium.

SEM images of decellularized porcine adipose tissue (pDAT) processed as solid foam

The porcine adipose tissue was extracted and the decellularization was performed using isopropanol and Triton X-100, a non-ionic detergent. Once the decellularization process concluded, in order to be used as a biological scaffold for cell cultures *in vitro*, the solid foam was formed by freeze-drying method (Figure 26A, B). SEM images demonstrated the highly interconnected porous structure. The pore size varied between 50 and 100 µm (Figure 26C, D).

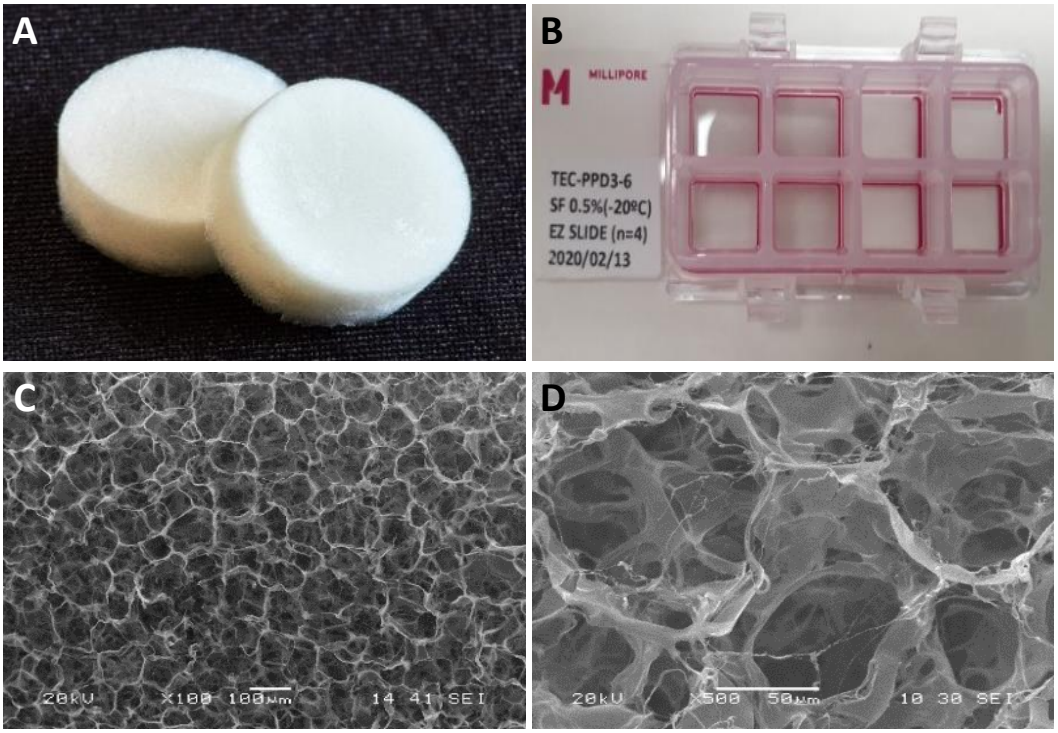


Figure 26. Images of decellularized porcine adipose tissue for *in vitro* use and porous structure details by SEM. Macroscopic images of decellularized porcine adipose tissue for *in vitro* use in combination with cells cultures (**A, B**). SEM images of the porous structure of pDAT showing 50-100 µm prous size (**C, D**).

Viability of hDPSCs cultured on plastic and pDAT by Calcein-AM/ popidium iodide

To exclude the cytotoxic effect of pDAT, 15.000 hDPSCs cells were seeded on both empty well (plastic) and pDAT solid foams for 4 days. Cell viability assay was performed culturing the hDPSCs in presence of live cell dyer Calcein-AM (green fluorescence) and death cell dyer propidium iodide (red fluorescence). Fluorescence microscope images demonstrated that the hDPSCs grown on both surfaces had almost 100% live cells, with all the cells dyed with green fluorescence. Only sporadically were cells found dyed in red with propidium iodide showing the lack of cytotoxicity of pDAT (Figure 27). The main difference we found between this two surfaces was the higher amount of cells found in control plastic (Figure 27A, B). Higher amount of hDPSCs that apparently we had in plastic could be due to monolayer growing they have in plastic in comparison to the 3 dimensional pDAT surface.

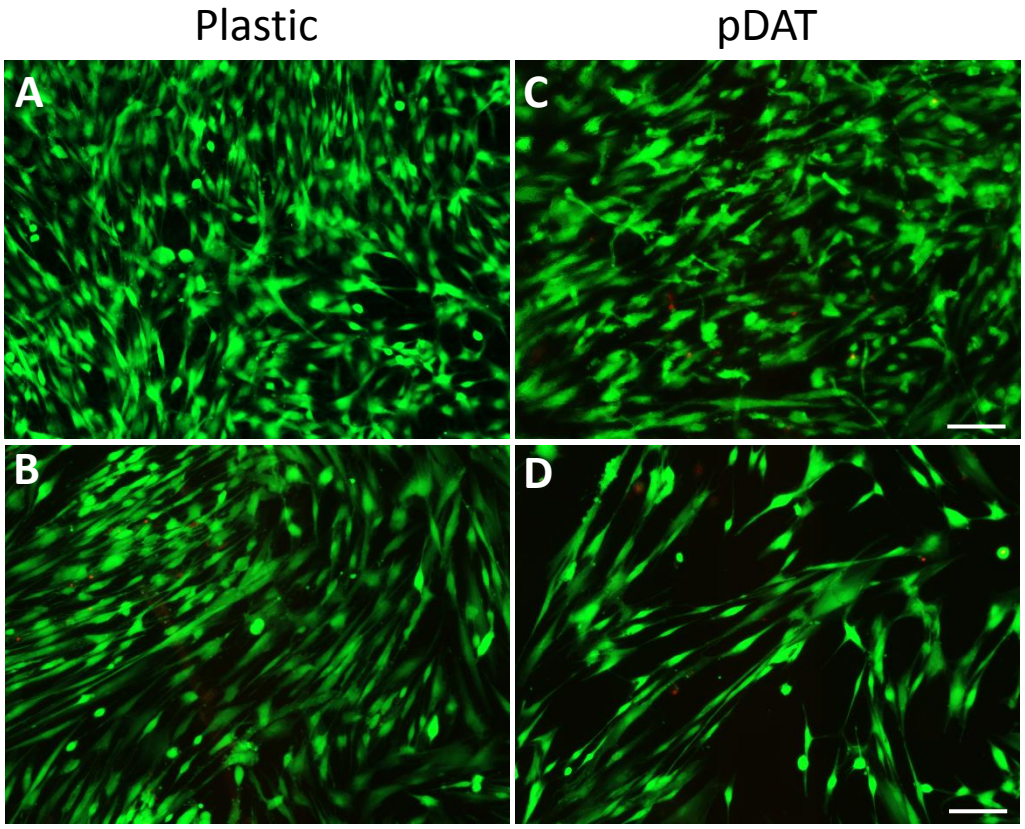


Figure 27. Calcein-AM/ propidium iodire images of hDPSCs cultured on plastic and pDAT solid foams. HDPSCs were seeded at 15.000 cell/well for 4 days and stained with live cell marker Calcein-AM (green) and death cell marker propidium iodide (red). The cells grown on plastic (**A, B**) showed higher amount of hDPSCs than pDAT (**C, D**) due to monolayer growing. Scale bar (A, C): 100 µm; (B, D): 50 µm.

hDPSCs culture on solid foam pDAT and bone-producing osteoblastic cell markers immunofluorescence.

pDAT solid foam was integrated to 8-well Minicell EZ slides (Merck Millipore PEZGS0816) for *in vitro* use in cell culture. The hDPSCs were seeded al 15.000 cell/ well density and grown for 14 days in presence of control media (DMEM) and osteoblastic differentiation media. Cell nuclei stained with DAPI showed less number of cells on pDAT porous structure than in plastic wells were the hDPSCs had grown as an adherent monolayer (Figure 28A). After the 14 days of culture, immunofluorescence assay was performed for the detection of osteoblastic differentiation markers Osteocalcin (BGLAP) and Osteonectin (SPARC). hDPSCs seeded in plastic and pDAT expressed this two

osteoblastic differentiation markers. The hDPSCs grown on pDAT scaffold had significantly higher amounts of BGLAP and SPARC relative intensity, both in control and osteoblastic differentiation conditions (Figures 28B, C; 29B, C).

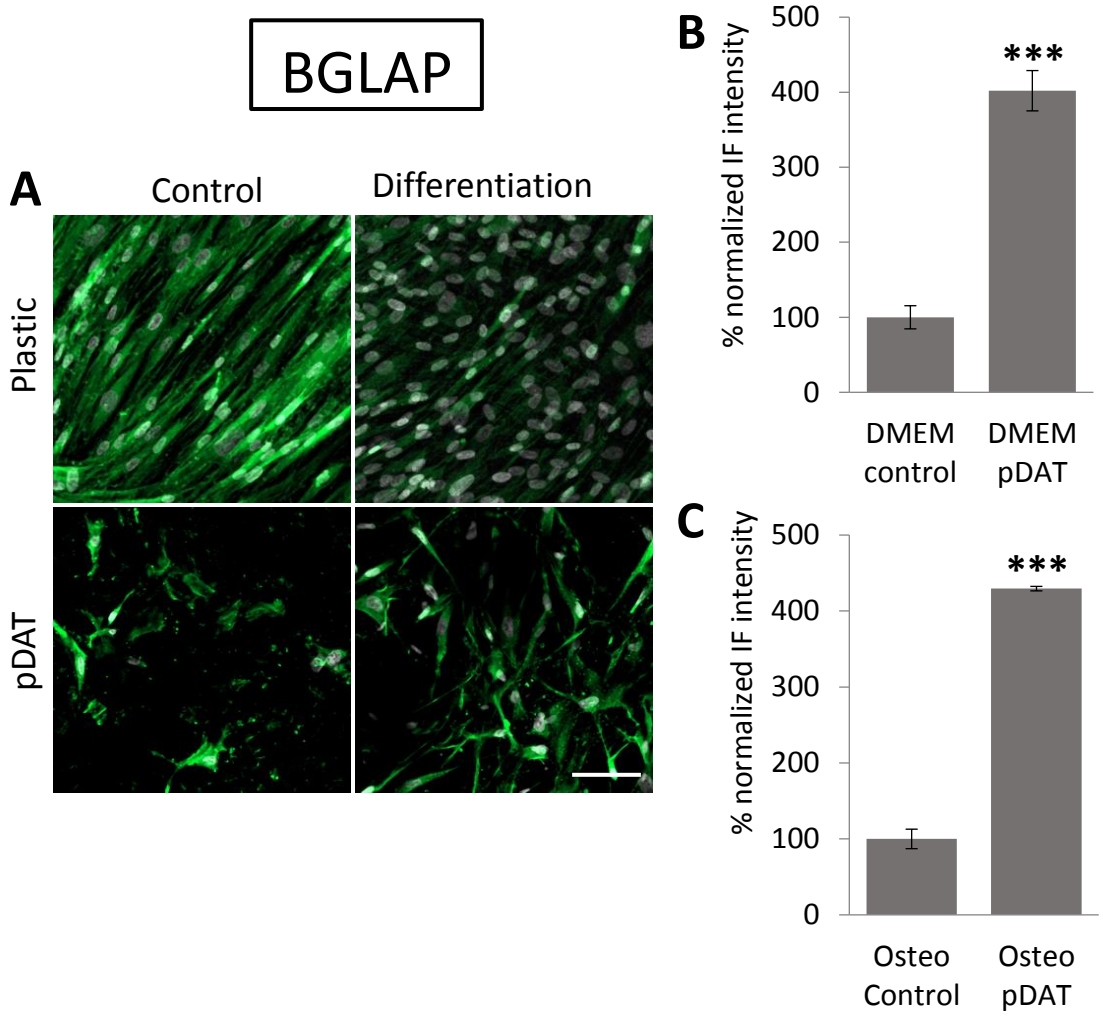


Figure 28. Osteocalcin (BGLAP) immunofluorescence images of hDPSCs cultured in plastic and pDAT in presence or absence of osteoblastic differentiation media and intensity quantification. Immunofluorescence images of hDPSCs seeded on plastic or pDAT-containing EZ-slide wells and cultured with control and osteoblastic induction media for 2 weeks (**A**). Quantification of relative BGALP IF labeling respect to the cell number in different conditions by ImageJ (**B, C**). Scale bar: 50 µm. Statistical significance was set at (***$p \leq 0.001$).

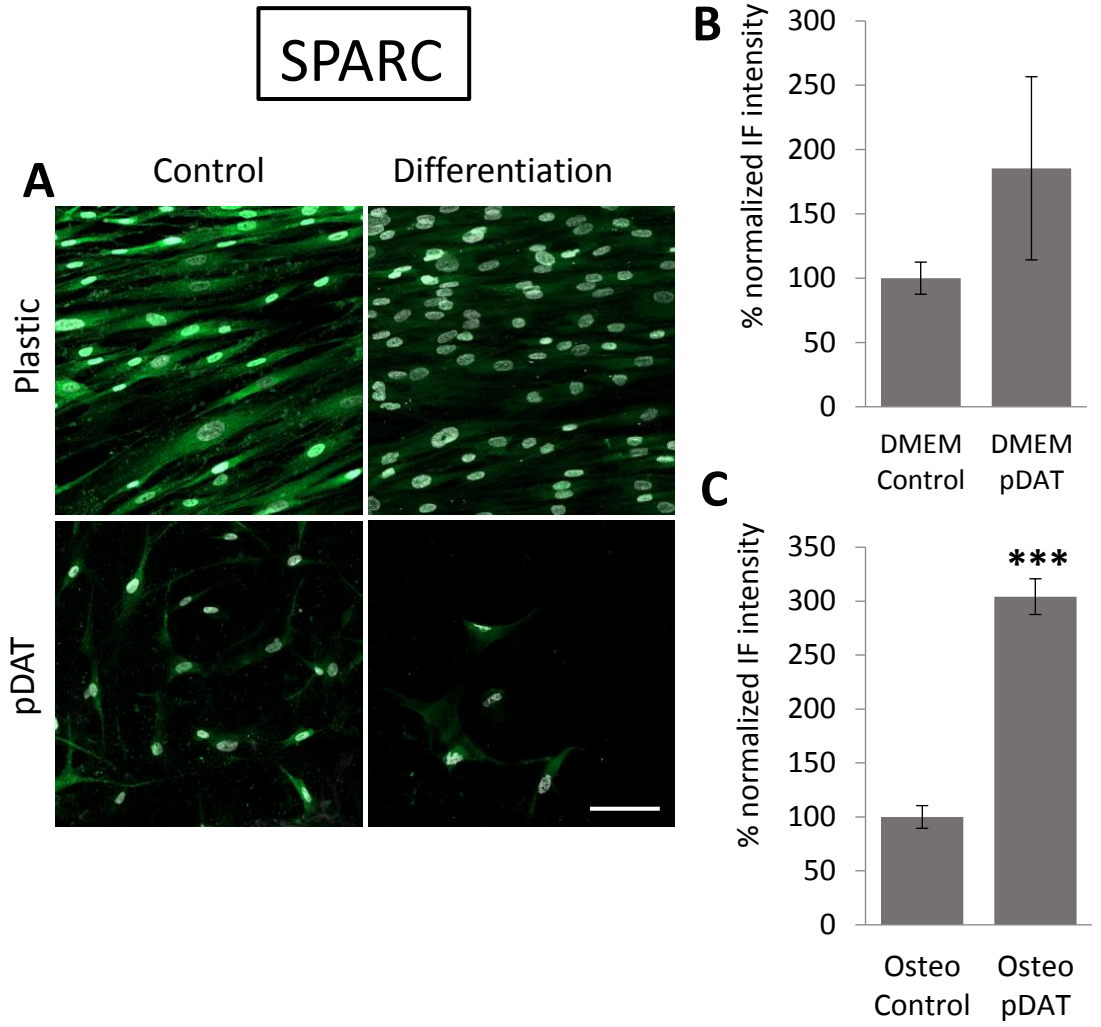


Figure 29. Osteonectin (SPARC) immunofluorescence images of hDPSCs cultured in plastic and pDAT in presence or absence of osteoblastic differentiation media and intensity quantification. Immunofluorescence images of hDPSCs seeded on plastic or pDAT-containing EZ-slide wells and cultured with control and osteoblastic induction media for 2 weeks (**A**). Quantification of relative SPARC IF labeling respect to the cell number in different conditions by ImageJ (**B, C**). Scale bar: 50 µm. Statistical significance was set at (***$p \leq 0.001$).

ALP staining and quantification of hDPSCs cultured on plastic and pDAT in presence or absence of osteoblastic differentiation media.

hDPSCs were seeded on plastic and pDAT for 14 days with control and osteoblastic differentiation media. After that period, the cells were dyed for alkaline phosphatase enzyme, mineralizing bone cells characteristic enzyme. The images showed

the hDPSCs had ALP enzymatic activity in both surfaces (Figure 30A). The quantification of ALP demonstrated being significantly higher in the cells grown in presence of osteoblastic differentiation media both in plastic and pDAT (Figure 30B, C).

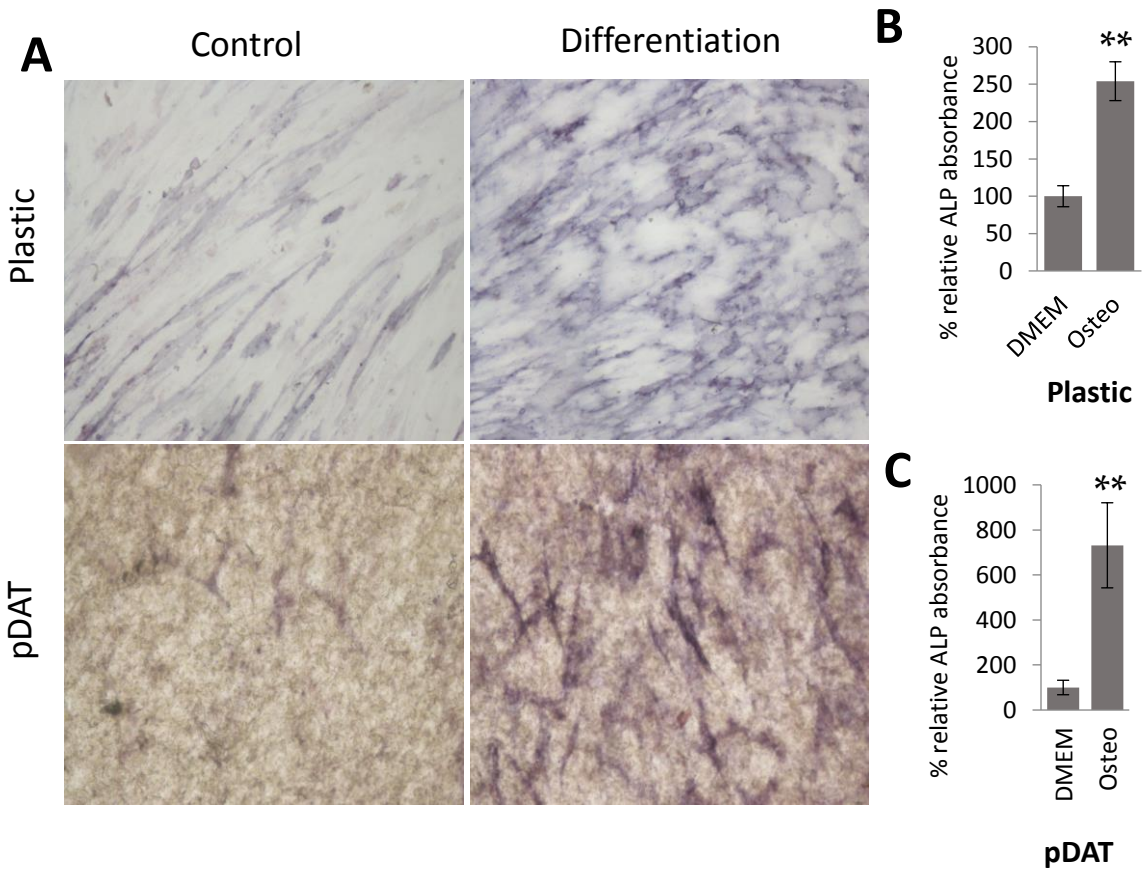


Figure 30. ALP activity quantification of hDPSCs cultured on plastic and pDAT and grown on presence or absence of osteoblastic differentiation media. The hDPSCs were cultured for 14 days on empty well (plastic) or pDAT-containing EZ-slide wells with control or osteoblastic differentiation media. ALP activity was detected by the purple/black precipitate detection (**A**). Quantification of ALP activity was performed measuring the relative ALP absorbance with ImageJ. Statistical significance was set at (**$p \leq 0.01$).

Alizarin red staining and quantification of hDPSCs seeded on plastic and pDAT solid foam grown in presence or absence of osteoblastic differentiation media.

Alizarin red is a calcium-binding dyer witch dyies in an orange/red colour the deposits of mineralized bone matrix. The hDPSCs were seeded in plastic and pDAT and grown with control and osteoblastic induction media for 4 weeks. After this time, the plastic wells ended with high cell confluence with occasional Alizarin red positive cells

(Figure 31A). Nevertheless, the highest Alizarin red precipitate density was found in the hDPSCs grown on pDAT (Figure 31A). Semi-quantitative measurement indicated that in both surfaces the osteoblastic differentiation media caused more intense staining (Figure 31B). Moreover, we used bright field images combined with immunofluorescence staining for DAPI in order to observe the relative cellular location respect to bone matrix deposits. The hDPSCs appeared to be more densely located within the borders of mineralized areas. Moreover, the few hDPSC cells found in close contact with calcified nodules had healthy-looking cell nuclei (Figure 31A).

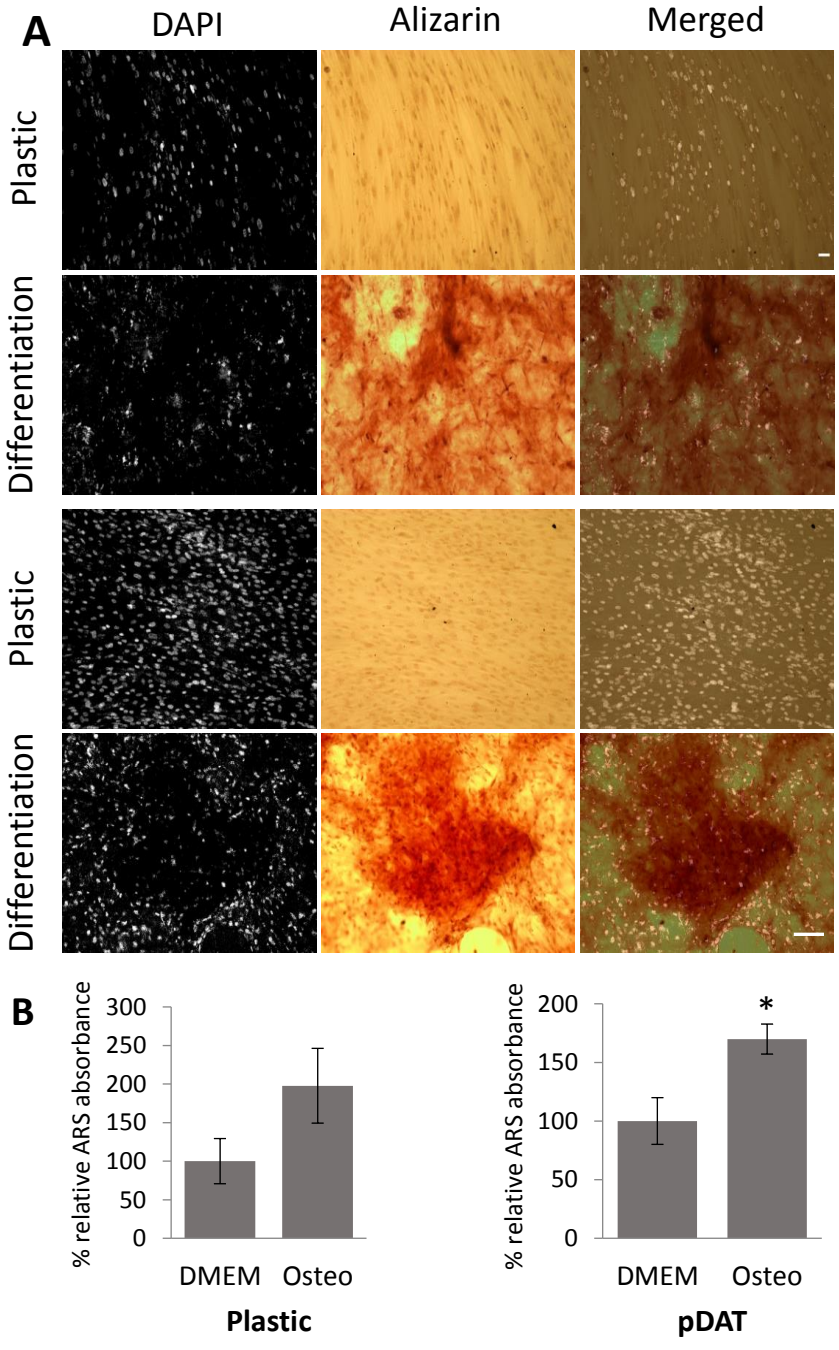


Figure 31. Alizarin red-DAPI double staining of hDPSCs cultured on plastic and pDAT solid foams in presence or absence of osteoblastic differentiation media. hDPSCs were seeded on empty wells (plastic) or pDAT solid foams with control media and osteoblastic induction media for 4 weeks before performing Alizarin red staining. The cells were counterstained with DAPI. The bright field images and immunofluorescence images were merged to study the hDPSCs location in mineralized bone matrix nodules areas (**A**). Quantification of relative Alizarin red absorbance after background subtraction (**B**). Scale bar: 50 µm. Statistical significance was set at (*$p \leq 0.05$).

TEM images of hDPSCs cultured in pDAT scaffolds crating large Sharpey-like collagen fiber bundles

With the aim of studying in detail the bone-like ECM ultra-structure the hDPSCs cultured in pDAT solid foams created, TEM images were taken. Small collagen fibers spread in large areas in the pDAT matrix scaffold were observed in control conditions beside some interspersed lipid droplets coming from the remains of decellularized adipose tissue. Interestingly, when hDPSCs were cultured on pDAT in presence of osteoblastic differentiation media, sharp transitions between thin collagen areas and thick and mineralized collagen areas were also identified (Figure 32; yellow dashed lines). This thick collagen fiber bundles had higher electron density and they were connected to the rest of the pDAT solid foam, which was composed by thinner and less calcified collagen fibers. In the electrodense collagen areas within the solid foam, intramembranous ossification sites were also found (arrowheads in figure 33A, B). Mineralized bone matrix was also observed anchored to the rest of the collagen fibers in electrodense areas by Sharpey-like fibers (asterisk in Figure 33B). This electrodense collagen areas and intramembranous ossification sites were not observed in solid foams without cell seeding (data not shown), indicating the *de novo* generation of these structures by hDPSCs.

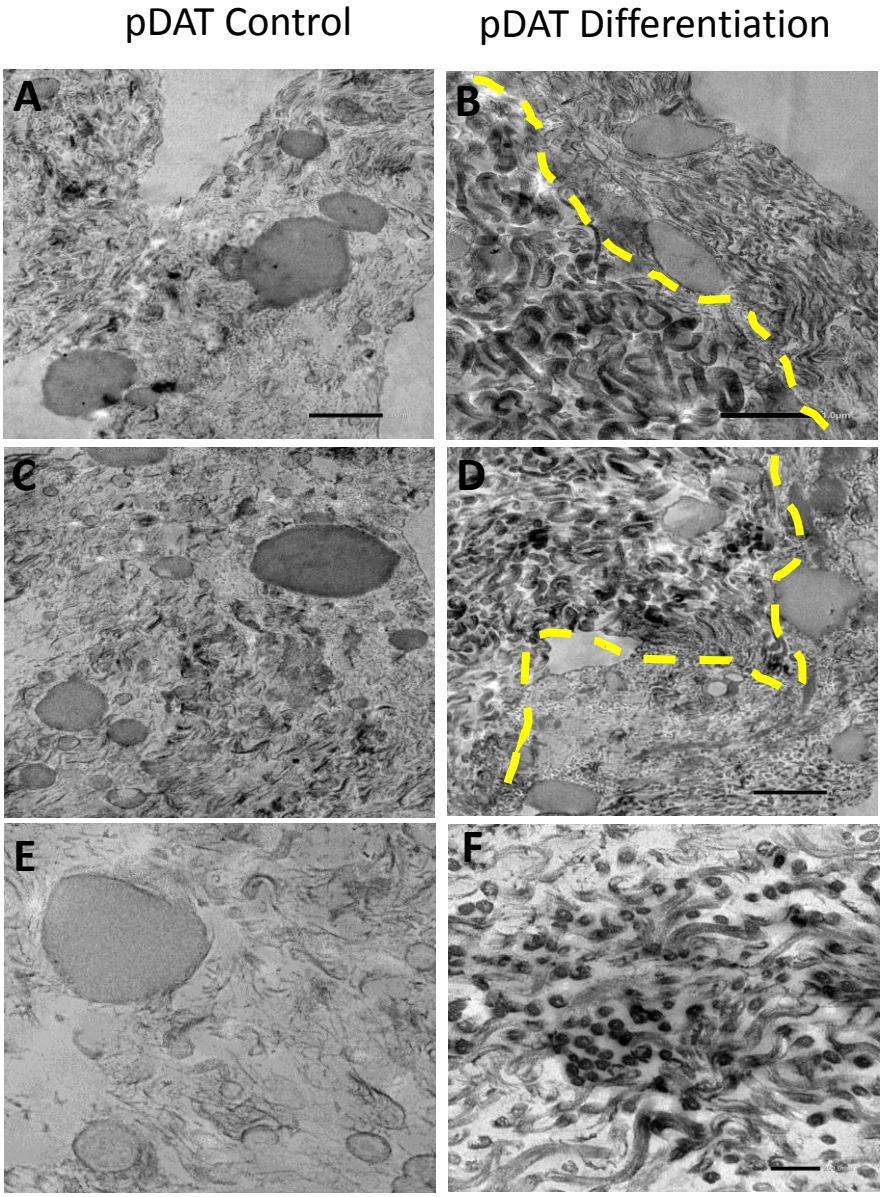


Figure 32. TEM images of the ultrastructure features of hDPSC cultured in pDAT solid foams. hDPSCs were cultured for 4 weeks with control media (left column) and osteoblastic differentiation media (right column). It coul be observed the transition area between hDPSCs produced bone ECM with thicker and more calcified collagen fibers and thinner collagen fibers of pDAT solid foam matrix (yellow dashed line). Higher amount of electrodense collagen fibers were found in osteoblastic differentiation conditions (bottom high-magnification images). Scale bars: 1 µm (A, C and D); 500 nm (B); 200 nm (E and F).

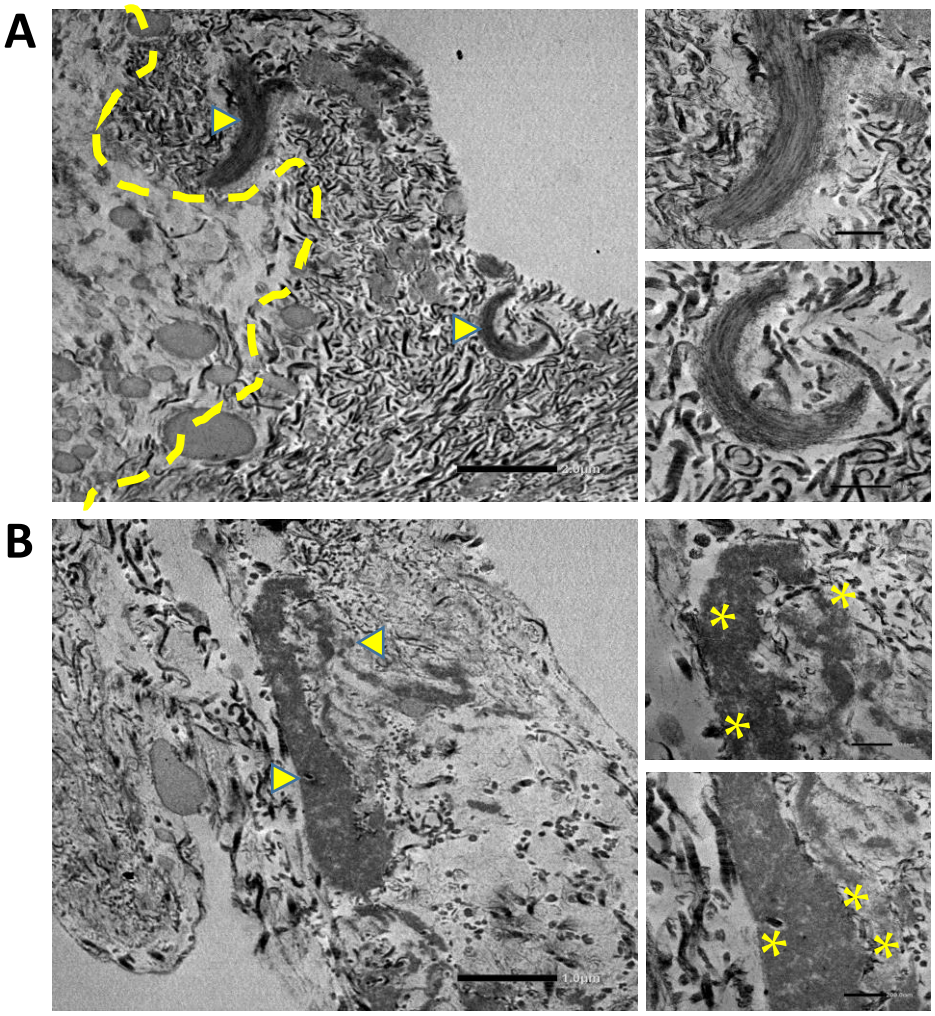


Figure 33. TEM images of intramembranous ossification sites and Sharpey-like fibers generated by hDPSCs cultured in the pDAT solid foams. Transition between non-calcified collagen areas and calcified thick collagen fiber bundle areas of hDPSCs seeded in pDAT solid foams (dashed line). Intramembranous ossification sites were found on this thick collagen containing electrodense structures. Magnification of arrowheads marked areas in the right panels (**A**). Presence of perforating Sharpey-like fibers on the edges of intramembranous ossification sites (asterisk in right panels), showing anchoring to the rest of the collagen matrix. Magnification of arrowheads marked areas in the right panels (**B**). Scale bars: 2 µm (top left image); 1 µm (bottom left image); 500 nm (top right images); 200 nm (bottom right images).

Discussion

One of the aims of this study was the evaluation of the hDPSCs combined with autologous plasma components on biomimetic titanium dental implant materials for bone regeneration *in vitro*. Within this framework, this study was focused on testing the possible enhancements in the osteoblastic differentiation process by assessing the deposition of secreted bone-matrix of hDPSCs cultured on standard Ti6AL4V and biomimetic BAS^{TM} (Avinent implant system) titanium surfaces in combination of plasma-derived products PRGF and PRF. After Gronthos *et al.* isolated hDPSCs for the first time (Gronthos et al., 2000), a lot of different laboratories have confirmed the capability of those neural crest derived stem cells to differentiate into different cell lineages of different germ layers (Nuti et al., 2016). These differentiations include bone-producing osteoblastic cell differentiation being one of the most interesting in the field of implantology (Giuliani et al., 2013; Tatullo, 2017).

Mesenchymal stem cell differentiation into functional bone-matrix secreting osteoblasts and osteocytes is constituted at least by three stages. Each one of these steps are characterized by the expression of specific gene markers. In the first step, mesenchymal stem cells commit to osteo-chondroprogenitor cells, triggering the expression of the transcription factor *RUNX2*. The expression of this gene commits mesenchymal stem cells to become bone-linage osteoprogenitor cells. In the second stage, MSCs transform into secretory osteoblasts, producing and secreting calcium-binding proteins BGLAP (osteocalcin) and SPARC (osteonectin). The combination of these two proteins with extracellular Collagen I forms the organic (osteoid) part of the bone extracellular matrix. Afterwards, hydroxyapatite mineral starts being deposited on this immature bone matrix mineralizing it. The mineralization process by crystal nucleation and growth is carried out thanks to the reaction of Ca2+ and PO4- ions catalyzed by Alkalyne Phosphatase activity. At this point, MSCs also express fully mature differentiated osteoblast markers, like the transcription factor *OSTERIX*. The expression of this transcription marker is maintained in mature osteocytes, after the mature calcified bone matrix surrounds the osteoblasts. These mature osteocytes also keep expressing, albeit at lower levels, *SPARC* and *Collagen I* as well as other bone matrix proteins for bone matrix remodeling throughout time (Figure 34).

In the last decade, there have been important improvements of the biocompatibility of titanium materials for clinical use by changes on the composition and surface of dental titanium implants. These changes were made especially with the purpose of inducing osteoblast differentiation of MSCs and bone deposition around the titanium pillar. Previous studies had already demonstrated the potential of mesenchymal stem cells to adhere, proliferate and differentiate into osteoblasts when cultured on titanium surfaces even when seeded without supplementing the media with osteogenic differentiation factors (Olivares-Navarrete et al., 2010). The results of this study confirmed those previous reports, showing that mesenchymal stem cells, in our case hDPSCs derived from human healthy donors, could change their behavior depending on the substrate they were cultured on. The attachment of hDPSCs to the titanium surface on its own was enough to induce mature osteoblastic differentiation with the potential to create bone matrix mineralized deposits stainable with Alizarin red. These results differ from what is commonly found when hDPSCs are cultured on standard cell culture plastics, where the osteoblastic differentiation does not happen until the cells are grown for several days with β-glycerolphophate, ascorbic acid and dexamethasone (Langenbach and Handschel, 2013). The stimulation of mesenchymal stem cell osteoblastic differentiation is one of the most important advantages of titanium surfaces. It has been demonstrated that both the macro and micro roughness of titanium surfaces play an important role in cell attachment, proliferation and osteoblastic differentiation (Boyan et al., 2016b; Coelho et al., 2009). hDPSCs show osteoblast differentiation, production of considerable amounts of bone morphogenetic proteins, bone proteins and vascular endothelial growth factors when cultured on porous titanium surfaces (Perrotti et al., 2013). Recent works suggests that osteoinductivity is enhanced by chemically modified micro rough titanium surfaces, representing a big advantage for dental clinic application (Boyan et al., 2016b; DE Colli et al., 2018). In the present study, a small but significant difference was found in the levels of expression of *OSTERIX* when comparing hDPSCs grown with control media (no osteoblastic induction) on Ti6AL4V (smooth) and BAS (rough) titanium surfaces. However, osteoblastic differentiation of hDPSCs cultured on BAS titanium surface was greatly enhanced due to the presence of platelet rich fibrin (PRF), either combined or

not with osteoblastic differentiation media. In these conditions, *OSTERIX* mRNA expression levels were raised by more than one order of magnitude.

hDPSCs *in vitro* expansion and maintenance were also tested for both Ti6AL4V (smooth) and BAS (rough) titanium surfaces. Both surfaces preserved cell viability, allowing a good cell proliferation. This was assessed by the detection of Ki67 positive cells in basal conditions. The hDPSCs cultured on Ti6AL4V titanium surface showed higher cell mobility compared to BAS titanium due to the flat surface. Despite both plasma derived products allowed a good hDPSC growth, cell proliferation was significantly enhanced by the addition of 20 % PRGF to the culture media, but not by insoluble PRF. This difference may be caused by the different application method of both plasma products. PRF membranes were put into the culture wells and maintained over the duration of the experiment, releasing molecules gradually. On the contrary, the soluble plasma fraction PRGF was added to the culture media at 20 % concentration and renewed with each media change every 2-3 days. It is possible to hypothesize that high concentration of growth factors induced hDPSCs the high concentration of growth factors, caused by the addition of soluble PRGF to the culture medium, would induce the proliferation of hDPSCs. This would have a negative effect over mature osteoblastic differentiation, as both processes (cell proliferation vs differentiation) are antagonist to each other. In hDPSCs cultured with PRGF, osteoblastic pre-commitment marker expression *RUNX2* and *SPARC* mRNA levels were actually increased. But somehow these hDPSCs failed to differentiate into mature osteoblast cells, as seen by a lower expression of *OSTERIX* and a decreased amount of secreted bone matrix detected by Alizarin red. In contrast, hDPSC cultures supplemented with PRF membranes would arguably generate conditions where by a slow gradual growth factor release from the fibrin clot would favor the mature osteoblast differentiation of hDPSCs. It is noteworthy that the combination of plasma rich fibrin with biomimetic BAS titanium surface was the most effective condition to induce *in vitro* osteoblastic differentiation of hDPSCs grown on titanium surfaces. This is where BAS rough titanium surface demonstrated being a better option with respect to the smooth Ti6AL4V titanium, as *OSTERIX* expression levels could be substantially enhanced when we combined BAS titanium with PRF.

To achieve a full translation of this methodology to the dental clinic, more *in vivo* studies will be necessary. In spite of not being the most abundant source of mesenchymal stem cells in the human adult body, for the fields of implantology and craniomaxilofacial surgery, hDPSCs are particularly interesting cells. For oral and maxillofacial trauma patient who lose several teeth in an accidental episode, this holds especially true. They might very well benefit from an approach whereby clinicians took advantage of the lost dental pieces to extract autologous hDPSCs to aid in the reconstruction of the completely affected bone area. Whatever the case, when cultured on microporous biomimetic titanium surfaces such as BAS™, hDPSCs respond with efficacy to osteoblastic differentiation protocols, especially in combination with platelet rich fibrin clot.

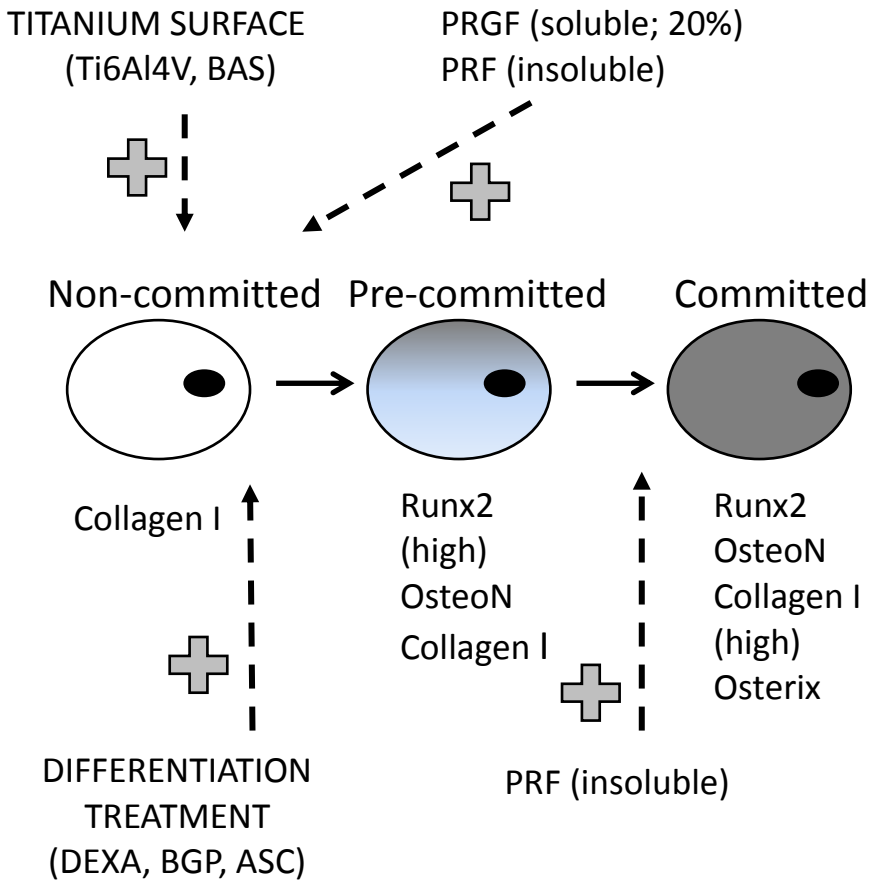


Figure 34. Summary of plasma derived product effects on hDPSCs osteoblastic differentiation processes on culture. Summary of the theoretical model of the different effects caused by the supplementation with pharmacological differentiation reagents, growth substrates (Ti6AL4V and BAS) and plasma derived products (PRGF and PRF) over the osteoblastic differentiation process. Mesenchymal stem cells grows *in vitro* in an undifferentiated state. They can be induced to commit osteoblastic differentiation where in the first stages cells will enhance the expression of the transcription factor *RUNX2*, as they will express progressively higher amounts of Collagen I and calcium-binding proteins like *SPARC*. In the final stage of osteoblastic differentiation, the cells acquire the ability to mineralize the extracellular matrix, forming the mature bone tissue. At the cellular/molecular level, this is characterized by the rise of the expression of *OSTERIX*, the mature osteoblast/osteocyte gene marker. Pharmacological reagents, plasma-derived PRGF and PRF as well as titanium surfaces appear all to influence the preosteoblast transition of hDPSCs. This is confirmed by the detection of higher levels of *RUNX2* and *SPARC* expression and/or phosphatase alkaline staining found in all these conditions. However, hDPSCs cultured with soluble PRGF did not achieve mature osteoblast stage, possibly due to proliferation/differentiation signaling conflict. On contrary, hDPSCs seeded in the presence of PRF clots showed the highest expression levels of osteoblastic differentiation, especially on BAS biomimetic titanium surface combined with osteoblastic induction reagents.

The previous study showed the good cell viability, proliferation and osteoblastic differentiation features of hDPSCs grown over titanium surfaces. Mesenchymal stem cells had demonstrated being a great option for their use in bone tissue engineering due to their capacity to adhere, proliferate and differentiate to different cell types when seeded on different biomaterials. However, with all the different mesenchymal stem cell sources found in the human adult body it is still unclear which one is the best option for the use on tissue engineering. For the effective development of bone and dental regeneration therapies, different types of post-natal stem cells need to be compared to determine the best MSC option. To bring light to this question, we next performed a comparative study between hDSPCs and hBMSCs, a promising MSC type due to its natural bone differentiation ability.

The aim of this comparative study was to compare the viability, proliferation and osteoblastic differentiation between hDPSCs and hBMSCs cultured on Ti6AL4V and BAS^{TM} titanium surfaces in the presence or absence of osteoblastic differentiation media. For the evaluation of these results, we have to take into account the patients age

difference between hDPSC and hBMSC donors for the evaluation of the results, which was 16-30 years for hDPSCs and 45 years for BMSCs.

Both hDPSCs and hBMSCs seeded on Ti6AL4V and BAS titanium discs showed no cytotoxic effect from the titanium surfaces. These two types of cells showed almost 100 % viability while being seeded on titanium surfaces where only isolated single cells were found stained by propidium iodide. On the contrary, the cell proliferation assay did exhibit significant differences between hDPSCs and hBMSCs. The comparative cell proliferation experiment showed a greater growth potential of hDPSCs compared to hBMSCs when seeded on both titanium surfaces after 24 and 48 hours. When the cells were cultured on coverslips this difference was only observed after the initial 24 hours of culture while at 48 hours both cells had similar proliferative activity. These results confirmed what was shown in other articles where the cells had been seeded in different biomaterials (Amid et al., 2021; Ponnaiyan and Jegadeesan, 2014).

The appearance of the mineral phase deposited by hDPSCs and hBMSCs on coverslips, TiAL4V and BAS titanium surfaces also differed. Alizarin red staining quantification demonstrated a higher bone mineral production of the hDPSCs compared to hBMSC. In addition, within different conditions the hDPSCs grown on Ti6AL4V and BAS titanium in the presence of osteoblastic induction media where the ones with the highest bone mineral production. As shown in other related studies, hDPSCs exhibited a higher mineralization ability than hBMSCs when cultured on these two titanium surfaces (Davies et al., 2015; Mohanram et al., 2020).

Apart from this, the RNA sequencing was analyzed following expression/ lack of expression criteria. To achieve this aim, we performed a comparative RNA expression analysis between pairs of different conditions to find out the genes that were expressed in one condition and not in the other. We chose a presence/absence (ON/OFF) comparison to detect specific sets of genes, whose expression would be specifically triggered in osteogenic differentiation enhancement conditions, to potentially identify new gene targets and signaling pathways involved in the osteoblastic differentiation of hDPSCs and hBMSCs.

The main difference in terms of genes expression between hDPSCs and hBMSCs grown in plastic with control media was the HOX gene family expressed by hBMSCs. HOX genes are responsible for embryonic MSC proliferation and for the regional specifications and related tissue-specificity (Ackema and Charité, 2008). Previous studies had demonstrated the differences in the HOX expression pattern in mandibular neural crest cells and adult mandibles, in contrast to distal mesoderm-derived mesenchyme and bones (Dong et al., 2014; Lee et al., 2015b; Wehrhan et al., 2011). It have been suggested that this gene family could be related to the site-specificity of MSCs, depending on the anatomical position (Wang et al., 2009). These HOX genes expression difference could explain the different cellular behavior associated to autogenous cell grafts of different embryonic origins (Leucht et al., 2008). The results obtained on this study are consistent with previous studies which demonstrated the HOX gene expression decrease in mandibles compared to that of long bones (Lee et al., 2015b; Leucht et al., 2008).

Focusing specifically on osteoblastic differentiation related genes, we found that the osteoblastic differentiation marker *OSTERIX/SP7* expression showed to be induced in hDPSCs when culturing them on titanium surfaces in the presence or absence of osteoblastic differentiation media. In the hBMSCs differences were not found because these cells already had a basal *SP7* expression in these control conditions. These results are consistent with the previous experiments where we observed that the *OSTERIX/SP7* expression could be enhanced by differentiation media as well as by the titanium surfaces. In addition, two *SPARC* related genes, *SPOCK2* and *SPARCL1*, showed to be silent in hBMSCs grown in control conditions while they were expressed when the cells were cultured on Ti6AL4V titanium surfaces with the osteoblastic differentiation media. Finally, *ZBTB16* is the last osteogenic differentiation gene we have to mention. *ZBTB16* is a zinc finger transcriptional factor that contains a domain for protein-protein interactions and nine Kruppel-type zinc finger domains for DNA binding. *OSTERIX/SP7* binds *ZBTB16* gene promotor to start its transcription (Figure 35). In this study, we found that neither hDPSCs nor hBMSCs had any expression of this gene when cultured with control media but both cell types showed a remarkable expression of *ZBTB16* when grown with osteoblastic differentiation media. These results are related with some

previous studies, which concluded that ZBTB16 gene is involved on dexamethasone mediated osteogenic differentiation. For this reason, *ZBTB16* was only expressed when the cells were cultured with osteoblastic differentiation media (Felthaus et al., 2014a; Felthaus et al., 2014b; Onizuka et al., 2016). Overall, the RNAseq analyses made over hDPSCs and hBMSCs in different culture conditions suggest a possible role of ZBTB16 as a master regulator of osteogenesis.

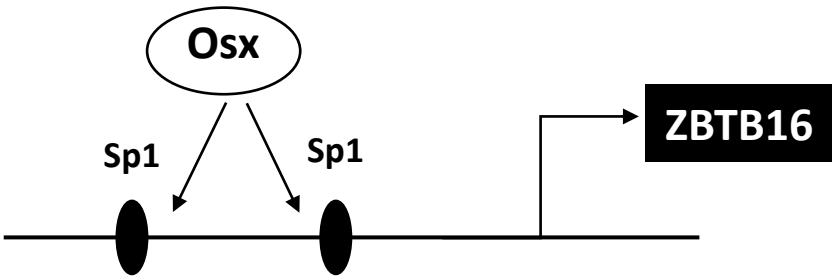


Figure 35. Osterix binds to the Sp1 sequence of the ZBTB16 promoter region.

Among other interesting differentially expressed genes, *NTRK3* and *GFRA2* genes were expressed in hDPSCs seeded on plastic with control media but this expression disappeared by the effect of the differentiation media and/or titanium surface. Neurotrophin receptor genes, and particularly *NTRK3*, had been suggested to be hDPSCs stemness markers, hence these results are consistent with previous studies (Luzuriaga et al., 2019b).

It is also important to mention the lack of difference in expressed genes between hDPSCs grown on Ti6AL4V and BAS titanium surfaces. The few genes that were differentially expressed in those conditions were few, and followed an inconsistent pattern. This result demonstrated the similar gene expression induction effects of both titanium discs. Therefore, we can conclude that whatever differences in the pattern of gene expression between cells cultured on these titanium surfaces, they would have to be sought in slight changes in gene expression levels, rather than in the triggering of the expression of any particular master gene related to osteogenesis.

After analyzing the differential expression of individual potential genes of interest, we resorted to a comprehensive Pathway Enrichment Analysis, to identify clusters of genes and signaling pathways that would be affected in hDPSCs and hBMSCs

in our different culture conditions. We used the connectivity map (CMAP) and gene ontology biological process (GOBP) to identify the most likely affected signaling pathways in each experimental comparison. We selected those pathways that presented at least two differentially expressed genes and a significant p value of <0.05. These databases showed the different biological processes and diseases where these genes are involved. Most of the outputs of the CMAP comparative analysis between hBMSCs and hDPSCs were related to HOX genes, being related to diverse processes ranging from acute myeloid leukemia to hematopoietic disorders. On the other hand, GOBP related the expression of different clusters of genes to anterior/posterior pattern specification, blood vessel development, cardiovascular system development, regulation of blood vessel size, hormone transport and hematopoietic stem cell differentiation. The significant differences detected on pathways related to anterior/posterior pattern specification and hematopoietic stem cell differentiation can be clearly related to the different embryonic origin and physiological function of hDPSCs and hBMSCs.

As seen in previous studies, even with higher expression of some osteoblastic differentiation related genes by hBMSCs, the bone mineral production is always higher in hDPSCs (Davies et al., 2015; Mohanram et al., 2020). These results were also confirmed in this investigation by the detection of mineral with Alizarin red. Moreover, cell proliferation was also higher in hDPSCs than in hBMSCs. It is also important to mention that the isolation methodology is different for these two types of cells. The procedure to obtain hDPSCs is easier and less aggressive than the one of hBMSCs. With all these data, we can suggest that hDPSCs represent a better source of mesenchymal stem cells for the use in bone and dental tissue engineering than hBMSCs (Amid et al., 2021; Mohanram et al., 2020).

As already mentioned, the dental implants have upgraded considerably over the last years, improving implant survival rate. This survival achieved around 95 % after 10 years post-loading (Moraschini et al., 2015) and almost 88 % after 20 years post-loading (Chrcanovic et al., 2018). However, with the increase of life expectancy and population

ageing in developed countries, the demand for very long-term durable dental implants seems likely to be maintained in the future.

Over the last decades, dental implant osseointegration has obtained big improving results; however, some significant challenges remain. One of the biggest problems in oral implantology is the Marginal Bone Loss (MLS), which is directly correlated to implant loss due to the mechanical stress that the alveolar bone suffers because of masticatory function in the absence of a functional PDL. The entire masticatory load is transferred to alveolar bone tissue because dental implants are directly anchored on it, which can lead to its resorption. There are many studies associating a high MLB with a high risk of periimplantitis and implant failure (Chrcanovic et al., 2018; Coli and Jemt, 2021; Galindo-Moreno et al., 2015). The most effective strategy to reduce periimplantitis and MBL has been proposed to be the reconstruction of PDL, which naturally acts like a cushion between alveolar bone and the dental piece by absorbing mechanical forces. However, PDL regeneration and engineering constitutes an extraordinary challenge (Lee et al., 2020). Any material used to substitute PDL should firmly anchor to both alveolar bone and the dental implant while conserving a highly vascularized tissue strip in between. In practice, this problem could only be solved by seeding osteogenic and vasculogenic stem cells on biomaterials.

In the context of PDL regeneration by stem cell therapy, the best scenario would be that the stem cells cultured on the biomaterial came from the patients themselves (autologous graft), with which under adequate manipulation we could avoid immune rejection issues. As previously mentioned, some of the most promising stem cell types for dental implantology are hDPSCs. These cells are particularly well suited for cryopreservation and autologous transplant (Ibarretxe et al., 2012b; Raik et al., 2020). Another important characteristic of hDPSCs is the high capacity of differentiation to a very large variety on both mesenchymal and non-mesenchymal cell types they have in the absence of xenogenic cell culture compounds like animal serum. Interestingly, the capacity of hDPSCs to differentiate to both vasculogenic and osteogenic cells has been shown in serum free media. We recently published and patented an animal serum free methodology to obtain vasculogenic cells (endothelia and pericytes) (Luzuriaga et al.,

2020; Pineda Martí et al., 2020). This method could be potentially applied to hDPSCs seeded on biomaterials to vascularize scaffolds as pDAT for their utilization in cell therapies.

In this investigation, we combined hDPSCs with pDAT solid foams obtained by following a patented decellularisation protocol (Madarieta Pardo et al., 2017). Adipose tissue represents a very accesible source of basement membrane adhesion proteins with biological activity (Yang et al., 2020). In the context of bone regeneration, it was very important to corroborate the differentiation of hDPSC to osteoblastic cells and production of mineralized bone matrix tissue was very important to corroborate when cultured on pDAT. We focused on the permissiveness of pDAT solid foam to support hDPSCs osteoblastic differentiation when these cells are seeded on this biomaterial. One of the most important problems to solve for PDL engineering will be the optimization of the pDAT solid foam culture system with the combination of two different previously differentiated hDPSC-derived cell types for the creation of differentiated vascular and bone tissue areas, which would imitate the structure and function of the lost PDL tissue.

The results obtained in this investigation confirms that pDAT solid foams could establish a very important biomaterial for PDL and dental bone regeneration. This formulation of pDAT significantly enhanced secreted bone matrix by hDPSCs, as evaluated by phosphatase alkaline and Alizarin red staining. The hDPSCs cultured on the scaffold showed no loss of viability under experimental conditions. hDPSCs were seeded at relatively low initial desities (15.000 cells/well; 21.428 cells/cm^2) and also low volume of pDAT scaffold (120 µl/well) to couple it for long-term cell culture *in vivo* in EZ-slides. Maintaining higher cell densities over longer periods would possibly require better nutrient and oxygen supply through a vascular network (Nakamura et al., 2019). Further *in vivo* experiments will be necessary to test the potential of this material on the healing of bone tissue and PDL lesions.

Finally, we also observed the production of thick calcified collagen fiber bundles and intramembranous ossification sites of hDPSCs cultured on pDAT. This calcification sties were attached to the pDAT scaffold by Sharpey´s fibres. All these results support

that the combination of hDPSCs, or other mesenchymal stem cells, with pDAT is not only a good osteoblastic differentiation induction ECM environment but could also help on the graft and surrounding hard tissue attachment. In the case of bone healing, this attachment has a particular interest, where physical anchoring of the scaffold to the bone tissue is necessary. Transplantation experiments *in vivo* will be needed to corroborate these findings, where apart of being a vehicle of stem cells, the scaffold would act as a bioinductive ECM which in the best scenario would enhance the calcified attachment to hard bone tissue and also bone mesenchymal stem cells recruitment and activation of the host bone tissue.

Conclusions

In the last decades, different mesenchymal stem cells had proven their potential to be used in bone tissue engineering. Their high proliferation and multi-linage differentiation ability make these cells ideal candidates for regeneration therapies. Moreover, the improvements on implants and scaffolds manufacturing give rise to different scaffold upgrades in porosity, roughness or durability. These modifications are focused on enhancing biocompatibility for stem cell growth and differentiation. Finally, the use of plasma derived growth factors showed to be a perfect solution for *in vitro* autologous stem cell proliferation and differentiation in bone tissue engineering therapies, avoiding non-desirable immune responses because of the use of animal derived serums in cell cultures.

The following conclusions have been drawn from the results obtained in this work:

1. hDPSCs showed non affected cell viability and proliferation when cultured on Ti6AL4V and BAS titanium surfaces.
2. Both titanium surfaces had osteoinductive effect on hDPSCs without the need of osteoblastic differentiation media.
3. The plasma derived product PRGF induced cell proliferation on *in vitro* hDPSC cultures while PRF maximized osteoblastic cell differentiation of hDPSCs and calcified bone matrix production.
4. The combination of BAS titanium surface with plasma derived PRF enhanced differentiation of hDPSCs to bone-producing cells. These results provide strong experimental support to the widespread use of plasma derived fibrin clots in common clinical practice to enhance bone production around microporous titanium implant surfaces.
5. The comparative study of hDPSCs and hBMSCs cultured on Ti6AL4V and BAS titanium surfaces suggested a higher cell proliferation and mineralization of hDPSCs compared to hBMSCs, but, more data are needed to make a firm conclusion. Due to the easier and less invasive isolation, higher proliferation and bone mineral depositions hDPSCs could be a better option than hBMSCs for bone regeneration therapies.

6. Porcine decellularized adipose tissue (pDAT) provided a good support to hDPSCs adhesion and viability.
7. pDAT supported hDPSCs osteoblastic differentiation with production of calcified bone matrix by intramembraneous ossification and formation of Sharpey fibre-like attachment structures.

Taking into account the relatively abundant and available source of human-derived raw adipose tissue material and that both DAT and hDPSCs can be isolated from human donors; this seems a great opportunity for their combined use in personalized clinical therapies for the healing of bone lesions and dental implantology.

Annex I

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(54) Title: CELLULAR AGGREGATES FOR USE IN VASCULARISATION THERAPY

(57) Abstract: Thc prcscnl invcnlion provides a scrum-frcc cndothclial cell diffcrcnlialion cullurc medium comprising (a) a basal cullurc medium and (b) an cndothclial cell diffcrcnlialion combination of EGF-FGFand VEGF prolcin, whcrcin lhc amount of EGF is highcr than lhc amount of FGF prolcin. Thc prcscnl invcnlion furlhcr provides a process for lhc preparation of cellular aggrcgalc suspensions comprising differentiated endothelial cells from dental stem cells using the serum-free medium, as well as the use of the rcsulling suspension in therapy.

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Annex II

Articles

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Dental pulp stem cells as a multifaceted tool for bioengineering and the regeneration of craniomaxillofacial tissues

Maitane Aurrekoetxea †, Patricia Garcia-Gallastegui †, Igor Irastorza, Jon Luzuriaga, Verónica Uribe-Etxebarria, Fernando Unda and Gaskon Ibarretxe**

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Dental pulp stem cells, or DPSC, are neural crest-derived cells with an outstanding capacity to differentiate along multiple cell lineages of interest for cell therapy. In particular, highly efficient osteo/dentinogenic differentiation of DPSC can be achieved using simple *in vitro* protocols, making these cells a very attractive and promising tool for the future treatment of dental and periodontal diseases. Among craniomaxillofacial organs, the tooth and salivary gland are two such cases in which complete regeneration by tissue engineering using DPSC appears to be possible, as research over the last decade has made substantial progress in experimental models of partial or total regeneration of both organs, by cell recombination technology. Moreover, DPSC seem to be a particularly good choice for the regeneration of nerve tissues, including injured or transected cranial nerves. In this context, the oral cavity appears to be an excellent testing ground for new regenerative therapies using DPSC. However, many issues and challenges need yet to be addressed before these cells can be employed in clinical therapy. In this review, we point out some important aspects on the biology of DPSC with regard to their use for the reconstruction of different craniomaxillofacial tissues and organs, with special emphasis on cranial bones, nerves, teeth, and salivary glands. We suggest new ideas and strategies to fully exploit the capacities of DPSC for bioengineering of the aforementioned tissues.

Keywords: DPSC, differentiation, tooth, bone, salivary gland, nerve, cell therapy

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Wnt/β-Catenin Regulates the Activity of Epiprofin/Sp6, SHH, FGF, and BMP to Coordinate the Stages of Odontogenesis

*Maitane Aurrekoetxea[1], Igor Irastorza[1], Patricia García-Gallastegui[1], Lucia Jiménez-Rojo[2], Takashi Nakamura[3], Yoshihiko Yamada[4], Gaskon Ibarretxe[1] and Fernando J. Unda[1]**

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Background: We used an *in vitro* tooth development model to investigate the effects of overactivation of the Wnt/β-catenin pathway during odontogenesis by bromoindirubin oxime reagent (BIO), a specific inhibitor of GSK-3 activity.

Results: Overactivating the Wnt/β-catenin pathway at tooth initiation upregulated and ectopically expressed the epithelial markers *Sonic Hedgehog (Shh), Epiprofin (Epfn), and Fibroblast growth factor8 (Fgf8)*, which are involved in the delimitation of odontogenic fields in the oral ectoderm.This result indicated an ectopic extension of the odontogenic potential. During tooth morphogenesis, *Fibroblast growth factor4 (Fgf4), Fibroblast growth factor10 (Fgf10), Muscle segment homeobox 1 (Msx-1), Bone Morphogenetic protein 4 (Bmp4), and Dickkopf WNT signaling pathway inhibitor 1 (Dkk1)* were overexpressed in first molars cultured with BIO. Conversely, the expression levels of *Wingless integration site 10b (Wnt-10b)* and *Shh* were reduced. Additionally, the odontoblast differentiation markers Nestin and *Epfn* showed ectopic overexpression in the dental mesenchyme of BIO-treated molars. Moreover, alkaline phosphatase activity increased in the dental mesenchyme, again suggesting aberrant, ectopic mesenchymal cell differentiation. Finally, Bmp4 downregulated *Epfn* expression during dental morphogenesis.

Conclusions: We suggest the presence of a positive feedback loop wherein *Epfn* and β-*catenin* activate each other.The balance of the expression of these two molecules is essential for proper tooth development. We propose a possible link between Wnt, Bmp, and Epfn that would critically determine the correct patterning of dental cusps and the differentiation of odontoblasts and ameloblasts.

Keywords: Wnt/β-catenin, tooth development, GSK-3, BIO-culture, Epiprofin/Sp6, odontogenesis

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n Dental Pulp Stem Cells to Neuralwww.cellphysiolbiochem.com

BDNF and NT3 Reprogram Human Ectomesenchymal Dental Pulp Stem Cells to Neurogenic and Gliogenic Neural Crest Progenitors Cultured in Serum-Free Medium

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Key Words: Serum-free culture media • Calcium imaging • Cell differentiation • Dental Pulp Stem Cells • Brain Derived Neurotrophic Factor

Abstract

Background/Aims: Human Dental Pulp Stem Cells (hDPSCs) are one of the most promising types of cells to regenerate nerve tissues. Standard DMEM+10% fetal bovine serum (FBS) culture medium allows a fast expansion of hDPSC as a surface-adherent cell monolayer. However, the use of FBS also compromises the clinical use of these protocols, and its longterm presence favors hDPSCs differentiation toward mesenchymal cell-derived lineages, at the expense of a reduced capability to generate neural cells. The objective of this work was to characterize the role of neurotrophin signaling on hDPSCs using a serum-free culture protocol, and to assess the neurogenic and gliogenic capacity of hDPSCs for future nerve tissue bioengineering and regeneration. ***Methods:*** We compared the different expression of neurotrophin receptors by RT-PCR, Q-PCR, and IF of hDPSCs cultured with different growth media in the presence or absence of serum. Moreover, we assessed the response of hDPSCs to stimulation of neurotransmitter receptors by live cell calcium imaging under these different media. Finally, we compared the osteogenic potential of hDPSCs by Alizarin red staining, and the differentiation to gliogenic/neurogenic fates by immunostaining for Schwann lineage

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hDPSCs with PRF and PRGF on biomimetic titanium DOI: 10.22203/eCM.v038a14 ISSN 1473-2262

ADHESION, INTEGRATION AND OSTEOGENESIS OF HUMAN DENTAL PULP STEM CELLS ON BIOMIMETIC IMPLANT SURFACES COMBINED WITH PLASMA DERIVED PRODUCTS

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Abstract

Dental implants are the usual therapy of choice in the dental clinic to replace a loss of natural teeth. Over recent decades there has been an important progress in the design and manufacturing of titanium implant surfaces with the goal of improving their osteointegration. In the present work, the aim was to evaluate the usefulness of hDPSCs (human dental pulp stem cells), in combination with autologous plasma components, for *in vitro* bone generation on biomimetic titanium dental implant materials. In this context, the combination of hDPSCs stimulated by PRGF or PRF and cultured on standard Ti6A14V and biomimetic BAS™ (Avinent Implant System) titanium surfaces were studied in order to evaluate possible enhancements in the osteoblastic differentiation process out of human mesenchymal cells, as well as bone matrix secretion on the implant surface. The results obtained in this *in vitro* model of osteogenesis suggested a combination of biomimetic rough titanium surfaces, such as BAS™, with autologous plasma-derived fibrin-clot membranes such as PRF and/or insoluble PRGF formulations, but not with an addition of water-soluble supplements of plasma-derived growth factors, to maximise osteoblastic cell differentiation, bone generation, anchorage and osteointegration of titanium-made dental implants.

Keywords: Dental pulp stem cells, titanium implants, osteoblast differentiation, platelet rich in growth factors, platelet rich fibrin, biomimetic advanced surface.

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Article

Vasculogenesis from Human Dental Pulp Stem Cells Grown in Matrigel with Fully Defined Serum-Free Culture Media

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Abstract: The generation of vasculature is one of the most important challenges in tissue engineering and regeneration. Human dental pulp stem cells (hDPSCs) are some of the most promising stem cell types to induce vasculogenesis and angiogenesis as they not only secrete vascular endothelial growth factor (VEGF) but can also differentiate in vitro into both endotheliocytes and pericytes in serum-free culture media. Moreover, hDPSCs can generate complete blood vessels containing both endothelial and mural layers in vivo, upon transplantation into the adult brain. However, many of the serum free media employed for the growth of hDPSCs contain supplements of an undisclosed composition. This generates uncertainty as to which of its precise components are necessary and which are dispensable for the vascular differentiation of hDPSCs, and also hinders the transfer of basic research findings to clinical cell therapy. In this work, we designed and tested new endothelial differentiation media with a fully defined composition using standard basal culture media supplemented with a mixture of B27, heparin and growth factors, including VEGF-A165 at different concentrations. We also optimized an in vitro Matrigel assay to characterize both the ability of hDPSCs to differentiate to vascular cells and their capacity to generate vascular tubules in 3D cultures. The description of a fully defined serum-free culture medium for the induction of vasculogenesis using human adult stem cells highlights its potential as a relevant innovation for tissue engineering applications. In conclusion, we achieved efficient vasculogenesis starting from hDPSCs using serum-free culture media with a fully defined composition, which is applicable for human cell therapy purposes.

Keywords: stem cells; DPSCs; neovasculogenesis; endothelial cells; Matrigel; vasculature

cells

MDPI

Article

Wnt-3a Induces Epigenetic Remodeling in Human Dental Pulp Stem Cells

Verónica Uribe-Etxebarria [1,2], Patricia García-Gallastegui [1], Miguel Pérez-Garrastachu [1], María Casado-Andrés [1,3], Igor Irastorza [1], Fernando Unda [1], Gaskon Ibarretxe [1,*,†] and Nerea Subirán [4,†]

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Abstract: Dental pulp stem cells (DPSCs) from adult teeth show the expression of a very complete repertoire of stem pluripotency core factors and a high plasticity for cell reprogramming. Canonical WntandNotchsignalingpathwaysregulatestemnessandtheexpressionofpluripotencycorefactor sin DPSCs, and even very short-term (48 h) activations of the Wnt pathway induce a profound remodeling of DPSCs at the physiologic and metabolic levels. In this work, DPSC cultures were exposed to treatments modulating Notch and Wnt signaling, and also induced to differentiate to osteo/adipocytes. DNA methylation, histone acetylation, histone methylation, and core factor expression levels where assessed by mass spectroscopy, Western blot, and qPCR. A short-term activation of Wnt signaling by WNT-3A induced a genomic DNA demethylation, and increased histone acetylation and histone methylation in DPSCs. The efficiency of cell reprogramming methods relies on the ability to surpass the epigenetic barrier, which determines cell lineage specificity. This study brings important information about the regulation of the epigenetic barrier by Wnt signaling in DPSCs, which could contribute to the development of safer and less aggressive reprogramming methodologies with a view to cell therapy.

Keywords: dental pulp stem cells; chromatin remodeling; cell cycle; pluripotency; DNA methylation; histone acetylation; histone methylation; Notch pathway; Wnt pathway

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Nanostructured scaffolds based on bioresorbable polymers and graphene oxide induce the aligned migration and accelerate the neuronal differentiation of neural stem cells

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Abstract

Withinthe field of neural tissue engineering,there is a huge need for the development of materials that promote the adhesion,aligned migration and differentiation of stem cells into neuronal and supportive glial cells. In this study, we have fabricated bioresorbable elastomeric scaffolds combining an ordered nanopatterned topography together with a surface functionalization with graphene oxide (GO) in mild conditions. These scaffolds allowed the attachment of murine neural stem cells (NSCs) without the need of any further coating of its surface with extracellular matrix adhesion proteins. The NSCs were able to give rise to both immature neurons and supporting glial cells over the nanostructured scaffolds in vitro, promoting their aligned migration in cell clusters following the nanostructured grooves. This system has the potential to reestablish spatially oriented neural precursor cell connectivity, constituting a promising tool for future cellular therapy including nerve tissue regeneration.

Key words: Micro- and nanopatterning; Neural stem cells; Migration; Cell differentiation; Graphene oxide; Biodegradable polymer

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Regeneration of the nervous system still remains very challenging due to its limited plasticity and poor ability to heal ments for this specific biomedical application play a pivotal role. Much progress has been made in determining the ideal features a biomaterial should have for its use as a neural replacement graft, and in understanding the interactions of growing axons within thesebiomaterials;however,theregenerationlevelsinducedbythe biomaterial usually do not match those obtained by nerve tissue autografts and the development of new and effective nerve regeneration therapies is still an urgent clinical need.[2,3]

The biomaterials for nerve tissue regeneration should be biocompatible and biodegradable, while providing structural cues that promote oriented axon regeneration and guidance signals from extracellular matrix (ECM)-like components. Additionally, they shouldalsopresentlong-termstoragecapabilityandeaseofhandling/ suturing.[4–6] One important aspect to take in consideration is that the

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Universidad del País Vasco

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ZELULEN BIOLOGIA ETA HISTOLOGIA SAILA

MEDIKUNTZA ETA ERIZAINTZA FAKULTATEA

EUSKAL HERRIKO UNIBERTSITATEA

HEZUR EHUN INJENIARITZAN ERABILTZEKO GIZA HORTZ MUINEKO ZELULA AMEN (gDPSCs) OSTEODESBERDINTZAPEN GAITASUNAREN AZTERKETA TITANIO, GANTZ EHUN DEZELULARIZATU ETA PLASMATIK ERATORRITAKO PRODUKTUEKIN KONBINATUA

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Leioa, 2021

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Edukien taula

Laburpena

Hezur ehun ingeniaritza, arlo erlatiboki berri eta multi-disziplinarra da eta mindu edo galdutako ehunen funtzioa mantendu, hobetu eta birsortzeko ingeniaritza eta bizi zientzien aplikazioan oinarritzen da ordezko biologikoak garatzeko. Ehun ingeniaritzaren oinarrizko hiru zutabeak aldamioak (scaffold), ama zelulak eta hazkuntza faktoreak dira. Azken hamarkadetako teknologia aurrerapenekin eta material berri eta azaleren aldaketekin, hezur ehun ingeniaritzan eta hortz inplantologian erabiltzeko scaffoldek hobekuntza handiak jasan dituzte. Aldaketa hauen, MSC-en desberdintzapen osteoblastiko indukzio ezaugarri onak erakutsi dituzte. Gainera, MSC-ek ugalketa handia eta desberdintzapen gaitasun onak dituztela erakutsi dituzte hezur ehun ingeniaritzan erabiliak izateko. Hala ere, zein MSC zelula mota den hezur birsorkuntza terapietarako aukerarik onena oraindik ez dago garbi. Ama zelula autologoen erabileraren arazorik handiena, zelulen *in vitro* hazkuntzetan ohikoak diren animali jatorriko serumen erabilera da, behi suero fetala (BSF) bezala. Ama zelulak txertaturiko pazienteen erantzun immunea ekiditeko, suero hauen ordezkapena beharrezkoa da.

Ikerketa honetan, giza hortz mamiko ama zelulen (gDPSC) itsaspen, ugalketa, bideragarritasuna eta desberdintzapen osteoblastiko gaitasuna aztertu dira zabalki erabilitako Ti6AL4V titanio eta berria den azalera porotsu biomimetikodun (BAS^{TM}) titanio gainean haztean, desberdintzapen osteoblastiko medioaren presentzian edo gabezian, plasmatik eratorritako hazkuntza faktoreetan aberatsa den plasma (PRGF) eta plaketetan aberatsa den fibrina-z (PRF) osaturik.

Emaitzek, gDPSC-en itsaspen ona eta kaltetu gabeko bideragarritasun eta ugalketa zutela frogatu zuten Ti6AL4V eta BAS titanio gainean haztean. Hare gehiago, bi titanio azalerek, gDPSC-engan desberdintzapen osteoblastiko indukzio efektua zutela erakutsi zuten desberdintzapen mediorik erabili gabe. Bestalde, plasmatik eratorritako bi produktuen emaitza interesgarriak erakutsi zizkiguten. Alde batetik, PRGF-ak zelulen ugalketa tasa handitu zuten *in vitro*, BSF-aren ordezkari ona izan daitekeela erakutsiz zelulen terapia autologoetarako. Beste aldetik, PRF-ak gDPSC-en desberdintzapen osteoblastikoa handitu zuen kaltzifikaturiko hezur matrize produkzioa handituz. Azkenik, BAS titanioa eta PRF-aren konbinazioak gDPSC-en hezur sortazile zeluletarako desberdintzapen osteoblastikoa maximizatu zuen. Lan honetan lorturiko emaitzek, gaur

egun klinika praktikan hortz inplanteen inguruko hezur sorrera bultzatzeko ohikoak diren fibrina koaguluen erabilera bermatzen du.

Behin titaniozko bi azaleren gDPSC-etan duten eragina aztertuta, bideragarritasun, ugalketa eta desberdintzapen osteoblastiko gaitasunaren ikerketa konparatiboa egin genuen hezur birsortze terapietarako interesgarrienak ziren bi ama zelula mesenkimalen artean, gDPSC eta gBMSC-ak. Emaitzen, gDPSC-en ugalketa eta hezur matrize mineralizazio handiagoa iraoki zuten gBMSC-ekin alderatuta. Nahiz eta emaitza hauek berresteko datu gehiagoren beharra dagoen, isolatze errazago eta ez ain inbaditzaile eta ugalketa zein desberdintzapen osteoblastiko gaitasunen ezberdintasunengatik, gDPSC-ak gBMSC-ak baino aukera hobea izan daitezke hezur birsortze terapietarako.

Azkenik, hortz inplantologiaren beste arazo bat, lotailu periodontalaren galera da. Ehun honen funtzioa, hezur albeolarra murtxikatze indar mekanikoetatik babestea da kuxin funtzioa betez. Gaur egun, periodonto birsortze terapiak hesi mintzen erabileran datza, hortz inplantea jarri baina lehenago kalteturiko lekuan hezur sorrera handituz. Helburu honetarako, txerri jatorriko dezelularizatutako ehun adiposoa (pDAT) aztertu dugu. Gainera, pDAT-an hazitako gDPSC-ek desberdintzapen osteoblastikoa erakutsi zuten, mintz-barneko osifikazio guneak eta Sharpey gisako itsaspen fibra egiturak formatuz. Gizakian aurki daitekeen ehun adiposo iturri handia kontuan izanik eta biak, pDAT eta gDPSC-ak, paziente berberetik lortuak izan daitezkenez, hauen konbinazioa aukera aparta izan daiteke hezur birsortze eta hortz inplantologia terapia kliniko pertsonalizatuetarako.

Hitz gakoak: hortz mamiko ama zelulak, DPSC, hezur muineko ama zelulak, BMSC, ehun ingeniaritza, scaffold, zelula desberdintzapena, plasmatik eratorritako produktuak, PRGF, PRF, titanioa, dezelularizatutako ehun adiposoa, birsortze medikuntza.

Laburdurak

ALP= Fosfatasa alkalinoa

αMEM= α minimal essential medium

AMTP= Medikuntzako terapia produktu aurreratuak

APC= Alofikozianina

ARC= Adbentizioko zelula erretikularrak

ARS= Alizarin gorria

α-SMA= α-muskulo leuneko aktina

AT-MSC= Ehun adiposoko MSC-ak

BAS= Azalera biomimetiko aurreratuak

BDNF= Burmuin jatorriko faktore neurotrofikoak

BGLAP= osteokaltzina

BMPxxx= Hezur morfogenetiko proteina xxx

BMSC= Hezur muineko zelula amak

BSA= Behi serum albumina

CDxxx= Cluster differentiation

CFU-F= Colony forming unit fibroblasts

CMAP= The Connectivity Map

DAPI= 4´,6-diamino-2-phenilindol

DAT= dezelularizatutako ehun adiposoa

DFSC= Hortz folikuluko zelula amak

DME= β-merkaptoethanola

DMEM= Dubbelco´s modifikaturiko eagle´s medioa

DPSC= Hortz mamiko zelula amak

DSP= Dentina sialoproteina

DSPP= Dentina sialofosfoproteina

ECC= enbrioiko kartzinoma zelula

EDTA= Ethylenediamine tetraacetic acid

EGF= Hazkuntza faktore epiermikoa

EMD= Esmalteko matrize deribatua

ESC= Zelula ama enbrionarioa

FBS= Behi serum fetala

FGF= Fibriblastoen hazkuntza faktorea

FITC= isotiozianato fluoreszeina

GDNF: zelula glial jatorriko faktore neurotrofikoak

GOBP= Gene Ontology Biological Process

HA= Hidroxiapatita

gBMSC= Giza BMSC-ak

HBSS= Hank´s balanced salt solution

gDPSC= Giza DPSC-ak

HGF= Hepatozitoen hazkuntza faktoreak

HSC= Zelula ama hematopoietikoak

IBMX= 3-isobutil-1-metilxantina

ICM= Blastozisto barneko zelula masa

IL= Interleukinak

iPSC= Induziturik zelula ama pluripotenteak

ISTC= Terapia zelularraren sozietate internazionala

ITSx= Insulin-transferrin-selenium-x

sESC= Sagu ESC-ak

MNC= Muineko zelula mononuklearrak

MSC= Zelula ama mesenkimalak

NBT/BCIP= 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium

NeuN= Neuronen proteina nuklearra

NGF= Nerbioen hazkuntza faktorea

NSE= Neuronen enolase espezifikoa

OSTERIX/SP7= 7 transkripzio faktorea

PBS= Phosphate buffered saline

tDAT= Txerri DAT-a

PDGF= Plaketetatik eratorritako hazkuntza faktoreak

PDL= Lotailu periodontala

PDLSC= Lotailu periodontaleko zelula amak

PE= Fikoeritrina

PFA= Paraformaldehidoa

PGA= Azido poli-glikolikoa

PLA= Azido poli-laktikoa

PRF= Plaketetan aberatsa den fibrina

PRGF= Hazkuntza faktoreetan aberatsa den plasma

PRP= Plaketetan aberatsa den plasma

RA= Azido erretinoikoa

RUNX2= Runt-related transcriptional factor 2

SCAP= Papila apikaleko zelula amak

SEM= Ekorkuntz mikroskopio elektronikoa

SHED= Hortz erorkorren zelula amak

SHxxx= Src homology xxx

SPARC= Osteonektina

SPB= Sorensen fosfato bufferra

SSEA-1= Stage specific embryo antigen 1

TEM= Transmisiozko mikroskopio elektronikoa

TCP= Trikaltzio fosfatoa

TGF-β= β hazkuntza faktore eraldatzailea

V-CAM 1= Vascular cell adhesion protein 1

VEGF= Endotelio baskulaturaren hazkuntza faktoreak

Sarrera

Ehun ingeniaritzari buruzko sarrera

Ehunen ingeniaritza terminoa 1988an sortu zen National Science Foundation tailerrean "ingeniaritza eta bizitza zientzien printzipio eta metodoen aplikazioa ugaztunen ehun normal eta patologikoen egitura-funtzioen erlazioak ulertu eta ehunen funtzioa berreskuratu, mantendu eta hobetzeko ordezko biologikoen garapenean" bezala. Ehun ingeniaritza diziplina anitzeko eta arlo erlatiboki berria da, zeinak medikuntza kliniko, materialen zientzia, ingeniaritza mekaniko eta genetika bezalako diziplinak interkonektatzen dituen (Berthiaume et al., 2011). Ehun ingeniaritzak, galdu edo mindutako ehun funtzioen berreskurapena planteatzen du zelulen ereinketa, hazkuntza faktoreak eta hiru dimentsiotako aldamioak erabiliz (Chaudhari et al., 2016), hauek "ehun ingeniaritza hirukotea" bezala ezagutzen dira, zeinak bio-erreaktoreetan konfiguratuak izan daitezke ingurumen kontrolatuan (Dlaska et al., 2015; O'Brien, 2011).

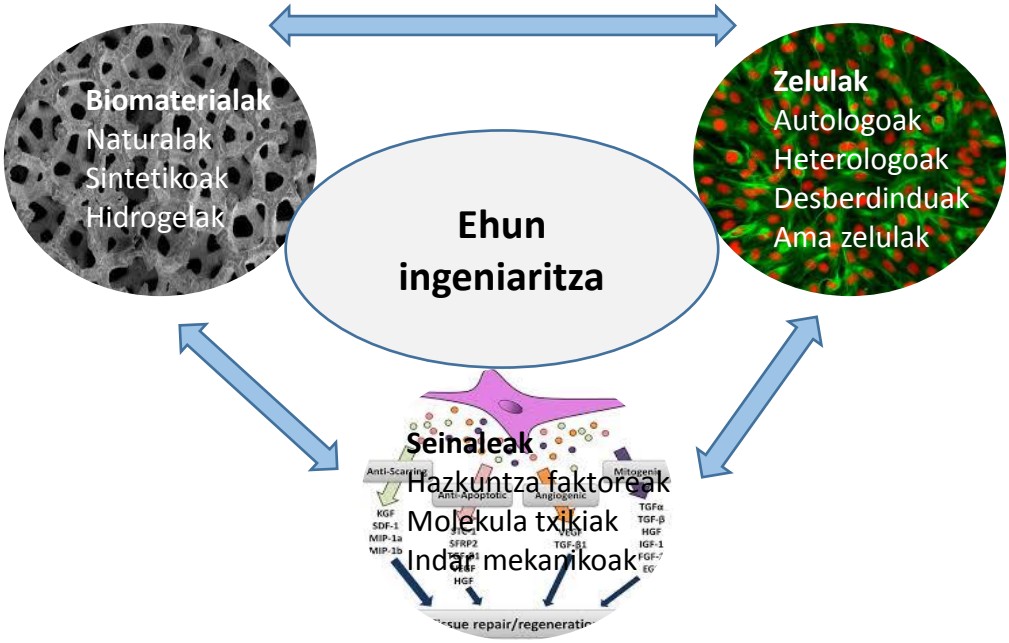
Ehun ingeniaritzaren helburu nagusia mindutako ehun edo organismoa berreskuratu, mantendu edo hobetzea da. Hezur ehunaren kasuan, ehun honek aurkezten dituen birmoldaketa naturala, birsorkuntza eta auto-konponketa abantaila handiak dira. Material berrien ikerketaren helburu nagusienetako bat material bateragarriak sortzea da, zeinak zelulei birsorkuntza seinaleak horni ditzaketen. Gainera, ehun ingeniaritza arloa, ingurumen naturalaren antzeko ezaugarriak dituzten material biomimetikoen aurkikuntzara bideratua dago, zelulen ugaltze, itsaspen eta desberdintzapena hobetzeko asmoz (Dhandayuthapani et al., 2011).

Odol transfusioen ostean, ehun transplante mota ohikoena hezur transplanteak dira, eta biztanleriaren zahartzea dela eta, mota onetako eskariak handitzen hari dira (Kattimani et al., 2016). Hezur ehun ingeniaritzak osteoblastoekin erlazionaturiko zelula amei begia bota die etorkizuneko terapietan erabiliak izateko (Stevens, 2008).

Hezur ehun ingeniaritzaren irizpideak kontuan izanik, garezur-aurpegiko ehun ingeniaritza pauso bat aurrerago doa hezur, listu guruin, lotailu periodontal, mukosako zementu eta dentina bezalako aho eta hortz ehunen birsorkuntzarako biomaterialen garapenean (Rahman et al., 2018).

Bereziki, periodontoaren birsorkuntza terapiek ongi informaturiko hezur/ehun birsorkuntza teknika gidatu dute, “mintzez babesturiko hezur birsorpena” bezala ezagutua. Teknika honen oinarria hesi-mintzen erabileran datza gandor albeolarreko akatsetan, hortz inplanteen inguruan kalteturiko hezurraren hazkundea areagotuz. Hezurra, inplante-hezur interfazea baino lehenago garatzen da, estabilitate mekanikoa emanez (Pilipchuk et al., 2015).

Laburbilduz, ehun ingeniaritzaren hiru zutabeak zelula amak, aldamioak, eta hazkuntza faktoreak dira (1. irudia).



1. Irudia. Ehun ingeniaritza hirukotea. Scaffoldetan ereindako zelula amak seinale biofisiko eta kimiko aproposekin koordinatua ehunen birsortzea bultzatzeko konbinazioa.

1. Zelula amak

Zelula amek, zelula ama bezala deituak izateko, hiru ezaugarri nagusi bete behar dituzte. Klonalitate, auto-berritze gaitasuna eta ehun eta organo ezberdinetako zelula helduetara desberdintzeko abilezia (Potten and Loeffler, 1990).

Deskribatutako lehen zelula amak, teratokartzinomatik isolaturiko enbrioi kartzinoma zelulak (ingelesez, embryonal carcinoma cells, ECCs) izan ziren 1950-ean (Stevens and Little, 1954). Hamalau urte beranduago, zelula hauen bi ezaugarri nagusi

deskribatu zituzten, auto-berritze gaitasuna eta hiru hozi-geruzetako zeluletara desberdintzeko abilezia *in vitro.* Zelula hauek zelula ama pluripotente bezala izendatuak izan ziren (Kleinsmith and Pierce, 1964). *In vivo* modeloetan deskribatuak izan ziren 1970-ean (Kahan and Ephrussi, 1970). Urte batzuk beranduago, 1981-ean, blastozisto barneko zelula masatik (ingelesez, inner cell mass of the blastocyst, ICM) isolatu zituzten sagu enbrioi zelula amak (mESCs) eta giza ESC-ak 1998-an (Martin, 1980; Thomson et al., 1998). Azken urteetan, zelula amen kontzeptu berri bat jaio zen, induzitutako zelula ama pluripotenteak (iPSCs). 2006-an saguetan deskribatuak izan ziren lehen aldiz eta 2007-an giza zeluletan (Takahashi et al., 2007; Takahashi and Yamanaka, 2006).

Garapen aldiaren arabera, zelula ama hauen desberdintzapen gaitasun ezberdina erakuts ditzakete. Honen arabera, bost taldeetan sailkatuak izan daitezke:

Totipotenteak: Mota honetako zelula amek gizabanako osoa sortzeko gaitasuna dute. Enbrioi oso zein denboraldiko sostengu ehunak (plazenta eta zilbor-hestea) sortzeko gaitasuna dute. Abilezia hau aldi blastomeriko arte mantentzen da (Posfai et al., 2021).

Pluripotenteak: blastozistoa sortu ostean, IMC-a osatzen duten zelula ama pluripotenteak hiru hozi-geruzetara desberdintzeko gaitasuna dute. Zelula ama hauek ehun eta organo heldu guztiak sor ditzakete bainan ez denboraldiko sostengu ehunak (Smith, 2001).

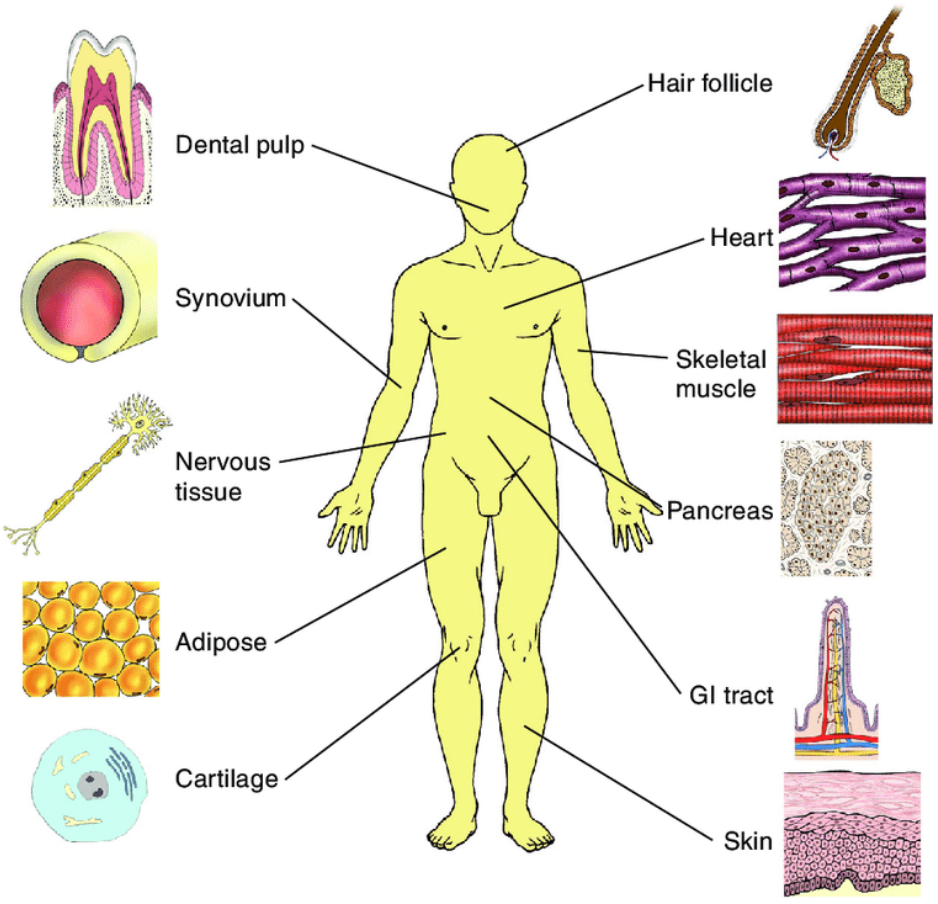
Multipotenteak: Zelula ama hauen desberdintzapen gaitasuna beraien ehun edo organoko jatorriari mugatua dago. Hauen funtzio nagusiak, ehun helduen konponketa eta mantentze-lanak dira (Slack, 2000).

Oligopotenteak: zelula ama hauek desberdintzapen abilezia oso mugatua dute zelula mota oso gutxietara desberdindu ahal izanik, adibidez, zelula mieloide eta linfoideak (Majo et al., 2008).

Unipotenteak: zelula ama hauek zelula mota bakarra sortuko dute, gihar zelula amak adibidez. Zelula ama gisa ezagutzen zaie auto-berritze gaitasuna dela eta (Seale et al., 2001).

1.1. Zelula ama helduak

Blastozistoa garatzen denean ICM-ko ESC-ak hiru hozi-geruzak sortzen dituzte: endodermoa, mesodermoa eta ektodermoa. Ehun eta organoen garapenaren ostean, zelula ama batzuk desberdintzapen terminalik gabe mantentzen dira hezur muin, odol, gibel, azal, burmuin, hortz edo giharretan bezala (Denham et al., 2005; Vats et al., 2005). Zelula ama hauen desberdintzapen plastizitate abilezia asko alda daiteke, zelula mota ezberdin askoetara desberdintzeko gaitasunetik hasita (multipotentea) zelula mota bakarrera desberdintzerarte (Unipotentea) (Almeida-Porada et al., 2001; Wagers and Weissman, 2004). Gainera, zelula hauen ugaltzeko gaitasuna asko alda daiteke ehunetik ehunera. Ugaltze handieneko zelulak zelula berritze altuko ehunetan aurki daitezke, hezur muin, azal edo hestean bezala (Baulies et al., 2020; Lenkiewicz, 2019; Yadav et al., 2020). Bestalde, beste zelula ama batzuk kalteturiko zelulen edo zauri baten erantzunean bakarrik ugalduko dira, gibel, bihotz edo nerbio sisteman bezala. (Angelini et al., 2004; Duncan et al., 2009; Mansergh et al., 2000) (2. irudia).



2. Irudia. Zelula amen lokalizazioa gizaki helduan. (Hodgkinson et al., 2009)

1.1.1. Zelula ama mesenkimalak (MSCs)

1990-ko hamarkadan zabaldu zen "zelula ama mesenkimal" kontzeptua Caplan-en erabilerari esker baina zientzia komunitateak ez zuen onartu 2000. urterarte (Caplan, 1991; Horwitz and Keating, 2000). Zelula ama mesenkimal bezala izendatzen dira ehun mesenkimal ezberdinetako zeluletara desberdintzeko gaitasuna duten zelula ama ez hetatopoietikoak. Zelula hauek gihar, kartilago, hezur, lotailu, gantz-ehun eta baskulatura ondoko guneak bezalako ehun edo organo ezberdinetatik isolatuak izan daitezke (Chamberlain et al., 2007; Crisan et al., 2008). Baita zilbor-heste, odol menstrual, plazenta, heste-lodi eta hortz egituretatik ere (Du et al., 2016; Ma et al., 2014; Portmann-Lanz et al., 2006; Tirino et al., 2011).

Zelula ama mesenkimalek zenbait baldintza bete behar dituzte horrela kontsidera daitezen. Terapia Zelularrentzako Elkarte Internazionala-k (TZEI) 2005-ean ezarri zituzten baldintza hauek: *in vitro* hakundeetan plastikoan istaskorrak izan behar dira eta kondrozito, adipozito eta osteozitoetara desberdintzeko gaitasuna eduki behar dute. Gainera, zelula hematopoietikoentzako zehatzak diren azalera markatzaileetarako (CD14, CD34 eta CD45) negatiboak izan behar dira, eta positiboak CD13, CD44, CD73, CD90 eta CD105-entzako (Dominici et al., 2006).

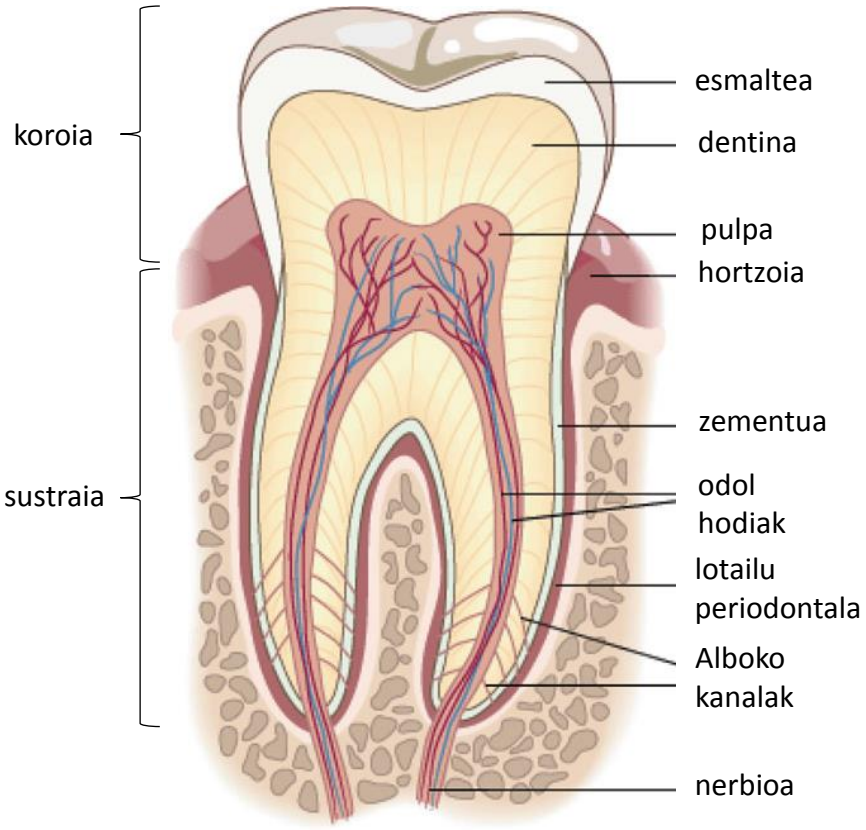
Gorputz helduan MSC populazio ezberdinak deskribatuak izan dira. Hezur muineko zelula ama mesenkimalak (ingelesez, bone marrow stem cells, BMSCs) izan ziren lehenik deskribaturiko MSC-ak (Anjos-Afonso and Bonnet, 2007; Friedenstein et al., 1976). Hauei jarraituz, zilbor hesteko zelula ama mesenkimalak (ingelesez, umbilical cord mesenchymal stem cells) 1991-n aurkituak eta 2004 urtean baieztatuak MSC bezala (McElreavey et al., 1991; Wang et al., 2014), hortz mamiko zelula ama mesenkimalak (ingelesez, dental pulp stem cells, DPSCs) (Gronthos et al., 2000), zelula ama mesenkimal kardiakoak (Beltrami et al., 2003), biriketako zelula ama mesenkimalak (Griffiths et al., 2005), odol periferiko-ko zelula ama mesenkimalak (Cao et al., 2005) edo gantz-ehuneko zelula ama mesenkimalak (ingelesez, Adipose tissue mesenchymal stem cells, AT-MSCs) (Fraser et al., 2006) deskribatu ziren. Naiz eta lehen deskribaturiko zelula moten jatorria hozi-geruza mesenkimala izan, aho barrunbeko zelula ama askoren jatorria gandor neuralean kokatzen da, DPSC-en kasuan bezala. Zelula hauek ama zelula mesenkimal

moduan sailkatuak izan diren arren, hozi-geruza ektodermal jatorriak neurona-antzeko zeluletara desberdintzeko gaitasun handiagoa ematen diote MSC-ekin alderatuz, hori dela eta zelula ama ektomesenkimal bezala deituak izan daitezke (Ibarretxe et al., 2012a).

1.1.1.1. Hortz mamiko zelula amak (DPSCs)

a. Ezaugarri orokorrak

Lehen esan bezala, DPSC-ak gandor neuraleko jatorria dute. Enbriogenesian zehar, gandor neuraleko zelula ectodermikoak aho barrunbera migratzen dira garezur-aurpegiko egitura ezberdinak sortzeko. Egitura hauen adibide dira hortz mamia, mingaina, garezur-aurpegiko nerbioak, lotailu periodontala, hezurrak eta giharrak (Ibarretxe et al., 2012a; Shyamala et al., 2015; Vega-Lopez et al., 2017). Ezaugarri honi esker, DPSC-ek leinu mesenkimaleko zelulez gain leinu neuraleko zeluletara desberdintzeko gaitasuna dute (Ibarretxe et al., 2012a).



3. irudia. Hortzaren egitura orokorrak. (Yildirim, 2013)-etik moldatua**.**

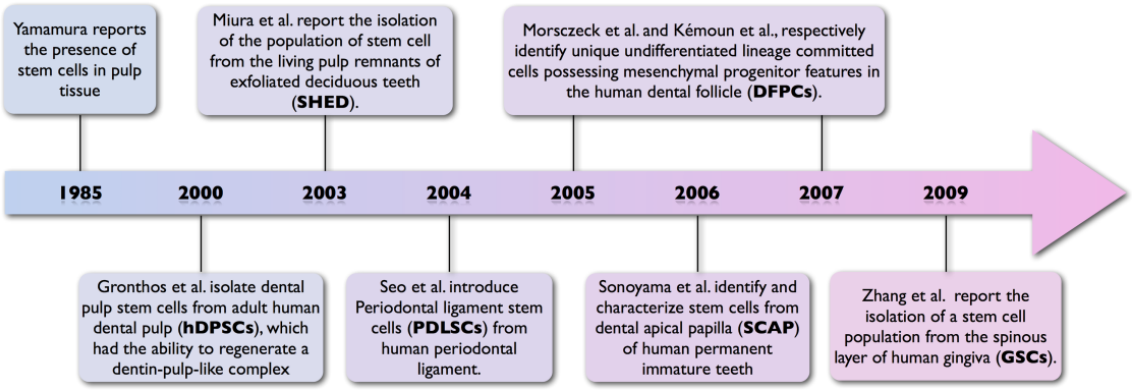
DPSC-ak 2000. urtean deskribatu ziren lehen aldiz hortz mamiko zelula ama mesenkimal moduan, BMSC-en antzera (Gronthos et al., 2000). Bi urte pasa ziren hortz mamiko zelula ama gisa deituak izan ziren arte, ezaugarritzat klonogenizitate eta ugalketa maila altuko zelulak izanik (Gronthos et al., 2002). Zelula hauek "mami ganbera"-n kokaturik daude, hortz koroiaren barnean (3. irudia). Hortz mamia konposizio heterogeneoko ehun konektiboa da eta odonto/osteoprogenitore zelula, zelula neural, baskulatura zelula, fibroblasto eta granulozito eta makrofagoak bezalako zelula inmuneak bezalako zelula mota ezberdinez osaturik dago (Goldberg and Smith, 2004). Onez gain, garrantzitsua da ikerketa batzuk DPSC-ak perizito baskularraren konpartimentuan kokatu dituztela esatea (Shi and Gronthos, 2003).

Hortz mami moduko ehun biguinez gain, hortz helduak zementu, dentina eta esmaltea bezalako ehun gogorrez osaturik daude. Hortzen sorreraren hasiera MSC-en eta aho zelula ektodermal epitelialen arteko elkarrekintzekin hasten da. Dentina da sortzen lehena eta honi jarraituz, esmaltea eta hortz folikulua. Zelula epitelialetatik eratorritako ameloblastoek sortzen dute esmaltea. Beste alde batetik, MSC-ak dira dentina, zementu, lotailu periodontal eta hortz mamiaren sortzaile (Sedgley and Botero, 2012; Thesleff and Aberg, 1999).

Gorputz helduan, hortz mamiaren funtzio nagusia dentinaren konponketa eta mantentze-lana da. Txantxarra moduko kalte larrietan DPSC-ak kalteturiko ingurumenera migratzen dute eta odontoblastoetara desberdindu ostean dentina konpontzailea sortzen dute (Dimitrova-Nakov et al., 2014; Tziafas et al., 2000) (4. irudia).

Nahiz eta DPSC-ak izan ikerketan ondoen ezaguturiko eta normalki erabilitako hortz zelula amak, ezaugarri ezberdinetako zelula ama populazio ezberdinak aurki ditzakegu. Hortz folikuluko zelula amak (ingelesez, dental follicle stem cells, DFSCs) hortz garapenean ehun enbrioniko ektomesenkimaletik isolatu daitezke non hortz-hozia inguratzen hari diren (Zhang et al., 2019). Zelula periodontaletaz gain, beste zelula leinu batzuetara desberdinduak izan daitezke *in vitro* (Honda et al., 2010). Hauez gain, papilla puntako zelula amak (ingelesez, stem cells from apical papilla, SCAP), garapenean dauden hortzen sustrai erpinetan kokatzen diren hazkuntza ratio handiko zelulak dira (Bakopoulou et al., 2011). Talde honetako beste zelula amen antzera, beraien jatorri

ektomesenkimala dela eta, zelula angiogeniko eta neuraletara desberdintzeko ahalmena dute (Dagnino et al., 2020; Nada and El Backly, 2018). Honez gain, lotailu periodontaleko zelula amek (ingelesez, periodontal ligament stem cells, PDLSCs) DPSC-en antzerako pluripotentzia markatzaile eta desberdintzapen gaitasuna dute (Liu et al., 2018; Seo et al., 2004). Azkenik, hasieran esfoliaturiko hortz erorkorretako zelula amek ere (ingelesez, primary exfoliated deciduous teeth stem cells, SHED) markatzaile pluripotentzial zein neuralak eta ugaltze ratio handia erakusten dute (Kerkis et al., 2006; Miura et al., 2003). Beste hortz ama zelulek gisa, SHED-ek zelula mesenkimal eta zelula neuraletara desberdintzeko abilezia erakutsi dute (Karbalaie et al., 2021; Sordi et al., 2021; Zhang et al., 2016) (4. irudia).



4. irudia. Hortzekin erlazionaturiko zelula amen historiaren denbora-lerroa. (Karamzadeh and Eslaminejad, 2013)**.**

b. DPSC-en azalera markatzaileak

DPSC-ek markatzaileen arteko ezberdintasuna erakutsi dute hortz mamiko zelula amen subpopulazioen eraginez (Alraies et al., 2020; Kawashima, 2012; Simonović et al., 2019).

Zelula hauen jatorri ektomesenkimala dela eta, garrantzitsua da markatzaile mesenkimal (Vimentina, I. Kolagenoa), neural (Nestina, GFAP) eta pluripotentzialen (OCT4, Nanog, Sox2) adierazpena aipatzea (Bae et al., 2021; Ibarretxe et al., 2012a; Wang et al., 2020).

Hare gehiago, DPSC subpopulazio barietateari esker, azalera markatzaileen adierazpena ez dago oso ongi ezarria. Orokorki, zelula hauek MSC-en mota gisa ezagutzen dira CD73 (5´-eknonukleatidasa), CD90 (glikosilfosfatidilinositolari loturiko glikoproteina) eta CD105 (endoglina) azalera markatzaileen adierazpen positiboarengatik. Gainera, DPSC-ek adierazpen positiboa dute CD27, CD29, CD44, CD146, CD166, CD271, V-CAM-1, SSEA4 eta STRO-1 azalera markatzaileentzat. Bestalde, adierazpen negatiboa dute CD14 (monozito eta makrofago markatzailea), CD19 (B zelula markatzailea) eta CD34 zein CD45 markatzaile hematopoietikoentzako (Gang et al., 2007; Gronthos et al., 2003). Nahiz eta honako hau izan DPSC-en azalera markatzaileen adierazpen ohikoena, CD34 markatzailearentzako positiboa diren zelulak ere ikertuak izan dira (Carnevale et al., 2018) (1. tabla).

	DPSCs	**SHED**	**PDLSCs**	**DFPCs**	**SCAPS**	**GSCs**	**BMSCs**
CD (+)	STRO-1	STRO-1	STRO-1	STRO-1	STRO-1	STRO-1	STRO-1
	CD29	CD29	CD29	CD29	CD29	CD29	CD29
	CD44	CD44	CD44	CD44	CD44	CD44	
	CD73	CD73	CD73	CD73	CD73	CD73	CD73
	CD105	CD105	CD105	CD105	CD105	CD105	CD105
	CD146	CD146		CD146	CD146	CD146	
		CD166			CD166	CD166	CD166
CD (-)	CD14	CD14					CD14
	CD19						
	CD34*	CD34	CD34	CD34	CD34	CD34	CD34
	CD45	CD45	CD45	CD45	CD45	CD45	CD45

1. taula. Hortzekin erlazionaturiko zelula amen eta hezur muineko zelula amen azalera markatzaile profila. DPSC: Hortz mamiko zelula amak, SHED: Hortz erorkorretatik lorturiko zelula amak, PDLSC: lotailu periodontalaren zelula amak, DFPC: hortz folikuluaren zelula aitzindariak, SCAP: hortz papila apikaleko zelula amak, GSC: hortzoi-ko zelula amak eta BMSC: hezur muineko zelula amak. CD34*: DPSC-en zenbait azpi-populaziok CD34 adierazi zuten (Carnevale et al., 2018) (autoreak egina).

c. DPSC-en desberdintzapena

Lehen aipatu bezala, DPSC-ek jatorri ektomesenkimala dute. Ezaugarri hau dela eta, osteo/odontoblasto, adipozito, kondrozito eta muskulu zeluletara desberdintzeko gaitasuna dute (Gronthos et al., 2002; Kawashima, 2012). Honez gain, gandor neural jatorriari esker, MSC zelulek baina neurona-antzerako zeluletara desberdintzeko ahalmena handiagoa dute (Luzuriaga et al., 2021). Gainera, ikerketa batzuk endodermoko zeluletara desberdintzeko abilezia dutela iradoki dute, DPSC-ek pluripotentzi-antzeko ezaugarria dutela esanez (Atari et al., 2011). Zoritxarrez, naiz eta endodermo-antzeko zelulak lortu, DPSC-en pluripotentzi gaitasuna ez da eztabaidaezin-ki frogatua izan.

Desberdintzapen osteogenikoa, DPSC-en desberdintzapena ezagunena da. Nahiz eta ikerkuntza ezberdinetan osteo-desberdintzapen indukzio koktelak aldaketak jasan ditzakeen, dexametasona, β-glizerol fosfatoa eta L-azido askorbikoa denbora guztian errepikatzen diren osagaik garrantzitsuenak dira. Molekula hauek ezinbestekoak dira I motako kolagenoa, fosfatasa alkalinoa, Run-erlazionaturiko 2 faktorea (RUNX2), osterix, osteopontina, osteokaltzina eta osteonektinaren adierazpena aktibatzeko. Gene hauen adierazpenak nahitaezkoak dira DPSC-en osteo-desberdintzapenean (Ajlan et al., 2015; Atari et al., 2012a; Bhuptani and Patravale, 2016; Goto et al., 2016; Riccio et al., 2010). Gene hauen artean, *RUNX2* da adierazten lehena. Osteo-desberdintzapenaren hasierako etapan *RUNX2*-aren adierazpenak dentina sialoproteina (DSP) eta dentina sialofosfoproteinaren (DSPP) espresioa eragiten du (Han et al., 2014; Vimalraj et al., 2015; Xu et al., 2015).

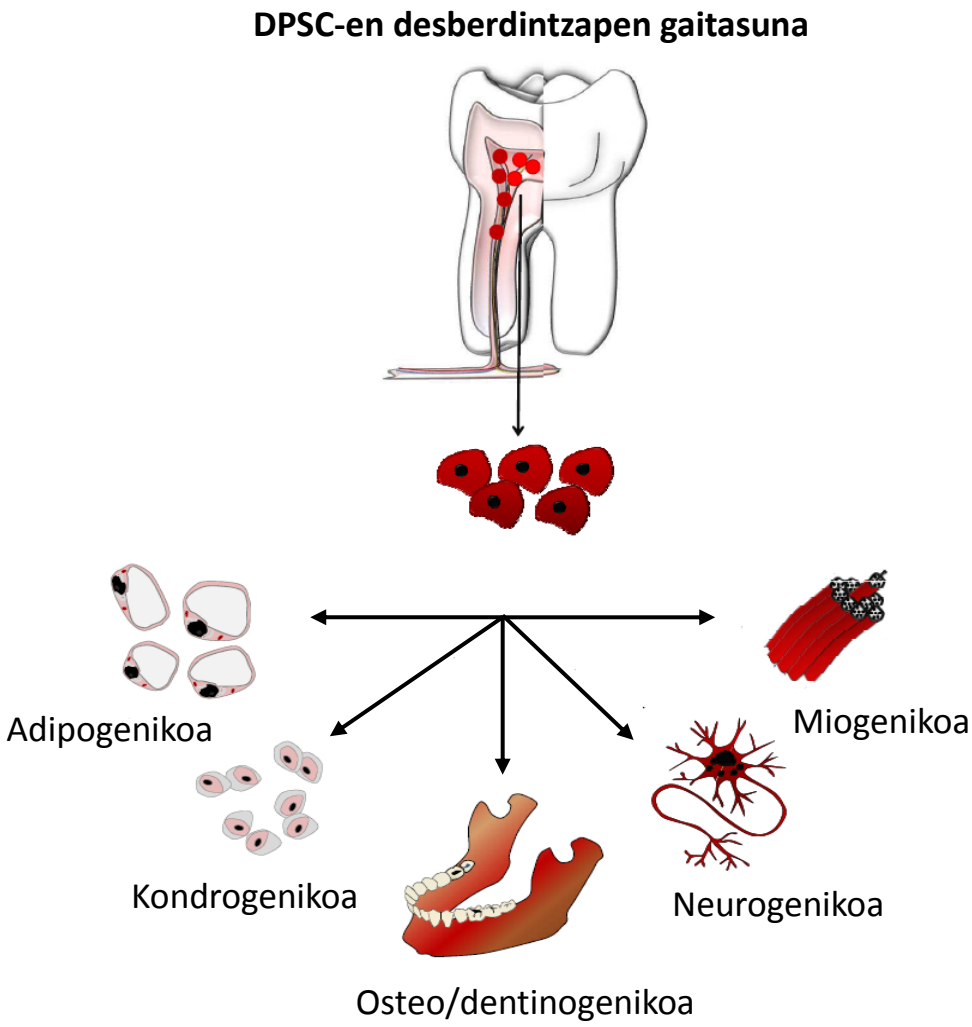
Desberdintzapen kondrozitikoaren kasuan, gehien erabilitako hazkuntza medioaren osagarri nagusiak L-prolina, L-azido askorbikoa, dexametasona, intsulina-transferrina-selenio (ITSx), sodio pirubatoa eta TGF-β3-a dira (Hilkens et al., 2013; Jang et al., 2016; Nemeth et al., 2014). Kondrozitoetarako desberdintzapen prozesuan proteina superfamilia garrantzitsuena TGF-β da, zeinak kartilago desberdintzapen/des-desberdintzapen prozesua kontrolatzen duten (Dexheimer et al., 2016). DPSC-ek beste MSC batzuk baino desberdintzapen kondrozitikorako gaitasun txikiagoa dute; hau,

DPSC-en populazio heterogeneo eta *in vitro* hazkuntzen oxigeno maila handiengatik da (Pisciotta et al., 2020).

Desberdintzapen adipozitikoan, dexametasonaz gain hazkuntza medioa intsulina eta 3-isobutil-1-metilxantinaz (IBMX) osatua egoten da. Adipozito desberdintzapena berresteko gantz azido lotzaileen 4. proteina, 4. motako glukosa garraiatzailea, lipoproteina lipasa markatzaile adipogenikoa eta peroxisoma ugaritzaileak aktibatutako ϒ hartzaileen adierazpenak beharrezkoak dira. DPSC-ek beste MSC batzuk baina desberdintzapen adiposo abilezia txikiagoa dute (Grottkau et al., 2010; Lee et al., 2015a; Zhang et al., 2016).

Bestalde, DPSC-ak zelula endotelial (Luzuriaga et al., 2019a; Luzuriaga et al., 2020; Marchionni et al., 2009), kardiomiozito (Ferro et al., 2012) eta muskulu leuna (Song et al., 2016; Zhao et al., 2012) bezalako zelula motetara ere desberdindu daitezke. Gainera, duela gutxiko ikerketek, zelula endotelial desberdintzapena lortu dute serum-ik gabeko medioak erabiliz, aurrera pausu handia terapia zelular autologoen erabilerarako (Luzuriaga et al., 2019a; Luzuriaga et al., 2020).

Leinu mesenkimaleko zelulez gain, DPSC-en desberdintzapenaren ezaugarri interesgarriena gandor neural jatorrian datza. Jatorri honi esker, DPSC-ak neurona-antzeko zeluletara desberdintzeko ahalmena dute. Azkeen urteetan erabiliak izan diren neurona desberdintzapen medioak neurotrofina eta beste molekula txiki batzuez (NGF, GDNF, BDNF, EGF, FGF, sonic hedgehog, forskolina, heparina, NT-3 eta azido retinoikoa (RA)) osaturik daude. Baita ere garrantzitsua da ITSx, N2, B27 eta aminoazido ez-ezinbestekoen erabilera (Arthur et al., 2008; Chang et al., 2014; Gervois et al., 2015; Kanafi et al., 2014; Király et al., 2011; Luzuriaga et al., 2019b; Osathanon et al., 2014; Xiao and Tsutsui, 2013; Zhang et al., 2017) (5. irudia).



5. irudia. DPSC-en desberdintzapen gaitasuna. (Aurrekoetxea et al., 2015).

DPSC-ek endodermo leinuko zelulak sortzeko ahalmena erakutsi dute (Atari et al., 2012b). Leinu endodermikoko zelula pankreatikoetara desberdintzeko gaitasuna erakutsi zuten glukagoia, somatostatina, intsulina eta polipeptido pankreatikoa ekoizteko abilezia zuten zelulak lortuz. Desberdintzapen hau oberen lortu zutenak CD117+ ziren zelulen subpopulazioa zen. Desberdintzapen prozesu onetan WNT eta PI3K/AKT seinaleztapen bideak funtsezko rola zuela erakutsi zuen (Ishkitiev et al., 2013; Yagi Mendoza et al., 2018). Onez gain, dexametasona, ITSx, HGF eta onkostatina M-z osaturiko serum gabe edo serum bajuko (1 %-2 %) medioak erabiliz hepatozito-antzerako zeluletara desberdintzea lortu zuten (Chen et al., 2016; Han et al., 2017; Ishkitiev et al., 2012).

1.1.1.2. Hezur muineko zelula amak (BMSCs)

a. Ezaugarri nagusiak

Friedenstein eta bere lankideek aurkitu zituzten hezur muineko zelula ama mesenkimalak 1970-ean, plastikoan itsasten ziren hezur muinetik eratorritako zelulen isolamendu metodoari esker (Friedenstein et al., 1970). Zelula hauek plastikoa hasten direnean, ugaritze ratio handiko kolonia konpaktu gisa fibroblasto itxurarekin. Hauek kolonia osatzaile unitate fibroblastoak gisa izendatu ziren (ingelesez, colony forming unit fibroblast, CFU-F) (Castro-Malaspina et al., 1980; Gothard et al., 2013).

CFU-F-ak, hezur muineko zelula mono-nuklearren (ingelesez, bone marrow mononuclear cells, MNCs) maiztasun baxua erakutsi zuten, 1/10.000 eta 1/100.000 artean zirelarik (Castro-Malaspina et al., 1980). BMSC-en maiztasuna, hezur muinean dauden zelula ama hematopoietikoena (ingelesez, hematopoietic stem cells, HSCs) baino askoz baxuagoa da, hezur muineko MNC-en 1 % izanik (Civin et al., 1996).

Gainera, ziklo zelularraren ikerketek erakutsi dutenez, BMSC-en frakzio txiki batek besterik ez dute ziklo zelularra aktibaturik (10 % at S + G2 + M) eta beste guztiak G0/G1 fasean egonik (Conget and Minguell, 1999). Pazienteen aldakortasunaren arabera, BMSC-en potentzial hedagarria laugarren pasetik hamabost pase igarotzeraino irits daitezke (Digirolamo et al., 1999; Phinney et al., 1999). Naiz eta ziklo zelularra aktibaturiko zelulen frakzioa txikia izan, ugaltze ratio handia dute *ex vivo*. Ugalketa gaitasun honek aldaketak jasan ditzake zelulak lortzeko metodoen, BMSC-en frekuentziaren eta emailearen adinaren arabera (Blazsek et al., 1999; Koç et al., 1999). Duela gutxiko ikerketek BMSC-en *in vitro* hazkuntzak hobetu dituzte bio-erreaktoreak erabiliz (Bhat et al., 2021; Santos et al., 2011). Naiz eta zelula somatikoek hazkuntza eta desberdintzapen prozesuetan telomerasa aktibitatea galtzen duten zelulen heriotza eraginez seneszentzi bidez, BMSC-ek telomerasaren aktibitatea mantentzen dute (Pittenger et al., 1999). Hala ere, hazkuntza pase askoren ostean, apoptosis eta seneszentzi seinaleak adierazten dituzte (Dhanasekaran et al., 2013).

BMSC-en funtzio nagusiak estroma mikro-ingurumenaren formakuntza eta mantentze-lana dela iradoki da. Erregulazio seinale induktibo bidezko mikro-ingurumenaren modulazioa ez da beste BMSC-entzako bakarrik, baizik eta beste

estromako zelula ez-mesenkimatiko eta zelula hematopoietiko aitzindarientzat ere bai (Cheng et al., 2000). Teoria hau BMSC-ek ekoizturiko kolageno, laminina, fibronektina eta proteoglikanoak bezalako matriz molekula ezberdinengatik indartuta dago (Chichester et al., 1993; Connelly et al., 2008; Pittenger et al., 1999). Zelula-zelula elkarreragin itsaskorra eta matrizeari elkarturiko kontra-errezeptore molekulak ere adierazten dituzte.

Ikerketa batzuk, DPSC-ak bezala, BMSC-ak baskulaturaren inguruko guneetan kokatzen direla iradoki dute, odol hodien kanpoko gainazala estalduz (Shi and Gronthos, 2003; Short et al., 2003; da Silva Meirelles et al., 2006). Aldiz, beste ikerketa batzuk BMSC-ak hezur muin estromako zelula laguntzaileen (ingelesez, adventitial reticular cells, ARCs) berdinak direla esan dute. Emaitza gisa, BMSC-ak estromako zelula mesenkimal multipotente edo hezur muin estromako zelula ama bezala ere ezagutzen dira (Gronthos et al., 2003; Horwitz et al., 2005).

b. BMSC-en azalera markatzaileak

BMSC-en ezaugarrietako bat CD14 (monozito eta makrofago markatzaileak), CD31 (PCAM) eta CD34 zein CD45 markatzaile hematopoietikoen adierazpen negatiboa da (Conget and Minguell, 1999; Jones et al., 2002; Pittenger et al., 1999). Bestalde, zelula hauek adierazpen positiboa dute gihar leuneko α-aktina (α-sma), STRO-1, SH2, SH3 eta SH4-entzako (Haynesworth et al., 1992; Simmons and Torok-Storb, 1991). Hauez gain, CD54 (ICAM-1), CD102 (ICAM-2) eta CD166 (ALCAM-1) itsaspen molekulentzako ere adierazpen positiboa dute (Chichester et al., 1993; Conget and Minguell, 1999; Prockop, 1997). Baita ere CD13 (ANPEP), CD29 (β1 integrina), CD73 (NTSE), CD90 (THY1) CD105 (endoglina) eta SSEA-4-entzako (enbrioien etapa-espezifiko antigenoa) (Boiret et al., 2005; Delorme and Charbord, 2007; Gang et al., 2007; Jones et al., 2006). Azkenik, garrantzitsua da aipatzea CD271-ren (afinitate-baxuko nerbio hazkuntza faktore errezeptorea) adierazpena, hezur muineko subpopulazioen markatzaile bezala. Subpopulazioen arteko markatzaile honen adierazpen ezberdintasuna BMSC-en adierazpen maila altu eta hezur muineko beste zelula pupolazioen adierazpen baxuan datza (Bühring et al., 2007; Cuthbert et al., 2015).

c. BMSC-en desberdintzapena

Hezur muineko MSC-ak leinu-anitzeko desberdintzapen gaitasuneko zelula ez-hematopoietikoak dira. Ikerketa askok erakutsi dute kartilago, gihar kardiako, gihar eskeletiko, hezur eta ehun adipotsua bezalako zelula mesenkimatikoetara desberdintzeko zelula hauek duten gaitasuna (Arthur et al., 2009; Bianco et al., 2001; Conget and Minguell, 1999; Matsuda et al., 2005; Prockop, 1997; Zheng et al., 2013). Beraien jatorriko hozi-geruza mesenkima izanik ere, neurona-antzeko zelula neuroektodermikoetara ere desberdindu daitezke (Krampera et al., 2007; Sanchez-Ramos et al., 2000; Zhang et al., 2012). Hala ere, neurona-antzeko zelula hauetara desberdintzeko gaitasuna, DPSC-ena baina txikiagoa da (Isobe et al., 2016; Pagella et al., 2020).

BMSC-en desberdintzapen osteogenikoa dexametasona, L-azido askorbikoa eta β-glizerol fosfatoaz (edo beste fosfato iturri izan daitekeen molekularen bat) osaturiko medioetan oinarritzen da, DPSC-en antzera. Zelula hauetan ere molekula hauek *RUNX2* genearen adierazpena areagotzen dute, osteo-desberdintzapen prozesuaren hasiera izanik (Langenbach and Handschel, 2013).

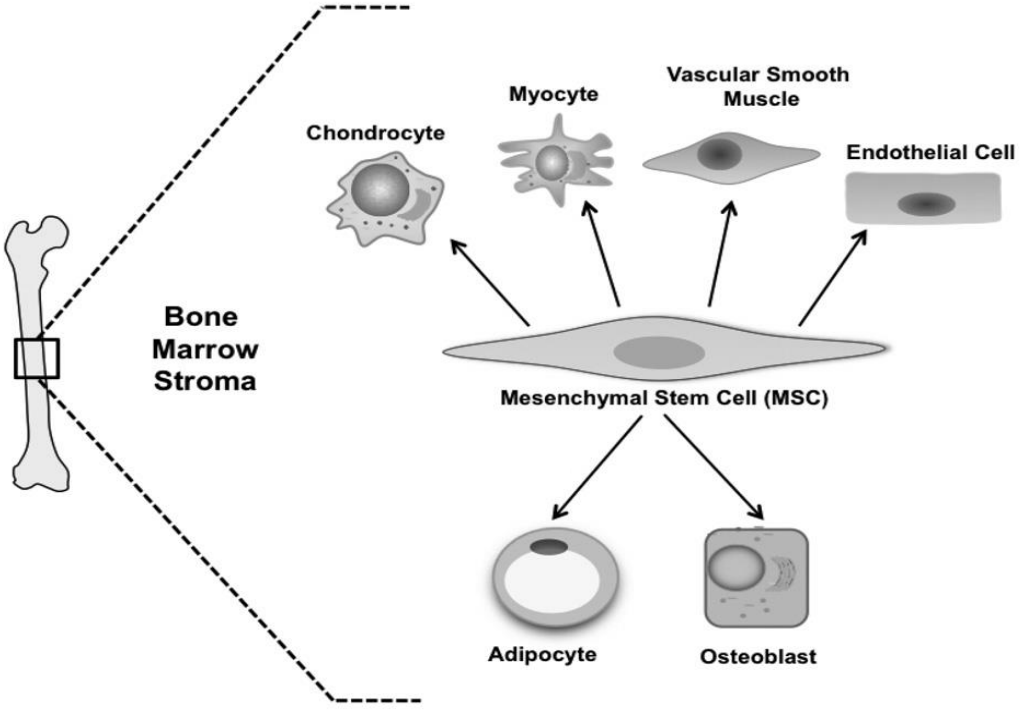
Beste alde batetik, desberdintzapen adipogenikorako dexametasonaz gain, indometazina eta IBMX-ez osaturiko medioak erabiltzen dira (Zheng et al., 2013).

Desberdintzapen kondrozitikorako aldiz, beharrezko molekulak dexametasona, sodio pirubatoa, L-azido askorbikoa, prolina, ITS eta TGF-β1 dira (Solchaga et al., 2011). Bi desberdintzapen hauentzako, adipogenikoa eta kondrogenikoa, BMSC-ek DPSC-ek baina desberdintzapen gaitasun handiagoa erakutsi dute (Isobe et al., 2016).

Hauez gain, BMSC-ak ere zelula kardiako, gihar eskeletiko, lotailu eta estroma hematopoietiko-laguntzailea bezalako zelula mesenkimaletara ere desberdin daitezke (Guo et al., 2018; Matsuda et al., 2005; Tian et al., 2010; Wu et al., 2018) (6. irudia).

BMSC-en jatorria mesenkimala denetik, orain arte aipaturiko desberdintzapenak “natural” kontsidera daitezke. Hala ere, zelula hauek jatorri ektodermikoko neurona-antzeko (neurona markatzaileren bat adierazten dute zelulak) zeluletara desberdindu

daitezke. Desberdintzapen honetarako gehien erabilitako bi hazkuntza koktel daude. Alde batetik oinarrizko fibroblasto hazkuntza faktorea (ingelesez, basic Fibroblast Growth Factor, bFGF) erabiltzen duen medioa dago. Zelula zein ehun mota ezberdinetan lau FGF errezeptore aurki ditzakegu adierazpen maila ezberdinekin (Eswarakumar et al., 2005). Errezeptore honek Src, Crk, fosfolipasa C-ϒ (PLC-ϒ) eta SNT-1/FRS2 bezalako seinale bideak aktibatzen ditu, hauetako batzuk desberdintzapen neuralean inplikatuak egonik (Powers et al., 2000; Reuss and von Bohlen und Halbach, 2003). Ikerketa batzuk ondorioztatu dutenez, SNT-1/FRS2 seinale bideak MAPK/ERK kaskada aktibatzen du, nahitaezkoa faktore neurotrofikoen jariapenerako (Abe and Saito, 2000). Beste aldetik, β-merkaptoetanolez (BME) osaturiko medioa dago zeinak 7 eguneko hazkuntzaren ostean neuronentzako espezifikoa den enolasa (NSE), NeuN eta M-neurofilamentuaren adierazpena bultzatzen duen (Khanabdali et al., 2016; Khang et al., 2012; Scintu et al., 2006).



6. irudia. Hezur muineko zelula amen desberdintzapen gaitasuna. (Muruganandan et al., 2009)**.**

2. Hezur ehun ingeniaritzarako aldamioak (Scaffold)

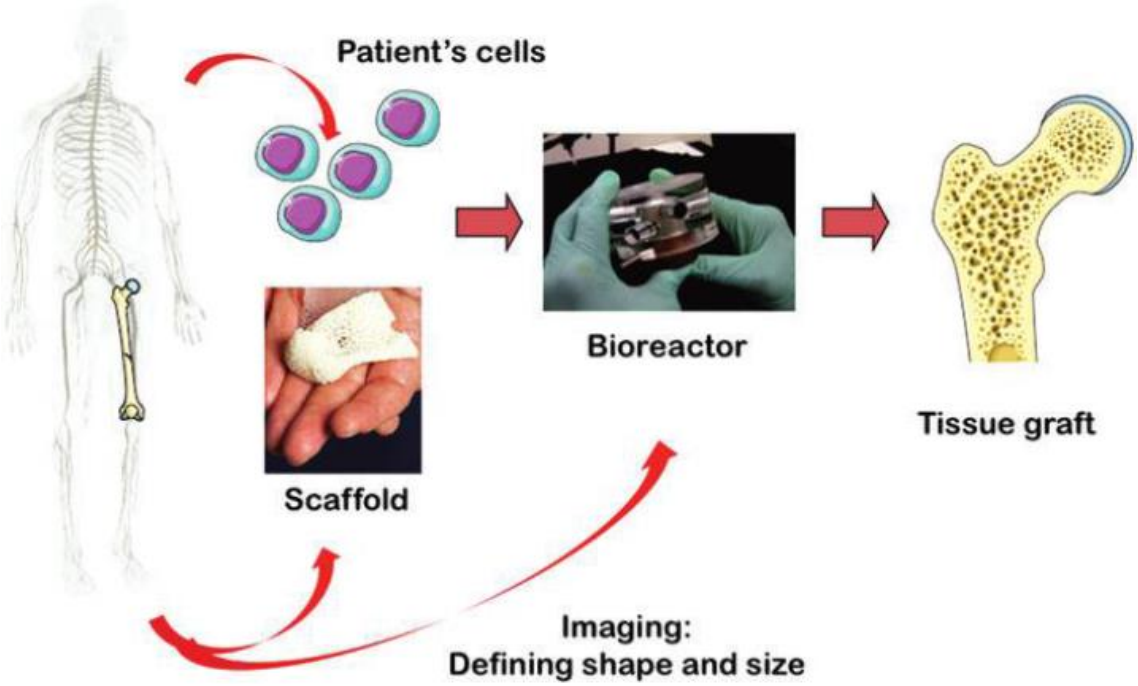
Scaffold hitza, ehun eta organoen erregeneraziorako ingurumen egokia ematen duten hiru dimentsiotako (3D) bio-materialei dagokio. Material berrien sorreren helburua, ingurumen naturalaren antzeko propietateak eta ezaugarri multi-funtzionalak dituzten scaffold berrien garapena da (Dhandayuthapani et al., 2011) (7. irudia).

Hezur ehun ingeniaritzan, scaffoldek hiru ezaugarri bete behar dituzte hezur injerto bezala erabiliak izateko: osteo-konduktibitatea, osteo-induktibitatea eta osteo-integrazioa (Albrektsson and Johansson, 2001). Injertoan hezurra hazi baldin badaiteke osteo-konduktibitate deitzen zaio, inplantearen azalera eta hezurraren arteko kontaktu zuzenari berriz osteo-integrazioa. Ezaugarri hauek ezberdinak izan daitezke materialaren arabera, inplante lekuaren arabera, etab. (Khan et al., 2005). Azkenik, osteo-induktibitate gisa ezagutzen da hezur sorrera eragin dezaketen zeluletan desberdintzeko ama zelula multipotenteei bultzatzeko gaitasunari (Roberts and Rosenbaum, 2012).

Historikoki, bio-materialen lehen belaunaldiaren ezaugarri nagusia bio-konpatibilitatea zen. Material hauen helburua ordezkatu behar zuten ehunaren antzeko ezaugarri fisikoak edukitzea zen. Zeramikak (zirkonioa eta alumina), metalak (titanioa, burdin herdoilgaitza eta aleazioak) eta polimeroak (kautxua, silikona, polipropilenoa eta polimetilmetakrilatoa) osatzen zuten hezur ehun ingeniaritzan erabiltzeko bio-materialen lehen belaunaldi hau. Hauetako scaffold batzuk erantzun immune ez-espezifikoak eragiten zituzten, azkenean ehun konektibo fibrotikoz inguratua izan arte. Honela, inguruko ehunetatik isolatuta geratzen ziren (Anderson, 2001).

Erantzun immune ez-espezifiko hauek saihestu nahian, bio-interaktibitatea izan zen bigarren belaunaldiko helburua. Honetarako, lehen belaunaldiko materialen azalerak hidroxiapatita (HA) edo β-trikaltzio fosfatoz estali ziren, mineralizazioa baimenduz. Belaunaldi honetan bio-degradagarriak ziren scaffoldak erabili ziren lehen aldiz. Scaffoldaren degradazio ratioa hezur sendaketa ratioarekin bateratuz, material hauen helburua hezurra sortzen zuten zelulei euskarria ematea zen gorputzak materiala xurgatzen zuen bitartean. Adibide ohikoenak polimero naturalak (azido hialuronikoa eta kitosanoa) eta sintetikoak (poliglikolidoa eta polilaktida) dira (Navarro et al., 2008).

Gaur egunean gauden hirugarren belaunaldiko scaffoldak material bio-sentikorrak dira. Bio-material hauek, gene espezifikoak aktibatzeko ahalmena dute, zelulen ugaltze eta desberdintzapena sustatuz. Belaunaldi hau ehun ingeniaritzan oinarritua dago, scaffold natural edo sintetikoak zelula amez eta hazkuntza faktoreez estaliz, hezur birsorkuntza eta baskularizazioa bultzatzeko asmoz (Amini et al., 2012; Hench and Polak, 2002; Rahman et al., 2018).



7. irudia. Hezur ehun ingeniaritza. Hezur scaffold-a eta zelula ama autologoak bioerreaktorean hazkuntza faktoreekin haziak hezur autologoaren sorrera bultzatzen dute *in vitro*, hezur konponketa aplikazioetarako *in vivo.*

Gaur egun, hezur ehun ingeniaritzarako scaffoldak polimero natural edo sintetikoz eta material inorganikoz osatuta daude.

Gehien erabiltzen diren polimero sintetikoak azido poli-glikolikoa (PGA), azido poli-laktikoa (PLA) eta beraien ko-polimeroak. Material hauen degradazio ratio kontrola eta ezaugarri mekaniko ezin hobeek, zelulak erein ostean ehun ingeniaritzan erabiliak izateko aukera ona eskaintzen dute (Dorati et al., 2017).

Bestalde, osagai biologiko naturalen artean proteinak (kolagenoa, fibrina gelak, soja eta zeta) eta polisakaridoak (alginatoa, kitin/kitosanoa, almidoia eta azido

hialuronikoaren eratorriak) aurki ditzakegu. Aukera onak dira zelulen itsaspena areagotzeko, hala ere, bio-degradazio eta ezaugarri mekanikoen kontrola murrizten dute. Gainera, immune erantzuna sor dezakete (Dorati et al., 2017).

Azkenik, metal, trikaltzio fosfato (TCP), beira bio-aktibo, hidroxiapatita (HA), wolastonita eta beraien arteko konbinazioak HA-TCP zeramika bifasikoa kasu, aurki ditzakegu material inorganiko gisa. Material hauen ezaugarririk interesgarriena hezur mineral fasearen antzekotasuna da (Kokubo and Takadama, 2006; Lakshmi and Sasikumar, 2015; Stevens, 2008). Desabantaila nagusia berriz, material hauen degradazio falta da, honi esker ezin dira xurgatuak izan ehun berrien sorrera eragotziz.

Azken ikerketetan ikusi denez, zelulen ugaltze eta desberdintzapenerako scaffolden porositatea erabakigarria da. Ikerketa hauen arabera 100 eta 400 µm arteko poro neurriak ezin hobeak dira hezur helduaren sorrerarako, baskularizazioa eta zelulen infiltrazioa hobetuz lortua (Boyan et al., 2016a; Murphy et al., 2010). Beste ikerketa batzuek berriz zelulen hazkunderako poro tamaina egokiena 800 µm direla iradoki dute (Roosa et al., 2010). Bestalde, 100 µm baina txikiagoak diren poroak ehun fibrotiko eta osteoide ez-mineralizatuaren sorrerarekin erlazionatu dituzte (Iviglia et al., 2019; Liu et al., 2018). Aldi berean, mikro-poroek azalera area handiagoa eskaintzen dute, ioien elkartruke eta zelula zein hezur itsaspen proteinak areagotuz (Abbasi et al., 2019; Diaz-Rodriguez et al., 2018; Morejón et al., 2019).

Kontuan edukitzeko scaffolden beste ezaugarri garrantzitsu bat materialaren forma da. Azalera ahurrek, azalera lau edo ganbilek baina ehun sorrera hobea erakutsi dute. Azalera ahurretan, zelulen lerrokatzea hobea da aktina eta miosina fibren dentsitate handiagoari esker, azalera ganbiletan berriz, ehunen hazkundea atzeratu egiten da (Bianchi et al., 2014; Knychala et al., 2013).

2.1. Titanioa

Azken mende erdian, hortzen ordezkapenerako titaniozko inplanteak aukerarik hoberen moduan ezarri dira, hauen erresistentzia, iraunkortasun eta osteo-integrazio ahalmen altuarengatik (Albrektsson et al., 1981; Breine and Brånemark, 1980). Hala ere,

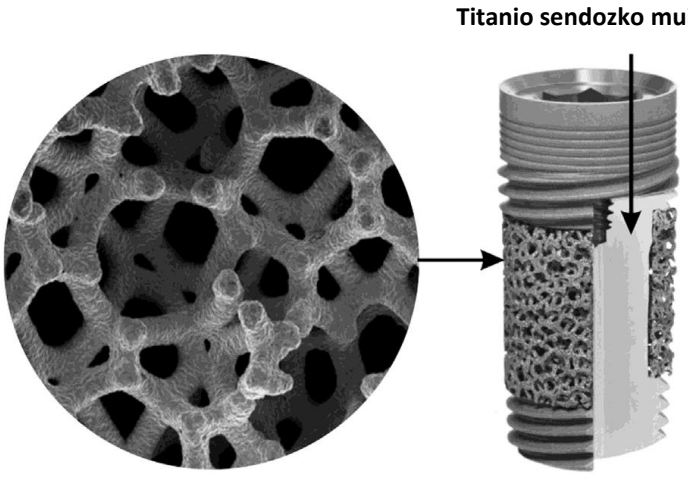
beharrezko masailezur faltagatik, bakterioen infekzioengatik (Periinplantitisa), inplante azaleren mikro-porositatearengatik edo inguruko zelula ama mesenkimalen aktibazio faltagatik inplante hauen efizientzia konprometitua izan da (Boyan et al., 2016a; Graziano et al., 2008). Horregatik, scaffold berri, azalera topografia eta zelula ametan oinarrituriko ikerketak, inplantologia klinikorako hobekuntza handiak ekar ditzakete masailezur eta barailezurren birsortze terapietarako.

Nahiz eta hortz inplanteetarako aukerarik hoberena titanioa dela ikusi den arren, azken hamarkadetan, inplante azalera ezberdinen diseinu eta fabrikazioak osteo-integrazioa hobetzean duen garrantzia frogatu da (Gasik et al., 2015; Rani et al., 2012; Rupp et al., 2018; Salou et al., 2015). Titanioaren konposizioaz gain, garrantzitsua da azalera hauen topografia kontuan hartzea inplantearen ainguraketa iraunkorra lortzeko (Annunziata and Guida, 2015; Jemat et al., 2015; Le Guéhennec et al., 2007; Naves et al., 2015).

Titanio azalera gogorrek, inguruko hezur albeolarrarekiko duten kontaktu azalera maximizatzeari esker, osteo-integrazio ahalmen handiagoa izatea frogatu dute titanio leunekin alderatuz (Coelho et al., 2009). Inplante hauen hezur integrazio hobe hau, titanio azaleren mikro-porositatearekin erlaziona daiteke, zeinak inguruko zelula ama mesenkimalen desberdintzapen osteoblastikoa bultzatzen duen. Azkenik, inplantea kokatu ostean, zelula mesenkimal hauek dira hezurra sendotu eta sendatzearen arduradun (Boyan et al., 2016a; Graziano et al., 2008).

Lehen aipatu bezala, ikerketa ezberdinek frogatu duten bezala, poro tamaina txikiek (<180 µm) zelulen desberdintzapena bultzatzen dute inplantearen sendaketaren hasieran, aldi berean, tamaina handiko poroek (>300 µm) zelulen ugalketa eta hezur sorrera bultzatzen dute (Coelho et al., 2015). Hala ere, porositateak materialen ezaugarri mekanikoetan eragin zuzena du, beraz, ezinbestekoa da oreka egoki bat mantentzea porositate eta gogortasunaren artean inplante motaren arabera. Hare gehiago, beste ikerketa batzuk erakutsi dutenez, titanio porotsuek korrosioaren aurkako erresistentzia txikiagoa dute gorputzeko fluidoen fluxu dinamikoaren ondorioz. Porositateak gorputzeko fluidoen fluxua errazten du, degradazioa azkartuz (Chen et al., 2017).

Titanio porotsuen garapenean hobetu beharreko beste ezaugarri bat inplanteen zurruntasuna txikitzea da. Titanio inplante zurrunetan ikusi denez, hezur sorrera eta birmoldaketa txikitzen dute estresa babesteak dakarren inplikazio negatiboengatik. Arazo hauek azkenean hezurraren birxurgapena eragiten dute, eta ondorioz inplantearen galera (Ma et al., 2006; Świeczko-Żurek, 2009) (8. irudia).



8. irudia. Titaniozko hortz inplanteen azalera porotsua. (Pałka and Pokrowiecki, 2018)**.**

Titanio aleazioak hiru taldeetan sailka daitezke:

1- A fase egonkortzaileak: karbono (C), oxigeno (O), aluminio (Al) eta nitrogenoa (N).
2- B fase egonkortzaileak:
    a. Isomorfoak: tantalo (Ta), molibdeno (Mo), banadio (V) eta niobioa (Nb).
    b. Euktoideak: silikona (Si), kromo (Cr), kobre (Cu), hidrogeno (H), manganeso (Mn), nikel (Ni) eta burdina (Fe).
3- Gehikuntza neutroak: estainu (Sn) eta zirkonioa (Zr).

Kalteturiko hezur albeolarraren inguruko zelula mesenkimalen aktibazioa ezinbestekoa da inplantearen integrazioa bultzatzeko, iraunkortasuna eta erresistentzia hobetuz eta bakterioen eraginez sorturik infekzioak ekiditeko. Arrazoi honengatik,

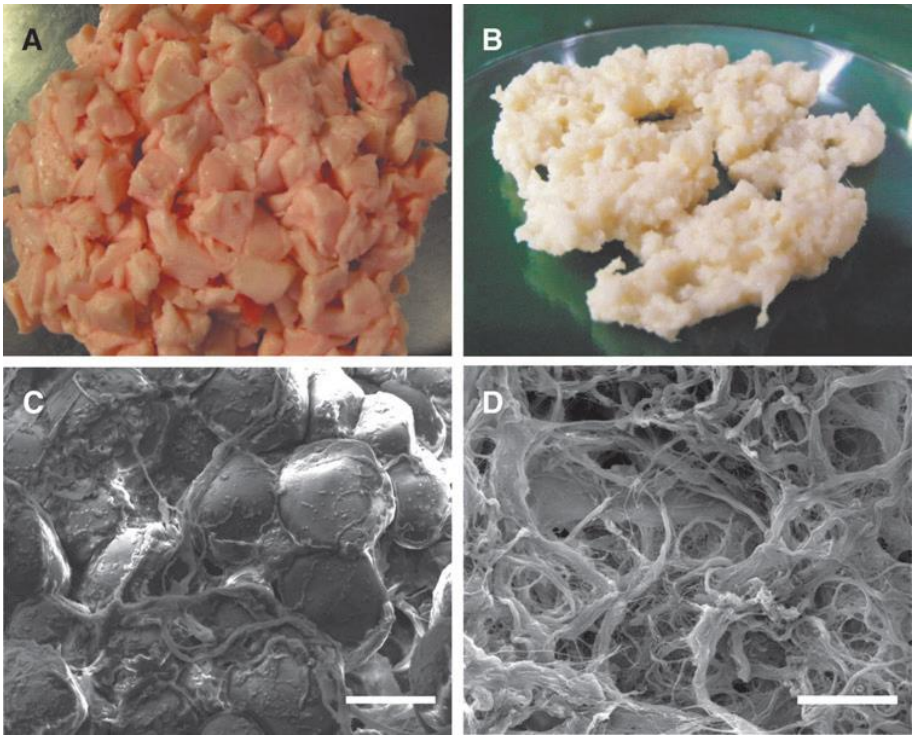
titanio inplante eta zelula aloinjertu/autoinjertuen arteko konbinazioak abantaila handiak eskaintzen ditu inplantologia klinikoan erabiliak izateko.

2.2. Dezelularizaturiko ehun adiposoa (DAT)

Gaur egun erabiltzen diren hortz inplante gehienak metal aleazio inerteak dira. Inplante hauek bakarrik erabiliz edota dezelularizatutako hezur matrize eta plasmatik eratorritako hazkuntza faktoreekin konbinatuz, ama zelula mesenkimalenosteo-desberdintzapena bultzatzen dute, inplante azaleran hezurra sortuz (Anitua et al., 2016b; Boyan et al., 2016a; Mayer et al., 2018; Zanicotti et al., 2018). Gaur egun, hortz inplanteen arazorik nagusiena periinplantitisaren garapena da. Periinplantitisa, murtxikatze naturalean inplanteek hezur ehunari transferitzen dion presio mekaniko jarraiaren ondorioz sortzen da. Kasu okerrenetan, periinplantitisak inplantearen galera eragiten du (Bertolini et al., 2019). Hortz naturaletan, arazo hau lotailu periodontalari (PDL) esker konpontzen da, inerbazio eta baskularizazio altuko ehun konektiboa da, kolageno sorta indartsuez osaturik dagoena. Kolageno fibra sorta hauek hortz sustrai zementu eta hezur albeolarrari ainguraturik daude kaltzifikazio arineko ertzei esker. Fibra hauei Sharpey fibra zulatzaileak bezala ezagutzen zaie (Aaron, 2012). PDL-aren funtzio nagusia mastekatzean sortzen den indarra xurgatzea da kuxin bezala jokatuz, honela hezur albeolarra babestuz

Dezelularizatutako ehun adiposoak (ingelesez, Decellularized Adipose Tissue, DAT) bio-material itxaropentsua dela ematen du malgutasun, irisgarritasun eta ezaugarri bio-aktiboak bezalako ezaugarriengatik (Yang et al., 2020). *In vivo* entseiuetan dagoeneko erakutsi dute bio-bateragarritasuna (Wang et al., 2013). DAT-aren ezaugarri interesgarrienetako bat, material honen leuntasuna da. Honi esker, ehun konektibo interfasea lortu daiteke hortz inplante sustraiaren eta hezur albeolarraren artean. DAT-a ehun adiposo zuritik lortzen da, hau, baskularizazio altuko ehuna da. Zelulen atxikipen eta integrazioari laguntzen dio oinarrizko mintz proteinen kontzentrazio altuari esker, hauen aretan aipagarrienak kolagenoa (I, III eta IV mota), perlekanoa (proteoglikano sulfatatua) eta laminina izango lirateke (Yang et al., 2020). Dezelularizazio prozesua ere hobetua izan da (Madarieta Pardo et al., 2017). Baita ere garrantzitsua da aipatzea DAT-

a modu ezberdinetan prozesatua izan daitekeela, aplikazioaren arabera. Hezur ehun birsorkuntzaren kasuan, formulazio itxaropentsuena apar sendo forma da. Honen porositate eta pisu ratioarekiko bolumen handiak, mantenugai, seinale eta zelulen mobilitatea baimentzen du materialaren matrizean barren (8. irudia).



9. irudia. Irudi makroskopiko (A, B) eta ekortzeko mikroskopio elektronikoko irudiak (SEM) (C, D), txerrien ehun adiposo ez manipulatua (A, C) eta dezelularizaturiko matrize extrazelularra (ECM) (B, D). Eskala barra: 50 µm. (Choi et al., 2012)**.**

3. Hazkuntza faktoreak

Azken hamarkadetan, hezur proteina morfogenikoak (ingelesez, bone morphogeniz proteins, BMPs) eta esmalte matrizearen deribatuak (ingelesez, enamel matrix derivate, EMD) bezalako molekulak erabili izan dira zauritu edo galduriko ehunen birsorkuntza bultzatzeko (Dumic-Cule et al., 2018; Miron et al., 2016; Rao et al., 2013). Hala ere, azken urteetan, plaketetatik eratorritako produktuek tresna itxaropentsua bihurtu dira zelulen hazkuntza eta desberdintzapena areagotuz ehun ingeniaritzan.

3.1. Plasmatik eratorritako produktuak

Kalteturiko gunearen sendaketa lokala bultzatzeko asmoz sortu zen bildutako plasma soluziotik eratorritako plaketa eta hazkuntza faktoreen kontzentratuaren kontzeptua (Anfossi et al., 1989; Fijnheer et al., 1990). 1990. hamarkadaren bukaeran jarri zitzaion plaketetan aberatsa den plasma (ingelesez, platelet rich plasma, PRP) izena (Jameson, 2007; Marx et al., 1998; Whitman et al., 1997). PRP-a plaketez osaturik dago, hauek aktibatzean hazkuntza faktoreak askatzen dituzte, mindutako gunean zelulen hazkunde, atxikipen eta migrazioa areagotuz (Jameson, 2007; Marx, 2004). PRP-aren ostean bigarren plasmatik eratorritako produktu bat formulatu zen antikoagulatzaileak erabiliz, honi plaketetan aberatsa den hazkuntza faktore (ingelesez, platelet-rich growth factor, PRGF) izena jarri zitzaion (Anitua, 1999). Gainera, bi produktu hauen arazorik handiena (manipulatzeko zailtasuna) saiheste arren, hirugarren belaunaldiko plasmatik eratorritako produktua sortu zuten, plaketetan aberatsa den fibrina (ingelesez, Platelet rich fibrin, PRF) (Anitua et al., 2016b; Anitua et al., 2016a).

Aktibaturiko plaketek askatzen dituzten hazkuntza faktoreen artean honako hauek sar ditzakegu: β1 hazkuntza faktore eraldatzailea (ingelesez, transforming growth factor β1, TGF-β1), plaketetatik eratorritako hazkuntza faktorea (ingelesez, platelet derived growth factor, PDGF), baskulatura endotelial hazkuntza faktorea (ingelesez, vascular endothelial growth factor, VEGF), fibroblasto hazkuntza faktorea (ingelesez, fibroblast growth factor, FGF), hazkuntza faktore epidermikoa (ingelesez, epidermal growth factor, EGF) eta IL-1β, IL-4 eta IL-6 moduko interleukinak (IL) (Dohan et al., 2006).

Nahiz eta PRP eta PRGF-aren prestaketa oso antzekoa den arren, PRGF-ak plasma proteina eta koagulazio faktore gehiago ditu, efektu handiagoa eraginez PRP-arekin alderatuz (Anitua, 1999).

Plasmatik eratorritako bi produktuek, PRGF eta PRF, dituzten hazkuntza faktore nahasketak inguruko zelula mesenkimalen ugalketa eta desberdintzapena osteoblastikoa bultzatzen dute. Produktu hauen ezaugarri hoberenetako bat terapia pertsonalizatu autologoetan erabili ahal izatea da (Anitua et al., 2013). Hauen erabilerak dakartzan abantailak frogatuak izan dira eta beraien erabilera asko zabaldu da

esperimentazioan bezain ehun birsortze terapia klinikoan, batez ere odontologiako inplante terapietan (Anitua et al., 2016b; Masuki et al., 2016) (10. irudia).

10. irudia. PRGF (esker) eta PRF-rean (eskuin) irudiak.

PRGF-a plaketetan aberatsa den plasma frakzioz osaturik dago. Pazientearen odola zentrifugatu ostean, plasma frakzio hori kaltzio kloruro bidez aktibatzean lortzen da. Kaltzio tratamenduak plaketen degranulazioa abiarazten du, honen ondorioz, polimerizaturiko fibrina koaguloa eta hazkuntza faktoreen nahasketa konplexua lortzen da (Anitua et al., 2009; Jovani-Sancho et al., 2016; Paknejad et al., 2012). Ondorengo zentrifugazioak fibrina koagulua hazkuntza faktoreetan aberatsa den frakzio disolbagarritik apartatuko du (Anitua et al., 2012).

PRF-a lortzeko, odola anti-koagulatzaile gabe ateratzen denez, zentrifugazio azkar bat beharrezkoa da odola atera ostean. Odol frakzioak bereiztu ondoren, leukozito eta plaketak dituen koagulua lautu egin daiteke mintz bat lortu arte, produktu honek duen manipulazio errazari esker. Ezaugarri interesgarrienetako bat hazkuntza faktoreen askapen progresiboa da denboran zehar plaketek duten degranulazioaren eraginez. Ebakuntzaren ondoren, ehunaren sendaketa hobetzeko, zelulen hazkuntza eta kimiotaxia bultzatzen dute hazkuntza faktore hauek. Fibrina koagulua erresistente, gogor, malgu eta manipulatzek erraza da. Ezaugarri hauek moldagarritasun handia ematen diote azalera anatomiko ezberdinetan erabiliak izateko (Khurana et al., 2017; Kumar et al., 2016).

Gaur egun, hazkuntza faktoreetan aberatsa den plasma (PRGF) eta plaketetan aberatsa den fibrinaren (PRF) erabilera klinikoa zabaldua izan da hortz erauzketa osteko titanio inplanteen inguruko hezurra sendatzeko. Produktu hauei plasmatik eratorritako terapia medikorako produktu aurreratu (ingelesez, plasma-derived advanced medical therapy products, AMTP) bezala ezagutzen zaie (Giannini et al., 2015; Kobayashi et al., 2016; Nishiyama et al., 2016).

Metodologia

gBMSC-en isolamendu eta hazkuntza

gBMSC-ak Zuritxeko unibertsitateko (Zürich, Suitza) Zelula eta Ehun Ingeniaritza Laborategitik (Ehrbarlab) lortu ziren ondorengo prozedura jarraituz: gBMSC-ak, gizaki emaile osasuntsuetan (45 urteko bataz besteko adina) eginiko hezur-muin aspiratuetatik lortu ziren, prozedura kirurgiko ortopedikoetan. Aspiratuak, tokiko batzorde etikoak ezarritako baimen informatuarekin lortu ziren (University Hospital Basel; Prof. Kummer; approval date 26/03/2007 Ref. Number 78/07). Zelula nukleatuen isolamendurako, aspiratuan lorturiko globulu gorriak lisatuak izan ziren 0.15 M NH_4Cl (Sigma, Suitza), 1 mM $KHCO_3$ (Sigma, Suitza) eta 0.1 mM Na_2EDTA-rekin (Fluka, Suitza) osaturiko bufferraz. gBMSC-ak, % 10 BSF (ingelesez, fetal bovine serum) (Gibco, NY, AEB), % 1 penizilina-estreptomizina (Gibco, NY, AEB) eta 5 ng/ml fibroblasto hazkuntza faktore 2-az (FGF2, PeproTech, NJ, AEB) osaturiko αMEM (ingelesez, minimal essential medium) (Gibco, NY, AEB) medioan hazi ziren 37 °C-tan % 5-eko CO_2-arekin. 3 eta 8 bitarteko zelula-paseak erabili ziren esperimentuetarako.

gDPSC-en isolamendu eta hazkuntza

Giza hortz mamiko zelula amak (gDPSC), hortz klinikan paziente gazte osasuntsuetatik (18-tik 30 urte bitartekoak) erauzitako hirugarren molarretatik isolatu ziren. Pazienteek baimen informatua sinatu zuten aldez aurretik onartutako M10_2016_088 protokoloa jarraituz (Euskal Herriko Unibertsitateko etika batzordea).

Hortz mamiaren erauzketa molarren apurketarekin egin zen. Lorturiko hortz mamiak, HBSS (ingelesez, Hank´s balanced solution) (Gibco, NY, AEB) medioan disolbaturiko 3 mg/ml kolagenasa I (Gibco, NY, AEB) eta 4 mg/ml dispasa-z (Sigma, MO, AEB) osaturiko digestio entzimakoaz tratatu ziren ordubetez 37 °C-tan % 5-eko CO_2-arekin. Digestio entzimatiko soluzioa neutralizatzeko, % 10-eko BSF-z (HyClone, UT, AEB) osaturiko DMEM-a (ingelesez, Dulbecco´s modified eagle medium) (Lonza, Suitza) erabili zen. Honen ostean, zelulak 1500 rpm-etan zentrifugatu ziren 5 minutuz. Lorturiko zelulak mekanikoki bereizi ziren 18G-ko orratz batekin (BD Microlance, Fisher Scientific, UK). Zelulak bereizi ostean, % 10-eko BSF, % 1 penizilina-estreptomizina (Gibco, NY, AEB)

eta % 1 L-Glutamina-z (Sigma, MO, AEB) osaturiko DMEM medioan erein ziren 37 °C-tan % 5-eko CO_2-arekin. 5 80-ko konfluentzia lortu ostean zelulak 75 zm^2-ko flasketara (Sarstedt, Nümbrecht, Alemania) pasa ziren. Esperimentuetarako 4 eta 10 pase bitarteko zelulak erabili ziren.

Desberdintzapen osteogeniko medioa

Titanio eta pDAT-zko apar solidoetan ereindako gDPSC-en esperimentuetarako erabili zen medio osteogenikoa, DMEM (% 10 BSF, % 1 penizilina-estreptomizina eta % 1 L-Glutamina), 50 µM azido askorbiko (Sigma, MO, AEB), 20mM β-glizerolfosfato (Sigma, MO, AEB) eta 10 nM dexametasona-z (Sigma, MO, AEB) osaturik zegoen. gDPSC eta gBMSC-en arteko ikerketa konparatiboetarako berriz DMEM (% 10 BSF, % 1 penizilina-estreptomizina eta % 1 L-Glutamina), 50 µM azido askorbiko (Sigma, MO, AEB), 10mM β-glizerolfosfato (Sigma, MO, AEB) eta 100 nM dexametasona-z (Sigma, MO, AEB) osatua. Esperimentu konparatibo hauetarako, kontrol zein desberdintzapen medioak 5 ng/ml EGF (AF-100-15-500UG, PeproTech, NJ, AEB) ere bazuen.

Titanio diskoen fabrikazioa

2 mm-ko lodiera duten Ti6AL4V diskoak, 5.5 mm-ko diametroa duen barra bat moztuz lortu ziren Avinent Implant System SLU (Bartzelona, Espainia) enpresatik. BAS™ titanio azaleraren fabrikaziorako, titanio diskoaren aurpegi bat korindoi zuriz (Al2O3) F60 eginiko “shot blastin” teknika erabili zen, 212-300 µm-ko tamainako partikulak proiektatuz. Ondoren, diskoak bainu ultrasoniko batean garbitu ziren 10 minutuz eta ur distilatuz garbitu ziren. Garbiketen ostean, anodizatuak izan ziren DC energia iturriko anodoari lotuz eta kaltzio eta fosforoa erabiliz ur soluzioko elektrolito gisa. 0.75 mA/mm^2-eko korronte dentsitatea aplikatu zen eta potentzialari libreki handitzen utzi zitzaion 130 V-raino heldu arte. Azkenik, bainu ultrasonikoan ur destilatuzko 10 minutuko garbiketekin bukatu zen fabrikazio prozesua. Ti6Al4V titaniozko diskoek ez zuten inolako leuntze edo tratamendurik jasan.

pDAT-ren dezelularizaio eta apar solido prozesamendua

Txerrietatik eratorritako ehun adiposoa tokiko janari enpresa batetik (Jaucha SL, Nafarroa, Espainia) lortu zen. Ehuna gela tenperaturan desizoztu, garbitu eta irabiagailu batez krematua izan zen. Ondoren, ehuna izotzetan homogeneizatu zen bi hagaxka ezberdinezko Plyton-a (PT3100) erabiliz 1200 rpm-etan 5 minutuz. Lipidoen fase separazioa lortzeko, homogeneizaturiko ehuna 900 g-etan zentrifugatu zen ur ultra purua erabiliz 5 minutuz. Lipidoak eskuz baztertuak izan ziren eta proteinak kontserbatuak. Proteina pelletak gauan zehar isopropanolez (Merk Life Science, Espainia) tratatuak izan ziren astintzaile orbital batez gela tenperaturan. PBS-ez garbitu ondoren, % 1 Triton X-100 eta % 0.1 amonio hidroxido-z (Merk Life Science, Espainia) tratatu zen 36 orduz gela tenperaturan astintzaile orbitalean. PBS-ezko garbiketen ostean, liofilizatua izan zen erabat lehortu arte. Lorturiko ehuna fresatu egin zen nahasgailu errota (Retsch MM400) erabiliz prozesatuak izateko grano fineko hautsa lortu arte. Lorturiko hautsa nitrogeno likidoz izoztu zen eta 4 ℃-tan gorde zen hutseko lehorgailuan.

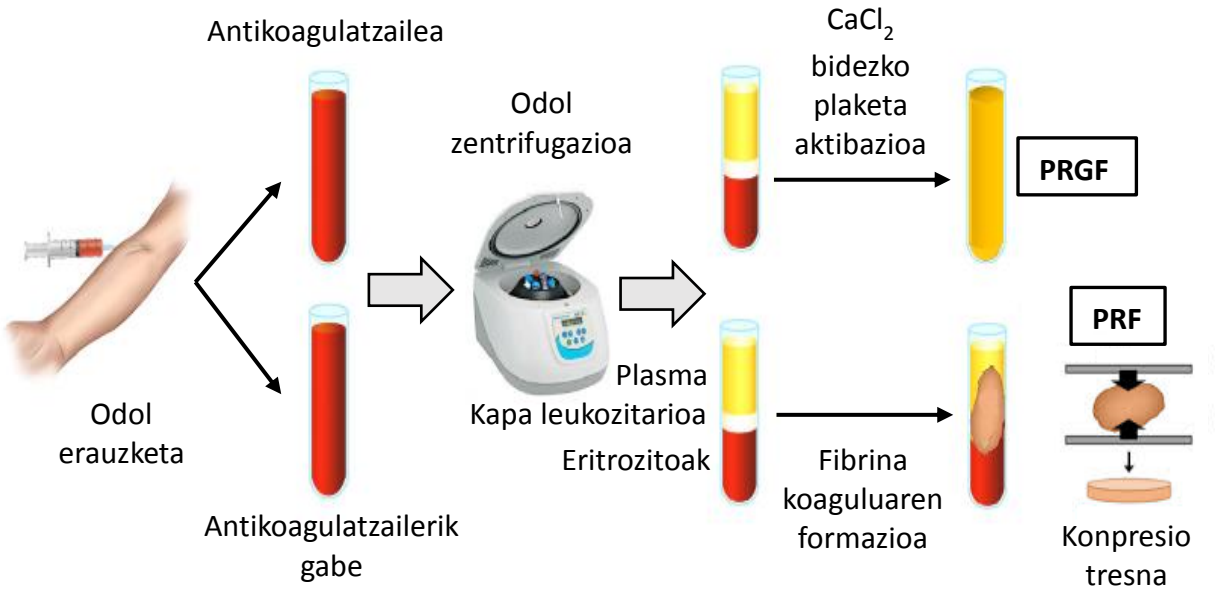
Apar solidoak pDAT izozte-lehortze prozesuaren bidez prestatu ziren. % 0.5 pDAT-ri 0.5 M azido azetiko gehitu zitzaion, lorturiko soluzioa irabiagailu magnetiko batez homogeneizatu zen 48 orduz gela tenperaturan. 20 mm-ko diametro eta 3 mm-ko lodiera duen tefloizko moldeak erabili ziren Ekorkuntz Mikroskopio Elektronikorako (SEM), 1 ml soluzio erabiliz. *In vitro* zelula kultiboentzako berriz, apar sendoak, 8 kristalezko hobitxodun Millicell EZ-portan (Merk Millipore) prestatu ziren 120 µl soluzio erabiliz. Lorturiko portak mikroskopio optiko eta fluoreszenteentzako bateragarriak ziren. Laginak -20 ℃-tan izoztu eta izoztu-lehortu ziren (0.63 mbar eta -10 ℃) apar sendoak lortzeko. Zelula hazkundeetan erabiliak izateko etileno oxidoz (Esterilizacion SL, Bartzelona, Espainia) esterilizatu ziren.

PRGF eta PRF-aren prestakuntza

PRGF-a prestatzeko, giza emaile gazte osasuntsuetatik lortu zen odola % 3.8 sodio zitrato antikoagulatzailea zuten 5 ml-ko odola biltzeko hodiak (BD Vacutainer, Plymouth, UK) erabiliz. 8 minutuz 580 g-etara zentrifugatu ondoren, plaketetan aberatsa den frakzioa 15 ml-lo falkon hodietara pasa zen. Plaketak, % 10 kaltzio kloruroz (Braun

medical, Melsungen, Alemania) aktibatuak izan ziren ordu batez 37 ℃-etan. Inkubazio honen ostean, plasma 3000 g-etan zentrifugatu zen 15 minutuz 4 ℃-tan eta filtratua izan zen 0.2 µm-ko poro tamainako iragazkiak erabiliz (Sarstedt, Nümbrecht, Alemania). Azkenik, plasma alikuotatu eta izoztuta gorde zen -20 ℃-tan erabili arte. PRGF-a medio aldaketa bakoitzarekin gehitu zen (10. irudia).

PRF-aren prestaketarako berriz, odola antikoagulatzaile gabeko hodietan (BD Vacutainer, Plymouth, UK) bildu zen. Laginak momentuan zentrifugatu ziren 580 g-etan 8 minutuz, odolaren koagulazio goiztiarra ekiditeko. Odol eta fibrinaren koagulazioaren ondoren, plaketak dituen fibrina koagulua hematokrito frakziotik bereizi zen bisturi eta pintzak erabiliz. Azkenik bi beiren artean konprimatu zen pisua erabiliz ordubetez gela tenperaturan. Fibrinazko koaguluak zm^2 bateko zati txikiagotan moztu ziren (10. irudia).



10. irudia. PRGF eta PRF-aren prestakuntza ilustrazioa. (Autoreak sortua).

gDPSC eta gBMSC-en hazkuntza titaniozko dizkoetan

Zelulek % 80-ko konfluentzia lortzean, % 0.05 Tripsina-EDTA erabiliz desitsatsi eta bildu ostean zentrifugatu eta kontatuak izan ziren. Esperimentuaren arabera zelula dentsitate ezberdinak erein ziren Ti6Al4V eta BAS™ titanio diskoetan. Zelulen itsaspena

areagotzeko, 15.000 zelula 70 µl-ko tantetan erein ziren diskoen gainean eta 3 orduz 37 °C-etan inkubatu ziren. Inkubazio honen ostean 700 µl DMEM medio gehitu ziren 24 putzuko plakentzako eta 4 ml 6 putzuko plakentzako.

gDPSC-en hazkuntza txerritik eratorritako ehun adiposo dezelularizatuan (pDAT)

Zelulek % 80-ko konfluentzia lortzean, % 0.05 Tripsina-EDTA erabiliz desitsatsi eta bildu ostean zentrifugatu eta kontatuak izan ziren. pDAT-a (Tecnalia, Donostia, Espainia) 24 putzuko plaketan (Sarstedt, Nümbrecht, Alemania) eta 8 putzuko Ibidi plaketan (Ibidi, Munich, Alemania) jarri zen. DMEM medioaz pDAT-a hidratatu ondoren, gDPSC-ak 10.000-20.000 zelula/putzu-ko dentsitatean erein ziren (esperimentuaren arabera). Hazkuntza medioa 2-3 egunero aldatu zen.

Kaltzeina/ propidio ioduro esperimentua

4 eguneko hazkundearen ostean, zelulak PBS-z garbitu ziren. Garbiketak egin eta gero, zelulak 3 µM kaltzeina-AM (Molecular probes, OR, AEB) eta 2.5 µM propidio ioduro-arekin (Sigma, MO, AEB) inkubatu ziren 30 minutuz 37 ℃-tan. Azkenik, zelulei 3 PBS garbiketa egin zitzaizkien eta argazkiak Axioskop mikroskopio fluoreszentea (Zeiss, Overkochen, Alemania) eta Nikon DS-Qi1 kamera erabiliz (Nikon, Tokio, Japonia) atera ziren.

Immunozitokimika

Zelulen hazkundearen ondoren, zelulak PBS-z garbitu eta % 4 paraformaldehidoz (PFA) fijatu ziren gela tenperaturan 10 minutuz. Honen ostean, zelulak 10 minutuz inkubatu ziren % 10 ahuntz seruma (Invitrogen, CA, AEB) erabiliz gela tenperaturan. Blokeo pausuari jarraituz, gau osoko inkubazioa egin zen 4 °C-tan % 0.1 Triton X-100/ % 1 BSA/ PBS erabiliz ondorengo antigorputzekin: BGLAP (ab93876, Rabbit polyclonal, Abcam, Cambridge, UK), Caspasa-3 (ab32351, Rabbit monoclonal, Abcam, Cambridge, UK), Ki67 (ab15580, Rabbit polyclonal, Abcam, Cambridge, UK), Osterix (ab22552, Rabbit polyclonal, Abcam, Cambridge, UK) eta SPARC (ab14174, Rabbit polyclonal, Abcam,

Cambridge, UK). Alexa 488 conjugated rabbit IgG (A11008, Abcam, Cambridge, UK) erabili zen antigorputz sekundario gisa antigorputz primarioak lokalizatzeko %1 BSA/ PBS erabiliz ordubetez gela tenperaturan. Zelulen nukleoak 4',6-diamino-2-fenilindol-ez (DAPI, Invitrogen, CA, AEB) tindatu ziren. Irudiak, Axioskop mikroskopio fluoreszentea (Zeiss, Overkochen, Alemania) eta Nikon DS-Qi1 kamera erabiliz (Nikon, Tokio, Japonia) eta Leica DM6000 B mikorskopioa (Leica, Wetzlar, Alemania), Leica DFC420 C kamera eta 3.3.3.16958 Leica application suite X (Leica, Wetzlar, Alemania) programaren bidez atera ziren.

Fluxu zitometria

gDPSC eta gBMSC-en fenotipoa fluxu zitometriaz analizatu zen. Antigorputz bakoitzarentzat 500.000 zelula desitsatsi ziren eta % 0.15 behi serum albumina (BSA, Sigma, MO, AEB) duen PBS-z inkubatu ziren 40 minutuz izotzetan ondorengo antigorputzekin: 1:50 CD45-APC (304011, Biolegend, CA, AEB), 1:50 CD73-APC (17-0739-41, eBioscience, MA, AEB), 1:50 CD90-FITC (328107, Biolegend, CA, AEB) eta 1:50 CD105-PE (12-1057, eBioscience, MA, AEB). % 0.15 BSA-dun PBS-z garbitu eta gero 500 µl PBS-tan bir-suspenditu ziren. Analisia, Gallios fluxu zitrometoa (Beckman Coulter, CA, AEB) erabiliz egin zen Kaluza 1.1 softwarearekin (Beckman Coulter, CA, AEB).

Ekorkuntz mikroskopio elektronikoa

Bi titanio azaleretan hazitako zelulak % 2 glutaraldehidoz (Sigma, MO, AEB) fijatu ziren 0.1 M Sorensen fosfato bufferra (SPB, Thermo Fisher Scientific, MA, AEB) erabiliz ordubetez 4 °C-tan. Pauso honen ondoren, laginak 3 aldiz garbitu ziren % 4-8 sakarosa zuen 0.1 M SPB bufferrarekin, eta gero beste 3 garbiketa 0.1 M SPB-rekin. Zelulak lehortzeko 15 minutuko inkubaketak egin ziren etanolean (% 30, % 50, % 70, % 90 eta % 100). Behin lehortuta, hexametildisilazanoz (Sigma, MO, AEB) garbitu ziren. Airean lehortu eta gero, 15 nm lodierako urre hautsez estali ziren laginak. Irudiak ekortzeko mikroskopio elektroniko (SEM, S4800, Hitachi High Technologies, Tokio, Japonia) bidez atera ziren. Zelularik gabeko diskoei ere mikroskopio berberaz atera zitzaizkien argazkiak.

Transmisiozko mikroskopio elektronikoa (TEM)

Laginak %2 glutaraldehidotan 15 minutuz fijatu ondoren Epon Polarbed erretxinean (Electron Microscopy Science, Hatfield, PA, AEB) txertatu ziren. 70 nm-tako sekzio ultra finak 150 kobrezko sarean (150 Square Mesh xopper 3,05 mm, AGAR Scientific, UK) jarri ziren. Post tindaketa ur destilatutan disolbaturiko % 2 uranil azetatoa (AGAR Scientific, UK) eta % 0.2 zitratoarekin (AGR1210, AGAR Scientific, UK) egin zen. Irudiak Philips EM208S transmisiozko mikroskopio elektronikoarekin atera ziren Jeol JEM 1400 Plus kamerari esker.

RNA erauzketa eta erretrotranskripzioa

Zelula peletak -80 °C-tan gorde ziren erabiliak izan arte. RNA, RNeasy mini kit-a (Qiagen, Hilden Alemania) erabiliz erauzi zen. RNA-ren kontzentrazio eta purutasuna 260/ 280 nm-ko absorbantziaz neurtu zen Nanodrop Synergy HT-ren bidez (Biotek, VT, AEB). cDNA-ren sintesia erretrotranskripzioz lortu zen 1000 ng-ko hasierako RNA-tik iScript cDNA kit-a erabiliz (Biorad, CA, AEB). Retrotranskripzio zikloak 25 °C-tako 5 minutuko pausoarekin hasi zen, honi jarraituz 46 °C-ko 20 minutu zikloa eta amaitzeko minutu bateko 95 °C-ko zikloarekin. Behin erretrotranskripzioa amaiturik 4 °C-tan mantendu ziren -20 °C-tan izoztu arte.

RNA sekuentziazioa

gDPSC eta gBMSC-ak plastiko, Ti6Al4V eta BAS titanio azaleretan medio kontrol edo osteoblastikoarekin hazi ziren 14 egunez. 24 disko erabili ziren kondizio bakoitzeko eta tripsinarekin zelulak desitsatsi ostean, RNA Quiagen RNA erauzketa kitarekin lortu zen. gDPSC eta gBMSC-etatik erauzitako RNA Zuritxeko Genomika zentroan sekuentziatu zen Illumina sekuentziagailuaz (Illumina, CA, AEB), 25.000 RNA irakurketa lortuz baldintza bakoitzeko. Gene hauen adierazpena presentzia/gabezia (ON/OFF) kriterioa jarraituz analizatu zen baldintza ezberdinen konparaketetan eta 10 irakurketa gordinetan jarri zen muga gene baten adierazpena positibotzat (ON) hartzeko. Aktibaturiko gene bideak “Pathway Enrichment Analysis” erabiliz analizatu zen, “The

Connectivity Map" (CMAP) eta "Gene Ontology enrichment Biological Process" (GOBP) datu baseak erabiliz CIC bioGUNE-n (CIC bioGUNE, Derio, Espainia).

Denbora-errealeko PCR kuantitatiboa (qPCR)

Power SYBR Green PCR Master Mix-a (Applied biosystems, CA, AEB) erabili zen qPCR-a egiteko. Anplifikatzeko programaren zikloak 10 minutu 95 °C-tan eta 95 °C 20 segundo eta 59 °C-ko minutu bateko 40 ziklorekin egin zen. Erabili ziren "etxezaintza" geneak β-aktina 5´-GTTGTCGACGACGAGCG-3´ eta 5´-GCACAGAGCCTCGCCTT-3´ eta Gapdh 5´ CTTTTGCGTCGCCAG -3´ eta 5´- TTGATGGCAACAATATCCAC -3´ izan ziren. Azterturiko geneak hauek izan ziren: kolageno I (*COL I*) 5´ -GGCCCCCTGGTATGACTGGCT-3´ eta 5´-CGCCACGGGGACCACGAATC-3´, osteonektina (*SPARC*) 5´-GAAAGAAGATCCAGGCCCTC-3´ eta 5´-CTTCAGACTGCCCGGAGA-3´, osteokaltzina (*BGLAP*) 5´-CGCCTGGGTCTCTTCACTAC-3´ eta 5´-CTCACACTCCTCGCCCTATT-3´, dentin sialofosfoproteina (*DSPP*) 5´-TGCCCAAATGCAAAAATATG-3´ eta 5´-GTGGGCCACTTTCAGTCTTC-3´, osterix (*OSX*) 5´-TGAGGAGGAAGTTCACTATG-3´ eta 5´-CATTAGTGCTTGTAAAGGGG-3´eta runx2 (*RUNX2*) 5´-CACTCACTACCACACCTACC-3´ eta 5´-TTCCATCAGCGTCAACAC-3´. Ikerturiko geneen adierazpena etxezaintza geneen adierazpenarekin normalizatuak izan ziren.

Fosfatasa alkalino tindaketa (ALP)

Zelulak minutu batez % 4 PFA-az fijatu ondoren 3 aldiz garbitu ziren % 0.05 Tween 20-PBS-z. ALP tindaketarako, 5-bromo-4-kloro-3-indolil fosfato/ tetrazolio nitro urdina (NBT/BCIP, Sigma, MO, AEB) erabili zen eta tindaketa 3 minuturo begiratu zen. Tindaketa amaitu ondoren, PBS-zko 5 minutuko 3 garbiketa egin ziren. Irudiak, Zeiss Stemi 2000-C mikroskopio estereoskopikoarekin (Zeiss, Alemania) atera ziren Canon PowerShot A80 kamera (Canon, Tokio, Japonia) erabiliz. ALP aktibitate entzimatikoa kuantifikatzeko, zelulak tripsina-EDTA-rekin desitsatsi ziren. Absorbantzia 420 nm-tan neurtu zen Synergy HT-ren bidez (BioTek, VT, AEB) Gen5 1.11 programa erabilita.

Alizarin gorri tindaketa (ARS)

gDPSC eta gBMSC-en potentzial osteogenikoa neurtzeko Alizarin gorria erabili zen zelulaz kanpoko kaltzio deposituak tindatzeko. 21 egunetako hazkuntzaren ondoren, zelulak fijatuak izan ziren % 4 PFA-z 10 minutuz. Ur distilatuz garbitu ostean, pH 4.2-dun % 2 ARS-arekin (Acros organics, Sigma, MO, AEB) tindatu ziren laginak ilunpean 45 minutuz gela tenperaturan. Ur distilatuz 3 aldiz 5 minutuko garbiketak egin ziren soberako ARS-a kentzeko. Irudiak Zeiss Stemi 2000-C mikroskopio estereoskopiko (Zeiss, Germany) eta Canon PowerShot A80 kamera (Canon, Tokyo, Japan) erabiliz atera ziren.

Analisi estatistikoa

Analisi estatistikoa IBM-ren SPSS software estatistikoa (v.26.0) erabiliz gauzatu zen, baldintza bakoitzaren media eta errore estandarra kalkulatuz. Esperimentu ezberdinetako balditzen datuak Mann Whitney testa edo ANOVA bidez analizatu ziren, Bonferroni edo Games-Howell testekin jarraituz, bariantzen homogeneitatearen arabera. Konfidantza tartea % 95 ($p < 0.05$), % 99 ($p < 0.01$) eta % 99.9-n ($p < 0.001$) ezarri zen.

Hipotesia

Azkeneko hamarkadetan, hezur ehun ingeniaritza eta hortz inplantologian erabiltzeko hortz inplante eta scaffold-ek asko hobetu dute. Material berriak, azalera topografia eta laztasunaren aldaketek, zelula ama mesenkimalen osteoblasto desberdintzapenean eragin zuzena dutela erakutsi dute. Scaffold berri guztiek hauen, porositate, laztasun eta formaren hobekuntzak kontuan izanik ere, erabilera klinikorako aukerarik hoberena zein den ez dago argi. Ugaritze eta desberdintzapen gaitasun handiari esker, MSC-ek hezur ingeniaritzaren etorkizuna direla dirudite; hala ere, hezur birsortze terapietarako MSC-rik hoberena aukeratzeko eztabaida oraindik abian dago. Azkenik, ehun ingeniaritzaren erronka nagusienetako bat zabaldua dagoen animali iturriko serumen erabilera da, behi serum fetala (BSF) bezala, zeinak ordezkatuak izan beharra diren immune erantzuna desaktibatzeko zelulak injertatutako pazienteetan.

Periinplantitisaren eragin dezakeen inplante galera ekiditeko, lotailu periodontala bezalako ehun garrantzitsua ordezkatzeko asmoz, gure hipotesiaren arabera titanio biomimetiko azalera BAS^{TM} eta dezelularizatutako ehun adiposoak (DAT), hezur ingeniaritzarako zelula amen desberdintzapen osteoblastiko bultzatzeko material eraginkorragoak izan daitezke. Gainera, erauzketa erraz, ugaritze altu eta osteo-desberdintzeko ahalmena duten hortz mamiko zelula amak (DPSC) zelula autologoez eginiko birsortze terapietarako aukera ona izan daitezke. Amaitzeko, plasmatik eratorritako hazkuntza faktoreetan aberatsa den plasma (PRGF) eta plaketetan aberatsa den fibrina (PRF) BSF-aren ordezkari onak izan daitezke *in vitro* zelula hazkundeetarako. Plasmatik eratorritako produktu hauek paziente berberetik lortu daitezkeenez, immune erantzun ez desiragarriak ekidingo lituzkete birsortze zelula terapietan.

Helburu nagusiak

Lan honen helburu nagusia, DPSC-en desberdintzapen osteoblastiko gaitasunak bio-materialekin duen elkarreragina aztertzea da hezur ehun ingeniaritzarako. Helburu hau lortzeko, Ti6AL4V zein BAS^{TM} titanio azalerak eta dezelularizatutako txerri ehun adiposo-aren (pDAT) efektua ebaluatu dugu plasmatik eratorritako produktu eta desberdintzapen osteoblastiko medioarekin konbinatua.

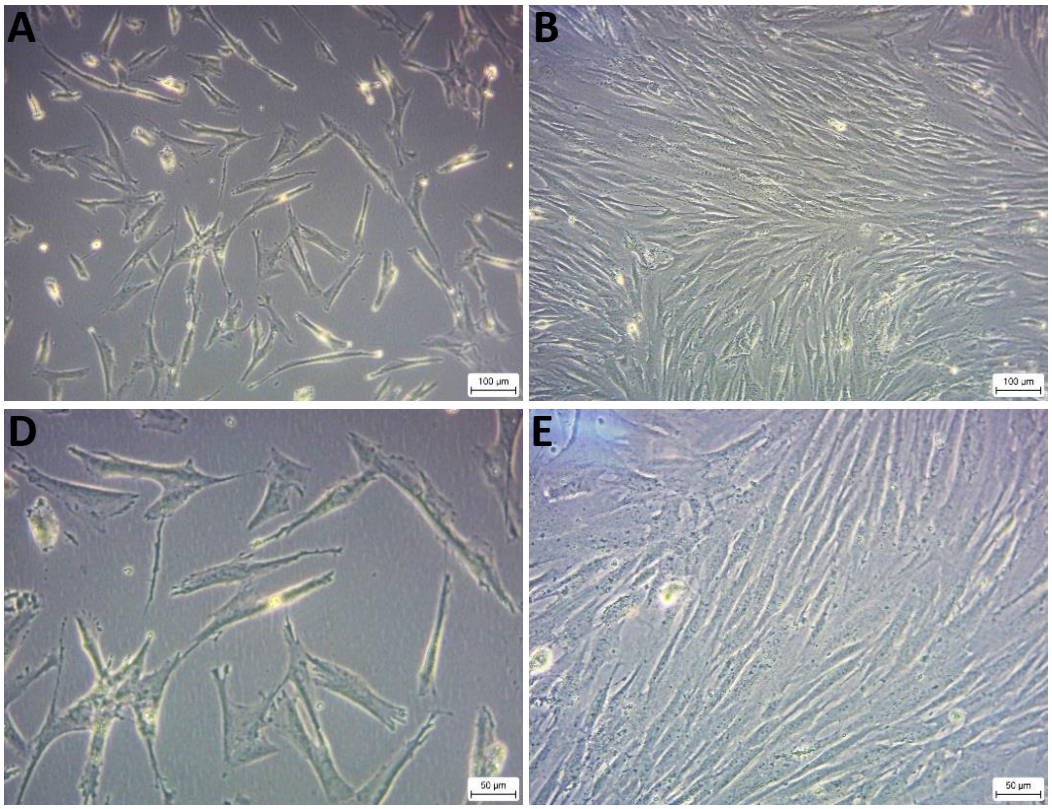
Helburu zehatzak

1. gDPSC-en bideragarritasun eta ugalketaren ebaluazioa Ti6AL4V eta BAS titanio azaleretan haziak.
2. PRGF eta PRF produktu plasmatikoek gDPSC-en ugaritze eta desberdintzapen osteoblastikoan duten eraginaren azterketa.
3. Plasmatik eratorritako produktuak, Ti6AL4V eta BAS titanioetan desberdintzapen medioaren presentzian edo gabezian hazitako gDPSC-en konbinazioak ugalketa eta desberdintzapen osteoblastikoan duten efektuen ikerketa.
4. gDPSC eta gBMSC-en arteko ugalketa eta desberdintzapen osteoblastiko gaitasunaren konparaketa Ti6AL4V eta BAS titanio azaleretan hazita desberdintzapen medioaren presentzian edo gabezian.
5. gDPSC-en itsaspen eta bideragarritasunaren ebaluazioa dezelularizatutako ehun adiposoan (DAT) haztean.
6. DAT scaffold-aren efektuen azterketa gDPSC-en desberdintzapen osteoblastikoan osteo-desberdintzapen medioarekin konbinatuta.

Emaitzak

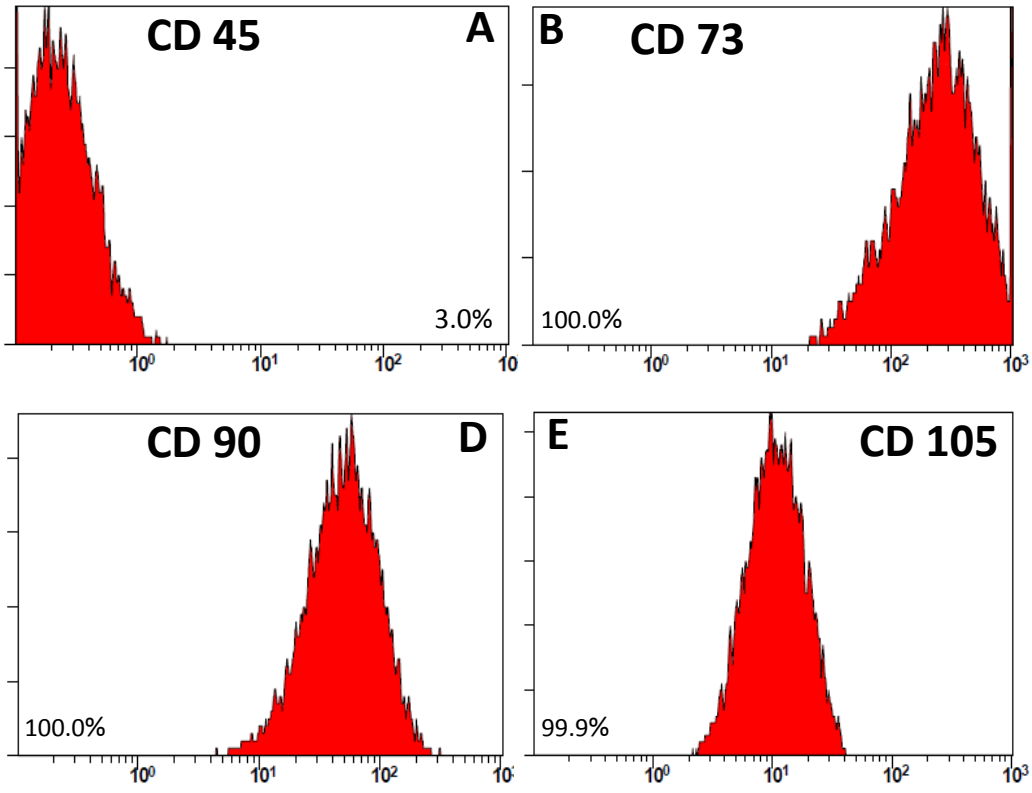
gDPSC-en isolamendu, hazkuntza eta karakterizazioa fluxu zitometria bidez

Giza hortz muineko zelula amak (gDPSC) hirugarren haginetatik isolatu ziren eta behi serum fetal (% 10), penizilina/estreptomizina (% 1) eta L-Glutamina-z (% 1) osaturiko DMEM medioan hazi ziren. 2-3 aste eta gero, zelulek kolonia atxikitu anitzak eratu zituzten, sub-konfluentzia lortuz. gDPSC-ek fibroblasto itxurako morfologia erakusten dute hazkuntza kultiboetan; zelulek ezaugarri mantentzen dute ugaltze maila altuarekin batera pase askotan (12. irudia).



12. irudia. gDPSC-en *in vitro* hazkuntza. gDPSC hazkuntzen fase kontraste mikroskopia irudiak egoera sub-konfluente (**A, D**) eta konfluentean (**B, E**) handipen txiki eta handiarekon. (Irastorza et al., 2019)-tik moldatua.

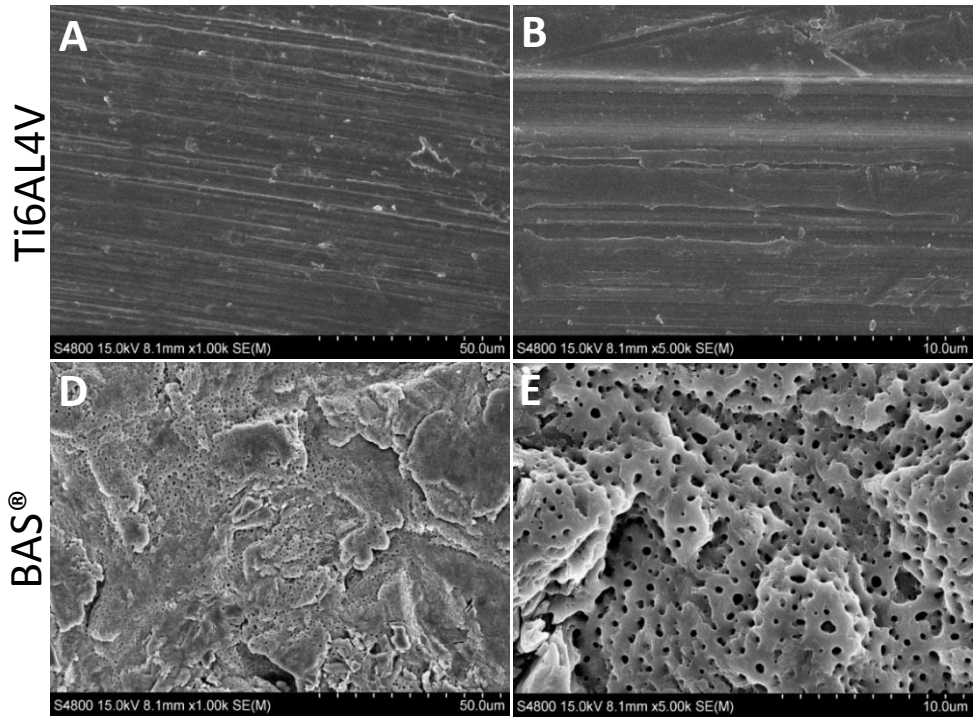
gDPSC-en markatzaile zelularren ebaluaketarako, fluxu zitometriaz analizatu ziren zelula markatzaileak ondorengo CD-en (cluster of differentiation) antigenoen aurkako antigorputzak erabiliz: CD45, CD73, CD90 eta CD105. gDPSC-ak negatiboak ziren CD45 markatzaile hematopoietikoarentzako. Bestalde, positiboak ziren zelula ama mesenkimalen markatzaile diren CD73 (% 100), CD90 (% 100) eta CD105-entzako (% 100). Emaitza hauekin, hortz mamiko zelula amen zelula ama mesenkimal fenotipoa ondoriozta dezakegu (Gronthos et al., 2003) (13. irudia).



13. irudia. Fluxu zitometria bidezko gDPSCen ama zelula markatzaileak. Fluxu zitometriak erakutsi duenez gDPSC-ak negatiboak ziren CD45 markatzaile hematopoietikoarentzat (**A**) eta positiboak CD73, CD90 eta CD105 (**B, D** eta **E**) zelula ama mesenkimal markatzaileentzat. (Irastorza et al., 2019)-etik moldatua.

Ekortze mikroskopio elektronikozko (SEM) Ti6AL4V eta BAS^{TM} titanio azaleren mikro-topografia analisia

Zelulak gabeko titanio azaleren SEM irudiak (Ti6AL4V) zirrikitu paralelo kontzentrikoak erakutsiz (14. irudia A, B), bestalde, titanio azalera latza (BAS^{TM}) hobi eta uniformeki sakabanaturiko mikro-poro txikiak (< 10 µm) erakutsiz azalera guztian zehar (14. irudia D, E).



14. irudia. Ti6AL4V eta BAS titanio azaleren SEM irudiak. Ti6AL4V (**A, B**) eta BAS (**D, E**) titanio azaleren ekortze mikroskopio elektronikoaren irudiak handipen txiki eta handiarekin. (Irastorza et al., 2019)-tik moldatua.

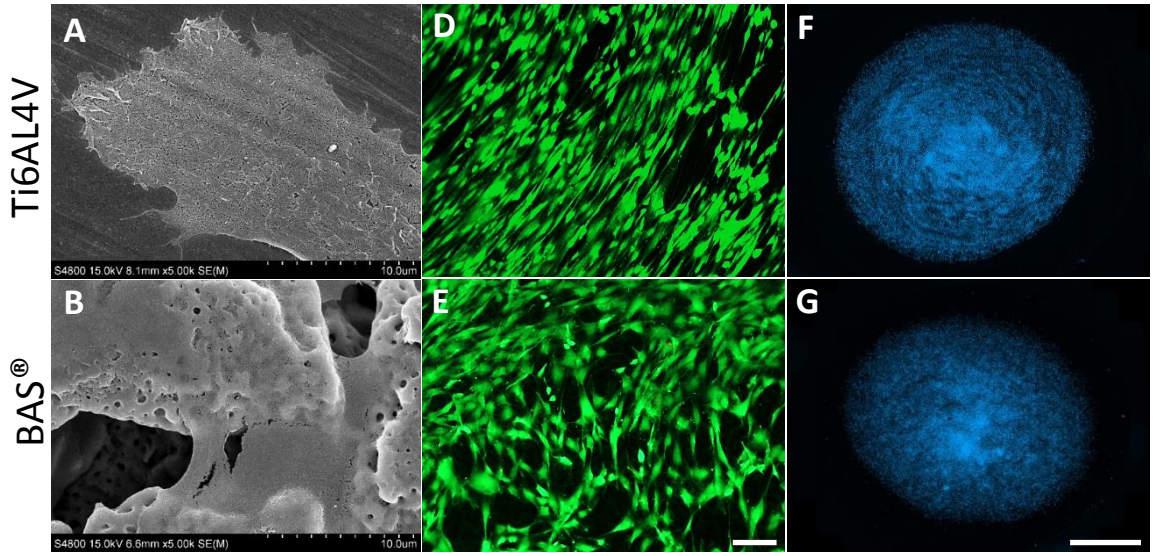
gDPSC-en zelula atxikimendu, migrazio eta bideragarritasuna Ti6AL4V eta BAS™ titanio azaleretan haztean

gDPSC-ak Ti6AL4V eta BAS™ titanio azaleretan erein ziren 20,00 zelula/diskoko dentsitatean 4 egunez. Zelulak sendoki atxikitu eta zabaldu ziren Ti6AL4V titanio leunaren azaleran zehar. Zelulak, diskoaren zirrikitu kontzentrikoak jarraituz orientaturik hazi ziren eta lamelipodio gisako zelula luzaketak erakutsi zituzten (15. irudia A). Bestalde, BAS titanio azaleran hazitako gDPSC-ak beraien morfologia moldatu behar izan zuten azalera porotsuan barneratzeko (15. irudia B). bi titanio azaleretan, zelulen atxikimendua hazkuntza denbora guztian zehar mantendu zen.

Zelulen bideragarritasun eta heriotza entsegua egiteko, titanio azaleretan hazitako gDPSC-ak kaltzeina-AM (fluoreszentzia berdea) eta propidio ioduroarekin (fluoreszentzia gorria) kultibatu ziren. Fluoreszentzia irudiek erakutsi zutenez, gDPSC-ak % 100 zeuden kaltzeinarekin tindatuak bi titanio diskoetan, hauen gainean hazitako gDPSC-en bideragarritasun handia erakutsiz (15. irudia D, E). Hare gehiago, gDPSC-ek ez

zuten PI fluoreszentzia gorririk erakutsi, hazkuntza hauetan zelula heriotza ez zegoela erakutsiz.

Azkenik, zelulen nukleoak fluoreszentzia urdinez (DAPI) tindatuak izan diren irudietan ikusten denez, Ti6AL4V titanio azaleran zelulak zirrikituak jarraituz espiral forman orientatzen zirela ikusi zen (15. irudia F), BAS titanioan aldiz, zelulek ez zuten inolako orientazio lehentasunik erakutsi (15. irudia G).

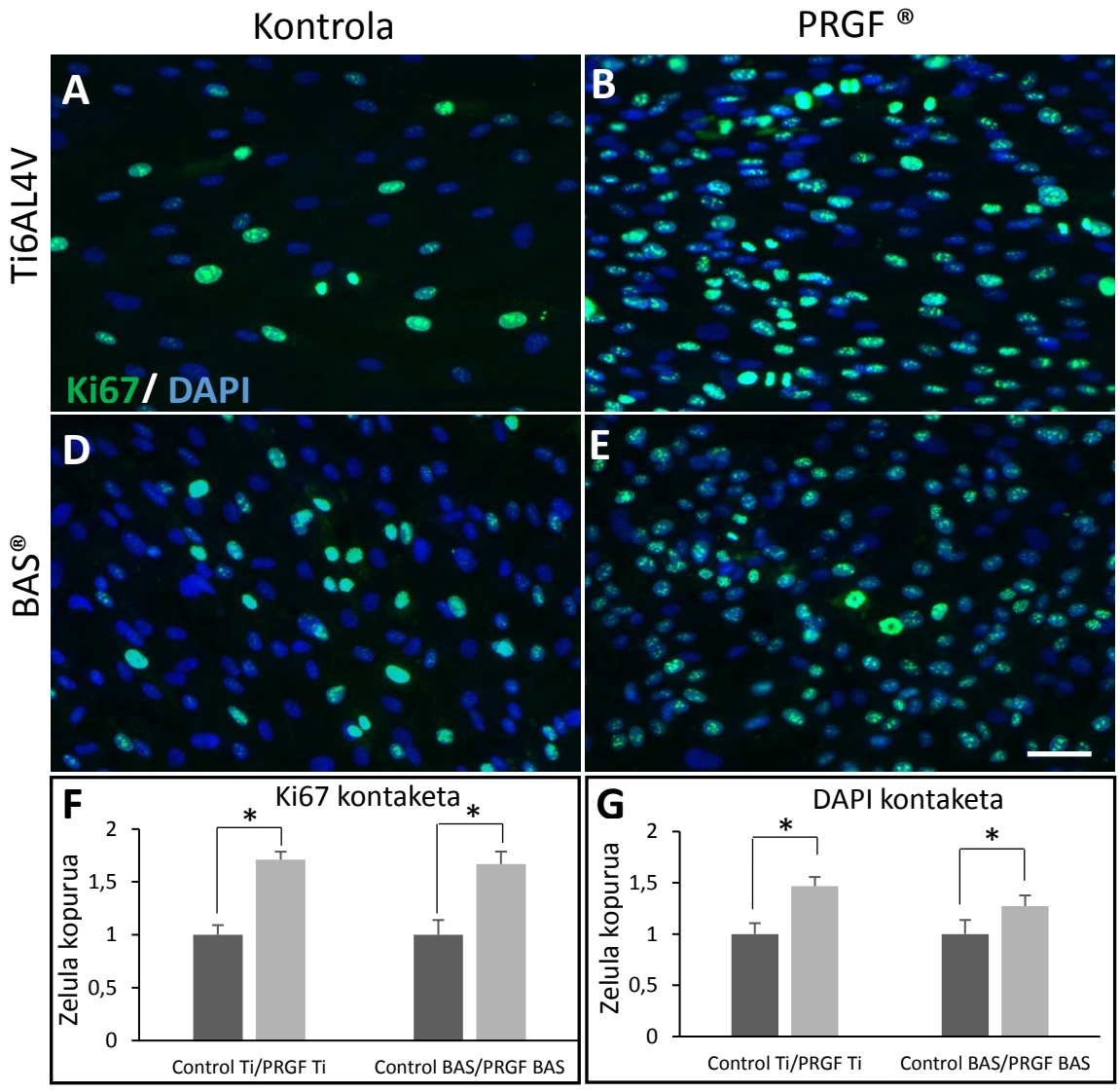


15. irudia. Titanio azaleretan hazitako gDPSC-en bideragarritasun, orientazio, mobilitate eta SEM irudiak. Ekortze mikroskopio elektronikoaren gDPSC-ak ereindako bi titanio azaleren irudiak (**A, B**). 4 egunez bi titanio azaleretan hazitako gDPSC-en fluoreszentzia irudiak bizirik dauden zelula (berde) eta hildakoak (gorri) erakutsiz (**D, E**). Eskala barra: 100 µm. Nukleoak DAPI-z urdinez tindaturiko gDPSC-ak, titanio disko osoko mosaiko fluoreszentzia irudietan. Ti6AL4V (**F**) eta BAS (**G**). Eskala barra: 2 mm. (Irastorza et al., 2019)-tik moldatua.

Ti6AL4V eta BAS^{TM} titanio azaleretan hazitako gDPSC-en ugaritzearen azterketa PRGF-arekin konbinatzean.

Ugaritzen hari diren zelulen kuantifikazio fotometriko/fluorimetrikoa ezin zen egin titanio diskoen opakotasunarengatik. Honengatik, bideragarriak ziren zelulen kopurua (interfase edo mitosian) nukleoen tindatzaile fluoreszente den DAPI-z tindaturiko nukleoen kontaketaren bidez egin zen. Irudiek erakutsi zutenez, nukleoen morfologia normala zen interfasean aurkitzen ziren zeluletan eta kromosoma kondentsatuak ikus zitezkeen zelula mitotikoetan. Espero bezala, gDPSC gehienak interfasean aurkitzen ziren, hala ere zelula batzuk mitosiaren etapa ezberdinetan

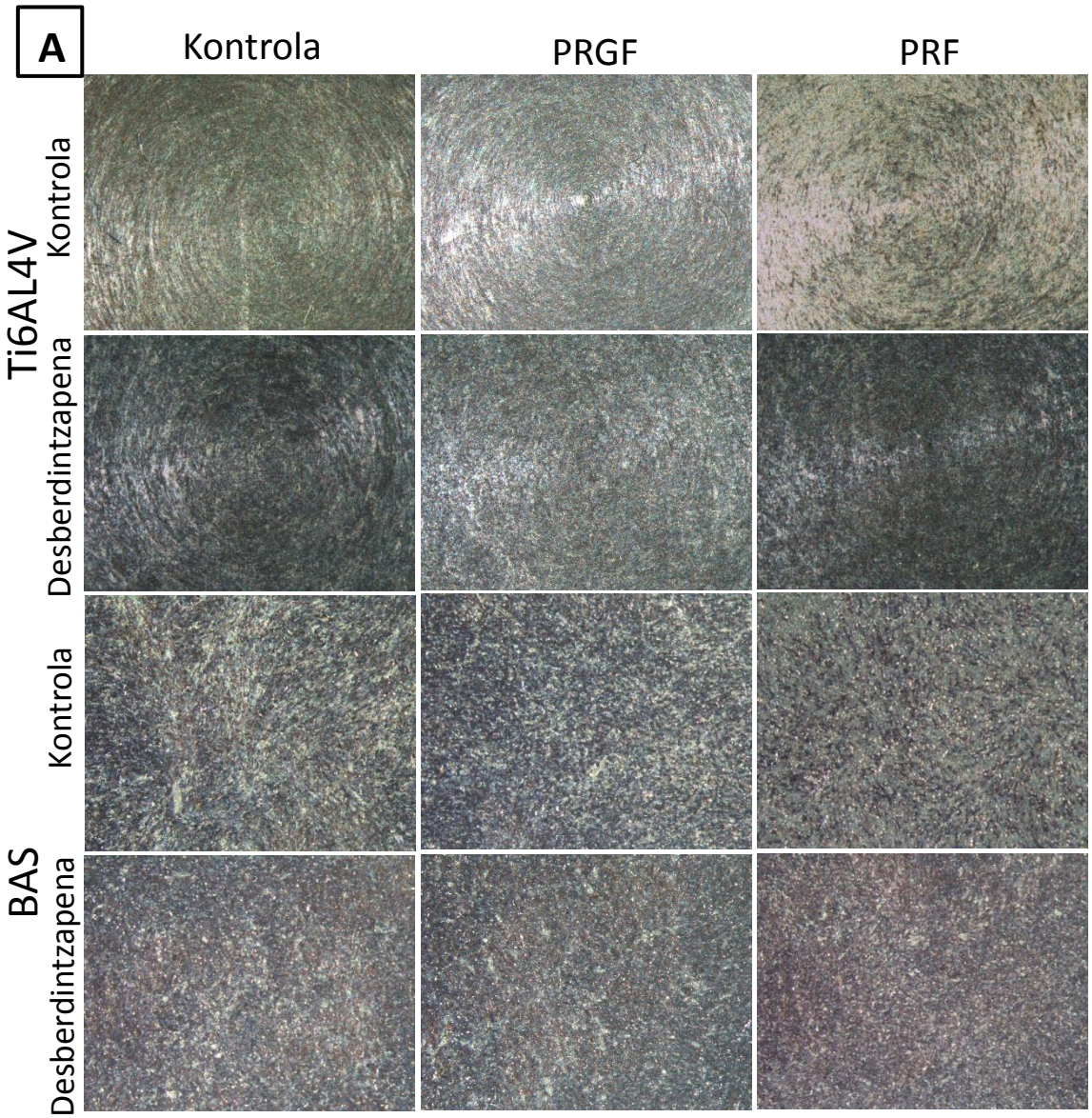
zeuden, gehienak metasafe eta anafasean. Emaitza hauek, gDPSC-en ugaltze normala erakusti zuten. Hala eta guztiz ere, gDPSC-ak PRGF solublearekin haztean, ugalketa esanguratsuki areagotua zuten (% 20-tik % 50-raino; $p < 0.05$) 4 egunek hazkundearen ostean kontrolekin alderatuta (16. Irudia G). Emaitza hauek erakusten dutenez, gDPSC-ak plasmatik eratorritako osagarriekin titanio azaleretan haztean, zelula ugalketa areagotuta dute. Emaitzan berresteko, Ki67 immunofluoreszentzi entsegua egin zen. Ki67-arekin tindaturiko gDPSC-ak, bi titanio azaleretan PRGF-arekin haziak, kontatuak izan ziren, berriz ere, ugaltzen hari diren zelula kopurua areagotua dagoela erakutsiz (16. irudia A-F).

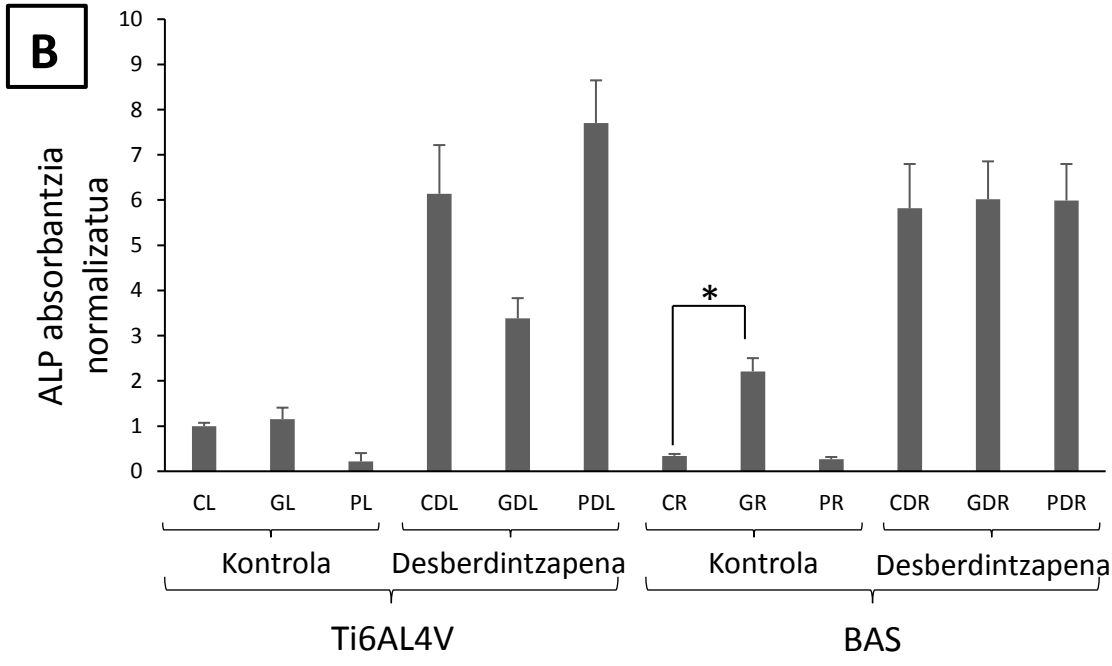


16. irudia. Titanio azaleren gainean PRGF-arekon hazitako gDPSC-en ugalketa entsegua. Ki67 ugalketa markatzaile (berde) eta DAPI-z nukleoak markaturiko (urdin) gDPSC-en immunofluoreszentzi irudiak baldintza kontroletan Ti6AL4V (**A**) eta BAS (**D**) titanio diskoetan, eta % 20 PRGF-rekin haziak Ti6AL4V (**B**) eta BAS (**E**) diskoetan 4 egunez. PRGF tratamenduak Ki67+ zelulen kopurua handitu zuen. Barra eskala: 50 µm. Titanio azaleren gainean kontrol eta PRGF baldintzetan mitosian dauden zelulen (Ki67+) arteko konparaketa (**F**) eta nukleo kopuru totalen konparaketa (DAPI; **G**). estatistikoki esanguratsua $p < 0.05$ denean. (Irastorza et al., 2019)-tik moldatua.

Ti6AL4V eta BAS™ titanio azaleretan hazitako gDPSC-en fosfatasa alkalinoaren aktibitatea

ALP aktibitate mailaren azterketa ohikoa da ama zelulen desberdintzapen osteoblastiko goiztiarraren markatzaile bezala. Fosfatasa alkalino entzima beharrezkoa da matrize extrazelularraren mineralizaziorako (Orimo, 2010). Desberdintzapen osteoblastikoa bultzatzeko, Ti6AL4V eta BAS titanio diskoetan ereindako gDPSC-ak 7 egunez hazi ziren erabilpen zabaleko desberdintzapen osteoblastikoa bultzatzen duen medioarekin. Hazkuntza medio hau dexametasona, azido askorbiko eta β-glizerol fosfatoaz osatua dago (Winning et al., 2019). Gainera, plasmatik eratorritako bi produktu ere, PRGF eta PRF, gehitu ziren medio hauetara. ALP aktibitate mailak nabarmen handitu ziren titanio azaleretan osteo-desberdintzapen medioarekin hazitako gDPSC-etan. Dena dela, naiz eta desberdintzapen medioarekin egon, PRGF-arekin hazi ziren gDPSC-ek ez zuten fosfatasa alkalino aktibitatea handitu (17. irudia A, B). Emaitza hauek PRGF-arekin hazitako DPSC-en ugaltze maila handituarekin bat datoz (16. irudia). Bestalde, titanio leunaren gainean PRF-rekin hazitako gDPSC-ek kontrolekin alderatuta, ALP aktibitatea areagotuta zuten. Gainera, desberdintzapen tratamendurik gabe ere, BAS titanioan PRGF-rekin hazitako gDPSC-ek ALP aktibitatea handituta zuten kontrolekin konparatuz. Lehen ikusi dugun PRGF-ak bultzaturiko zelula dentsitate handiaren ondorio izan daiteke emaitza hau (16. irudia).


A
Kontrola
PRGF
PRF
Ti6AL4V
Kontrola
Desberdintzapena
BAS
Kontrola
Desberdintzapena

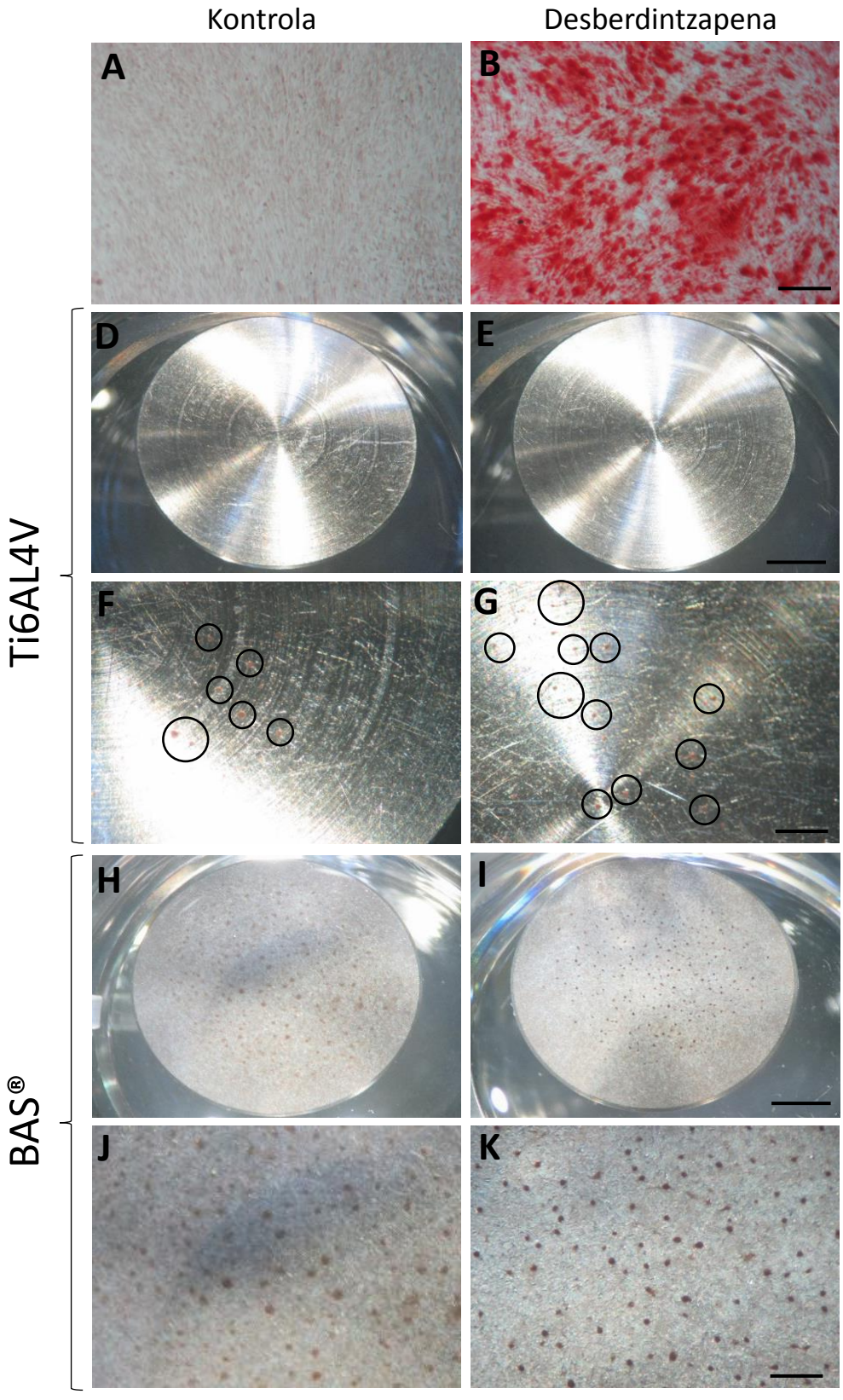

B
ALP absorbantzia normalizatua
10
9
8
7
6
5
4
3
2
1
0
*
CL
GL
PL
CDL
GDL
PDL
CR
GR
PR
CDR
GDR
PDR
Kontrola
Desberdintzapena
Kontrola
Desberdintzapena
Ti6AL4V
BAS

17. irudia. Bi titanio azaleretan, desberdintzapen osteoblastiko medioaren presentzia edo absentzian eta plasmatik eratorritako produktuekin (PRGF eta PRF) hazitako gDPSC-en fosfatasa alkalino entsegua. 14 egunez Ti6AL4V eta BAS titanio diskoetan, desberdintzapen osteoblastikoaren presentzian edo gabezian hazitako gDPSC-en fosfatasa alkalino tindaketa irudiak (**A**). ALP aktibitatearen kuantifikazio normalizatua erakusten duen grafiak (**B**). akronimoen esanahia: C (kontrola), G (PRGF), P (PRF), D (desberdintzapen medioa), L (Ti6AL4V titanioa) era R (BAS titanioa). Estatistikoki esanguratsua (*) $p< 0.05$ denean. Eskala barra: 1 mm. (Irastorza et al., 2019)-tik moldatua.

Ti6AL4V eta BASTM titanio azaleretan hazitako gDPSC-en desberditzapen osteogeniko terminalaren Alizarin gorri tindaketa

Zelula amen desberdintzapen osteogenikoaren froga nagusia hezur matrize nodulu kaltzifikatuen sorrera da. Tindaketa hau alizarin gorriaren bidez egin zen, zeinak kaltzifikaturiko hezur nodulu extrazelularrak gorriz tindatzen dituen. gDPSC-ak plastikoan, Ti6AL4V eta BAS titanio azaleretan erein eta desberdintzapen osteogenikoaren presentzian edo gabezian hazi ziren 14 egunez alizarin gorri tindaketaren aurretik. Mikroskopio estereoskopikoarekin ateratako irudiek erakusten dutenez, ez zegoen alizarin gorri tindaketarik plastikoan kontrol medioarekin hazitako gDPSC-etan. Bestalde, bi titanio azaleretan hazitako gDPSC-ek alizarin gorri tindaketa lekuak erakutsi zituzten, kaltzifikaturiko hezur matrize gordailuei dagokiona. Hezur matrize gordailu hauek maila makroskopikoan identifika daitezke kontrol kondizioetan, BAS titanio azaleran kontsistenteagoa izanik (18. irudia D-K). Horregatik, alizarin gorri tindaketak erakutsi duen bezala, gDPSC-en desberdintzapen osteoblastikoa bultzatzeko titanio azaleren eragina nahikoa dela erakutsi dute. Kontrolekin alderatuta, desberdintzapen osteoblastiko medioaren eraginak, tindaturiko matrize depositu gordailuetan ez zuela handipen osagarririk sortu ikusi zen gDPSC-ak titanio gainean haztean. Desberdintzapen osteoblastikoarekin hazitako gDPSC-en kaltzio deposituak intentsitate handiagoarekin tindatuta zeudela ematen zuen, batez ere BAS titanioan (18. irudia J, K). Desberdintzapen osteoblastikoaren efektuen frogarik handiena, plastiko gainean hazitako gDPSC-etan aurkitu zen. Baldintza honetan, dexametasona, azido askorbiko eta β-glizerol fosfatoz osaturiko desberdintzapen koktelak hezur matrize

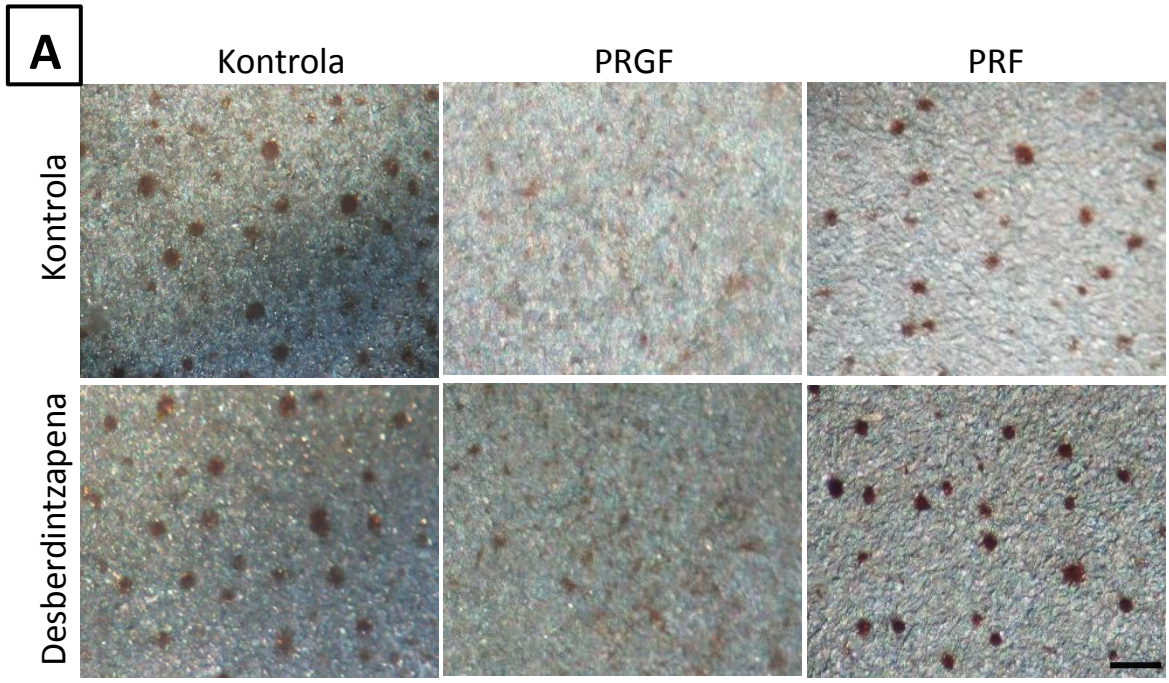
mineralizatu gordailuen handipena erakutsi zuten kontrolekin konparatuta (18. irudia A, B).

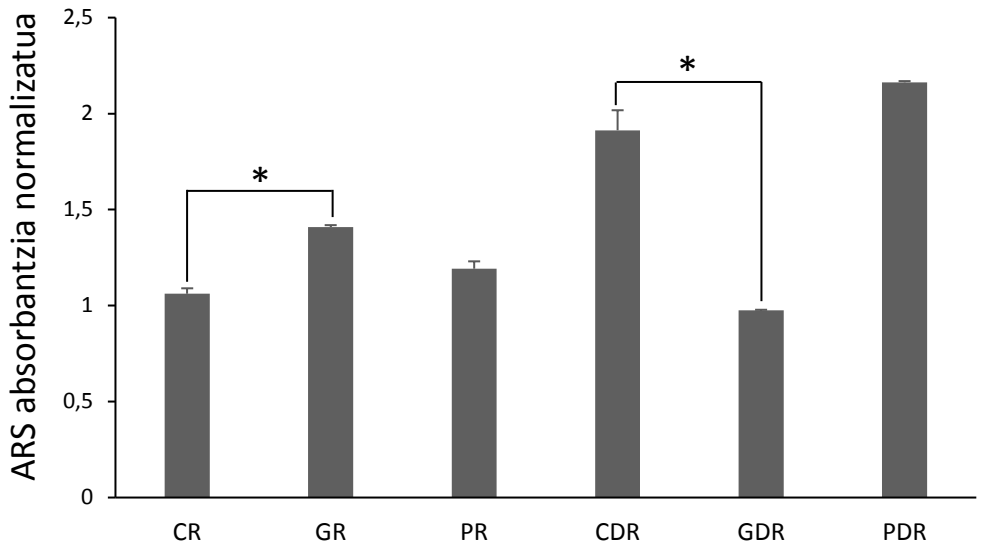


18. irudia. Ti6AL4V eta BAS titanio azaleretan hazitako gDPSC-en alizarin gorri tindaketa. 14 egunez, baldintza kontroletan alizarin gorriz tindatutako hezur matrize gordailuak plastikoan (**A**), Ti6AL4V (**D, F**) eta BAS-ean (**I, F**) haziak, desberdintzapen osteoblastikoarekin trataturiko plastiko (**B**), Ti6AL4V (**E, G**) eta BAS-etan (**J, K**) hazitako gDPSC-ekin konparatuta. Eskala barra (A, B): 100 µm; (D, E, H eta I): 2 mm; (F, G, J eta K): 0.5 mm. (Irastorza et al., 2019)-tik moldatua.

PRGF eta PRF osagarrien efektua desberdintzapen osteoblastikoarekin BAS^{TM} titanio azaleran hazitako gDPSC-engan

Bi titanio azaleretan hezur-sortzaileak diren zeluletara desberdintzeko gDPSC-en gaitasuna ikusi ostean, PRGF eta PRF-ak gDPSC-en desberdintzapenean duen efektua aztertu genuen. Efektu hau aztertzeko, BAS titanio azalera gainean desberdintzapen eta kontrol medioekin hazitako gDPSC-ei PRGF eta PRF gehitu zitzaien 21 egunez. gDPSC-ek sorturiko hezur matrize gordailuak baldintza guztietan aurkitu ziren PRGF eta PRF-arekin edo hauen gabezian haziak egonik ere. Hala ere, tindaturiko nodulu mineralizatuen gutxiagotzea nabarmena zen PRGF-arekin hazitako hDPSC-en bi baldintzetan (19. irudia A). bestalde, naiz eta ez erakutsi mineralizatutako noduluen kantitate handiagorik, PRF-rekin hazitako gDPSC-ek intentsitate handiagoko hezur matrize deposituak sortu zituzten. Emaitza hau kuantifikazio fotometrikoaren bidez egiaztatu zen alizarin gorri prezipitatuen disolbaketari esker (19. irudia B).





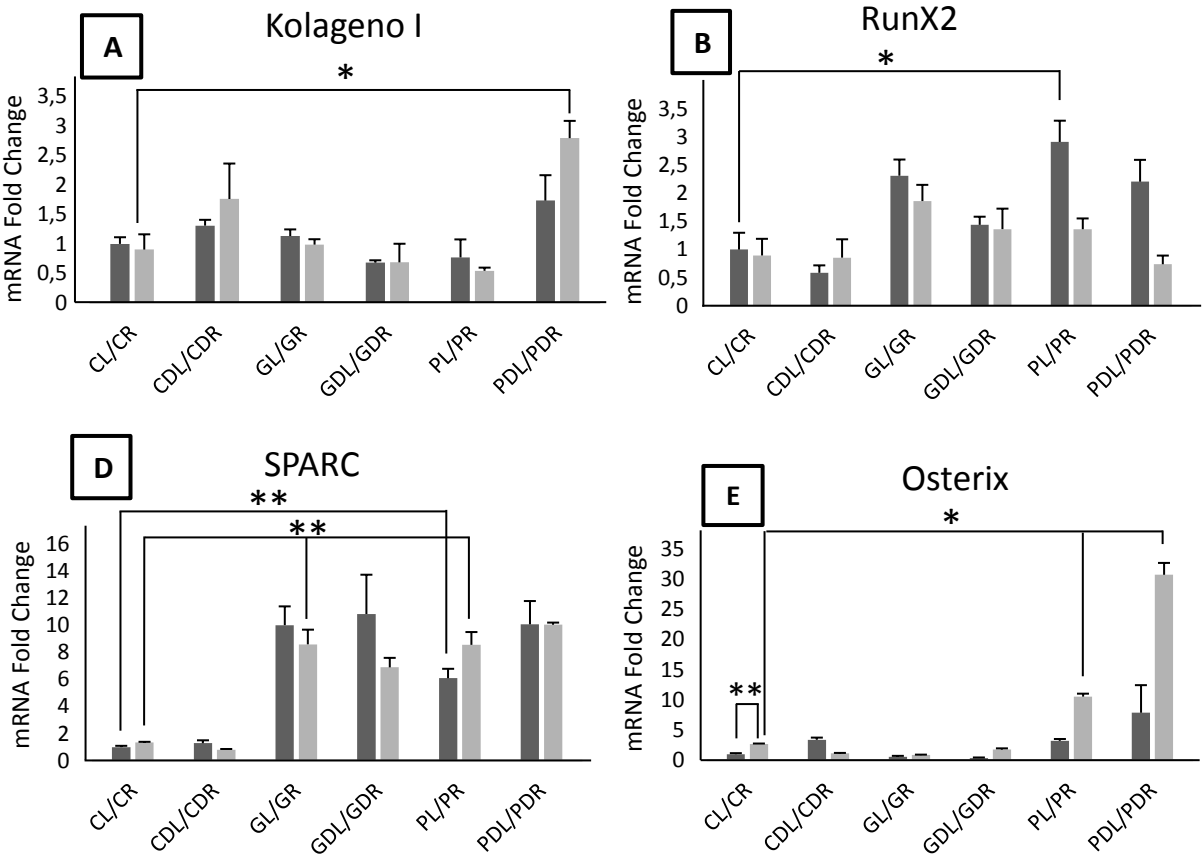
19. irudia. PRGF eta PRF-arekin BAS titanio azalera gainean hazitako gDPSC-en alizarin gorri tindaketa eta kuantifikazio fotometrikoa. Desberdintzapen eta control medioak eta PRGF eta PRF-rekin BAS titanio azalera gainean hazitako gDPSC-en alizarin gorri tindaketa 21 egunen ostean (**A**). Alizarin gorriaren kuantifikazio fotometriko normalizatua (**B**). Akronimoen esanahia: C (kontrola), G (PRGF), P (PRF) eta D (desberdintzapen medioa). Estatistikoki esanguratsua (*) $p< 0.05$ denean. Eskala barra: 250 µm. (Irastorza et al., 2019)-tik moldatua.

Ti6AL4V eta BAS^{TM} titanio azaleretan PRGF eta PRF-rekin edo hauen gabezian hazitako gDPSC-en desberdintzapen markatzaileen azterketa RT-QPCR-bidez

Alizarina gorriz eginiko hezur matrize deposituen tindaketak, gDPSC-en desberdintzapen osteoblastikoa erakutsi zuen PRGF eta PRF-arekin haztean. Hala ere, plasmatik eratorritako produktuek gDPSC-en desberdintzapen osteoblastikoaren seinalizazio bideei nola eragiten zien ez zegoen garbi. Galdera honi argia bota nahian, desberdintzapen osteoblastikoaren etapa ezberdinetan inplikatuta dauden gene markatzaileak aukeratu ziren. Gene markatzaile multzo hau Kolageno I (hezur matrize extrazelularraren konposatu organiko nagusia), RUNX2 (osteoblasto heldugabe

markatzailea), SPARC (osteonektina, tarteko osteoblasto jariatzaile markatzailea) eta OSTERIX/SP7-z (osteoblasto heldu markatzailea) osaturik zegoen.

RT-QPCR-aren emaitzek erakutsi zutenez, nahiz eta gDPSC-ak desberdintzapen osteoblastiko mediorik bage hazi, Kolageno I, RUNX2, SAPRC eta OSTERIX adierazten zituzten bi titanio azaleretan haztean (20. irudia). Gainera, PRGF edo PRF-arekin hazitako gDPSC-ek, baldintza gehienetan markatzaile hauen adierazpenak handituak zituzten. Kontrol eta plasmatik eratorritako produktuak zituzten medioen arteko Kolageno I-en adierazpen konparaketak, nolabaiteko aldakortasuna erakutsi zuen. Hala eta guztiz ere, PRGF eta PRF-rekin hazitako gDPSC-ek RUNX2 eta SPARC markatzaileen adierazpen handipen sendoa erakutsi zuten. Bi markatzaile hauen artean, SPARC aipatu behar genuke bereziki, honen adierazpen maila PRGF eta PRF baldintzetan 6-11 aldiz handituta zegoen kontrolekin alderatuz, desberdintzapen osteoblastikoaren presentzi edo gabeziko baldintzetan (20. Irudia). gDPSC-ek RUNX2 eta SPARC markatzaile pre-osteoblastikoen adierazpena handituta zuten arren PRGF eta PRF-ren desberdintzapen osteoblastikoren efektu bultzatzaileari esker, ezberdintasun interesgarriak aurkitu ziren bi plasmatik eratorritako produktu hauen artean. RUNX2 eta SPARC-en adierazpen handipenak pentsatuarazi zigutenaren kontra, OSTERIX osteoblasto helduen markatzailea PRF baldintzetan bakarrik zegoen handituta. Garrantzitsua da, kontrol baldintzetan Kolageno I, RUNX2 eta SPARC-en adierazpena aurkitu daitekeela esatea, naturalki egoera osteoblastiko pre-desberdintzatuan daudelarik. Nabarmena zen OSTERIX gene adierazpenaren handipen esanguratsua (10-30 aldiz) PRF-a zuten baldintzetan, desberdintzapen osteoblastikoa eduki edo ez eduki arren (20. irudia E). Dena dela, garrantzitsua da aipatzea PRGF-rekin hazitako balditzek ez zutela markatzaile honen adierazpena handitu, baizik eta kontrol baldintzetako adierazpen maila baino txikiagoak zituztela.

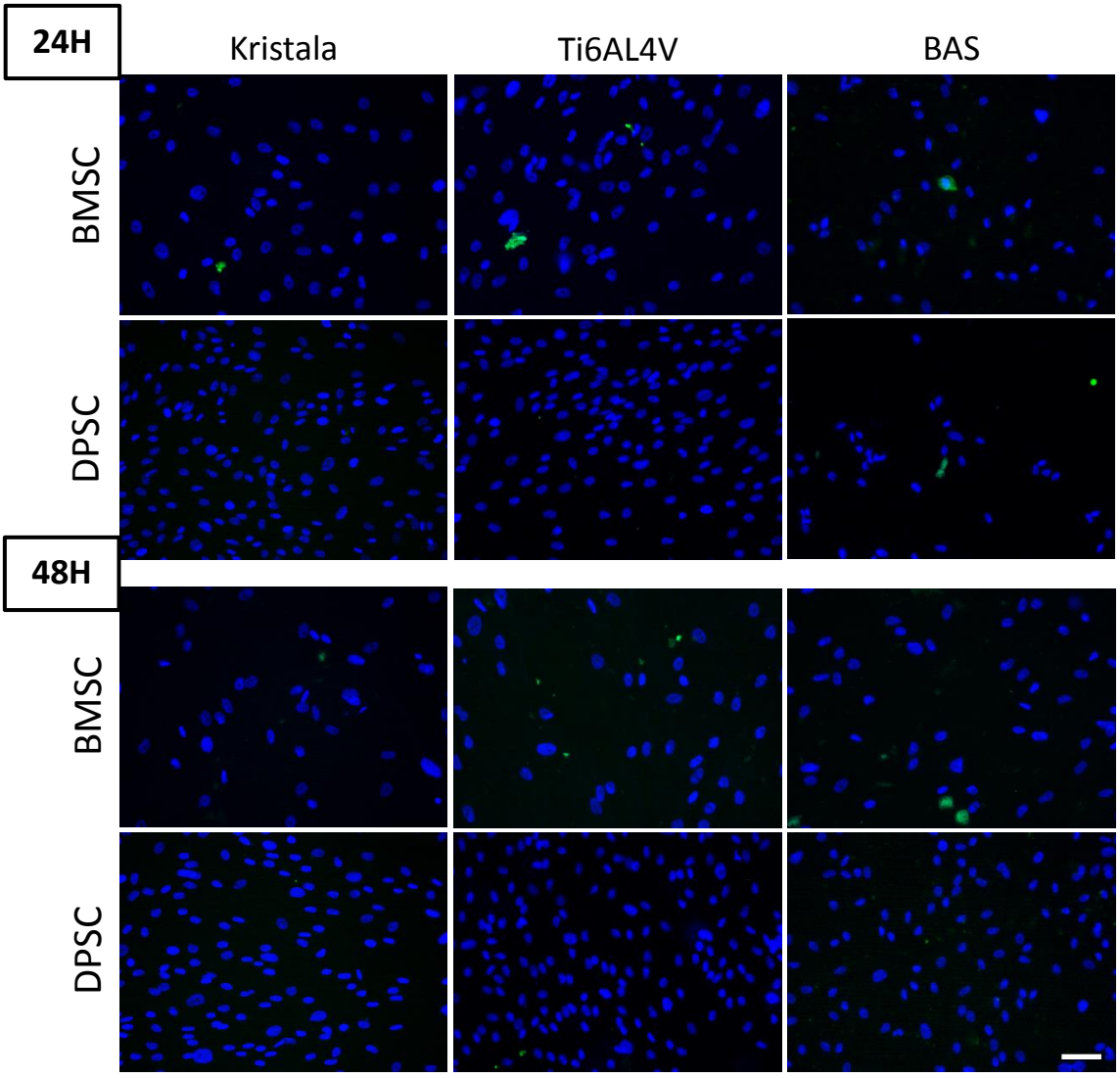


20. irudia. Ti6AL4V eta BAS^{TM} titanio azaleretan, PRGF eta PRF-arekin edo haue gabezian hazitako gDPSC-en desberdintzapen osteoblastikoaren gene adierazpenen QPCR-a. 14 eguneko hazkuntza eta gero eginiko Kolageno I, RUNX2, SPARC eta OSTERIX-en mRNA adierazpen normalizatua. Estatistikoki esanguratsua (*p< 0.05) eta (**p< 0.01) denean. Akronimoen esanahia: C (kontrola), D (desberdintzapen medioa), L (Ti6AL4V), R (BAS), G (PRGF) eta P (PRF). (Irastorza et al., 2019)-tik moldatua.

gDPSC eta gBMSC-en Kaspasa 3 bidezko zelula heriotz konparaketaren azterketa Ti6AL4V eta BAS^{TM} titanio azaleretan haziak 24 eta 48 orduz

BMSC-ak ongi ikerturiko desberdintzapen osteoblastiko gaitasuna duten MSC-ak dira. Gaur egun, hezur birsortze terapietan erabiltzeko zelula ama aukerarik aproposena ez dago oraindik garbi. Honengatik, bi zelula mota hauen arteko bideragarritasun, ugaltze eta desberdintzapen osteoblastikoaren arteko ikerketa konparatiboa gauzatu genuen. Kaspasa 3, apoptosiari esker gertatzen den zelula heriotza markatzailea da. gDPSC eta gBMSC-engan titanio azaleren eragin ditzaketen heriotza zelular ezberdintasunak aztertzeko, 24 eta 48 orduz hazi genituen bi zelula mota hauek titanio

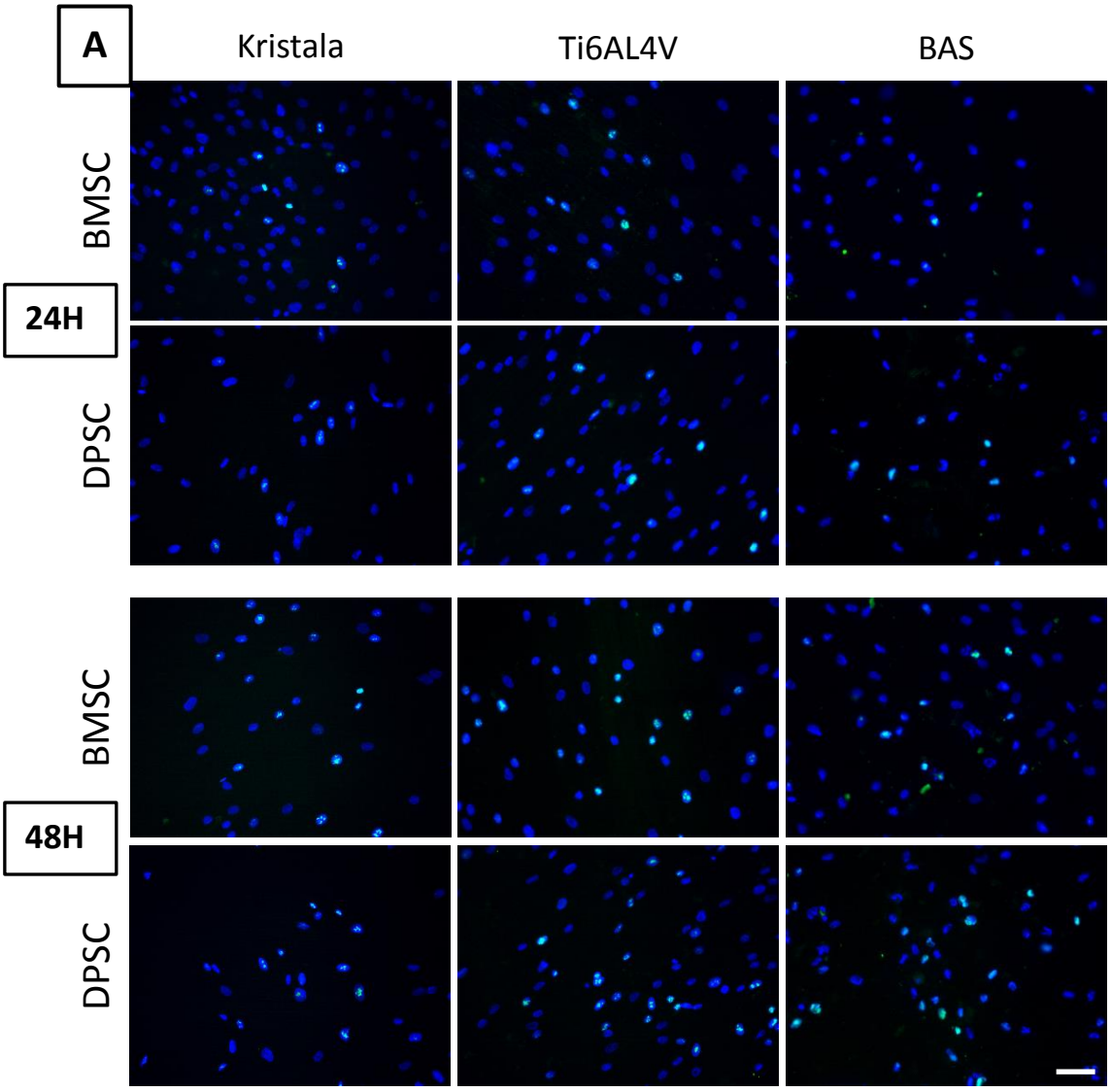
azaleren gainean. Denbora hau igaro ondoren, immunofluoreszentzi entseguak egin ziren zelula apoptotikoak antzemateko (Kaspasa 3+). Emaitzek erakutsi zutenez, bi titanio azalerek ez zuten inolako eragin zitotoxikorik izan gDPSC eta gBMSC-engan 24 eta 48 ordu igaro ostean. Zelula apoptotiko bat edo bi bakarrik aurkitu ziren titanio disko osoan bi zelula motetan, zelula heriotza tasa % 0.1 baina txikiagoa izanik. Zelulek nukleoak DAPI- tindatu ziren fluoreszentzia urdinez (21. irudia).

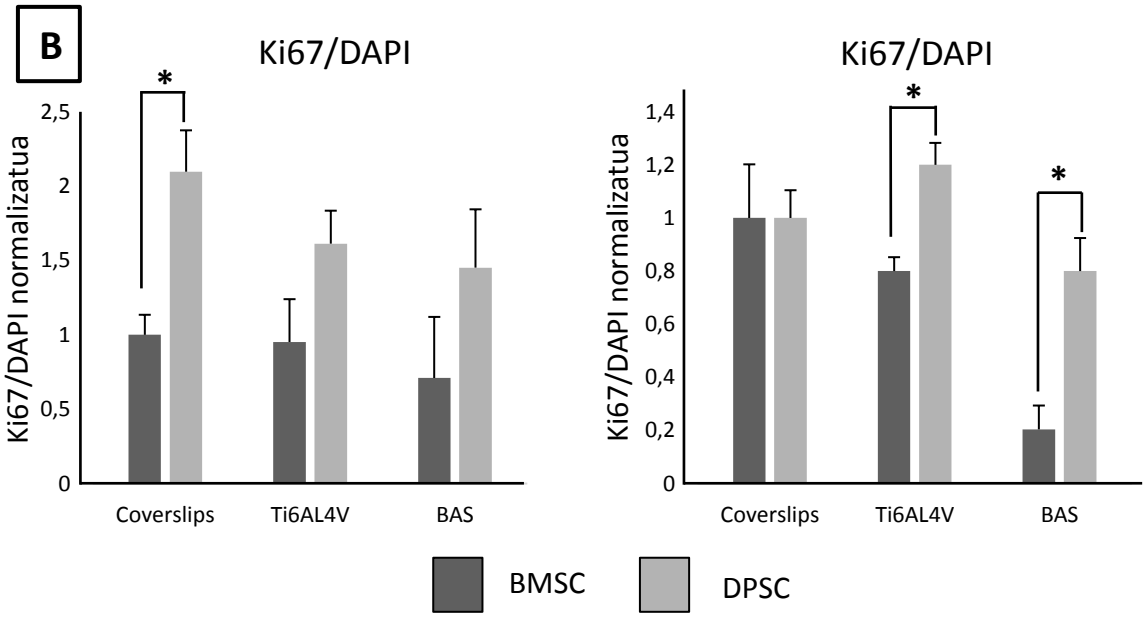


21. irudia. 24 eta 48 orduz kristal, Ti6AL4V eta BAS titanio azaleren gainean hazitako gDPSC eta gBMSC-en Kaspasa 3 heriotza zelular markatzailearen immunofluoreszentzia irudiak. Kristal, Ti6AL4V eta BAS titanio azaleretan hazitako gDPSC eta gBMSC-en Kaspasa 3 heriotza zelular markatzaile (berde) eta DAPI tindatzaile nuklearraren (urdin) immunofluoreszentzi irudiak 24 eta 48 orduren ostean. Bi titanio azalerek eragin zitotoxikorik ez zutela erakutsi zuten irudiek, bi zelula motetan Kaspasa 3 positibo diren zelula bakarrak aurkituz titanio disko osoan. Eskala barra: 100 µm.

Ti6AL4C eta BASTM titanio azaleretan 24 eta 48 orduz hazitako gDPSC eta gBMSC-en arteko ugalketa konparazio ikerketa

Kristal, Ti6AL4V eta BAS titanio diskoen gainean 24 eta 48 orduz hazitako gDPSC eta gBMSC-en arteko ugaltze ezberdintasunak ikertzeko, immunofluoreszentzi ikerketa egin zen zelula ugalketaren markatzaile den Ki67 (berde) eta DAPI nukleo markatzailea (urdin) antzemateko. Titanio diskoen opakotasunaren ondorioz, zelula kopuru totala (DAPI+) eta zelula proliferatzaile kopuru totala (Ki67+), eskuz kontatuz lortu ziren. Espero bezala, bi zelula moten gehiengoak interfasean ageri ziren, hala ere, ezberdintasun interesgarriak aurkitu ziren kristal gainean hazitako gDPSC eta gBMSC-en artean, eta baita titanio azaleren gainean hazitakoen artean ere (22. irudia A). Ki67/DAPI normalizatuaren grafiketan ikus zitzkenez, 24 orduren ondoren ezberdintasun esanguratsuak daude gDPSC eta gBMSC-en ugalketa proportzioen artean kristal gainean, gDPSC-ek izanik ugaltze mailarik handiena. Proportzio hau ere handiagoa zen gDPSC-etan Ti6AL4V eta BAS titanio diskoetan haztean ere, zertxobait handiagoa izanik Ti6AL4V titanioan bi zelula motetan BAS titanioan baino. 48 orduko hazkundearen ostean, gDPSC eta gBMSC-en ugalketa maila berdindu egin zela ikusi zen kristalean eta ez zen ezberdintasun estatistikorik aurkitu. Dena dela, Ti6AL4V titanioan, gDPSC-ek gBMSC-ak baina ugalketa maila handipen esanguratsua zutela frogatu zuten. Azkenik, BAS titanio azalerak ere emaitza interesgarriak erakutsi zituen, gDPSC-en ugalketa maila gBMSC-ena baino handiagoa izanik estatistikoki. Gainera, hDPSC-en ugalketa maila kontrol baldintzarenarekin parekatzea lortu zuten. Bestalde, gBMSC-ek BAS titanioaren gainean, 24 ordutan baino ugaltze maila txikiagoa erakutsi zuten, esperimentu osoko mailarik txikiena erakutsiz (22. irudia B).

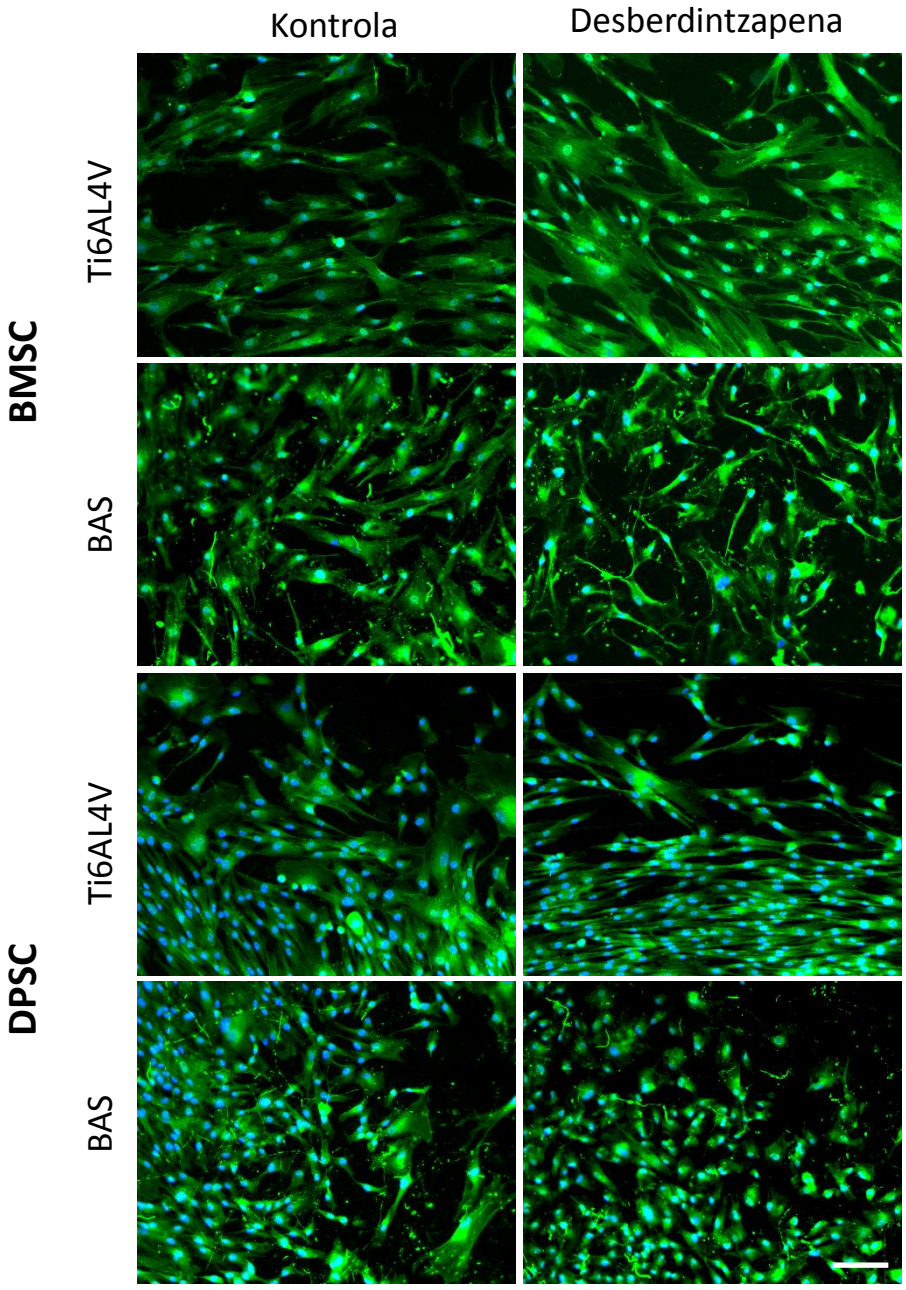

A
Kristala
Ti6AL4V
BAS
BMSC
24H
DPSC
BMSC
48H
DPSC


B
Ki67/DAPI
Ki67/DAPI normalizatua
2,5
2
1,5
1
0,5
0
*
Coverslips
Ti6AL4V
BAS
Ki67/DAPI
Ki67/DAPI normalizatua
1,4
1,2
1
0,8
0,6
0,4
0,2
0
*
*
Coverslips
Ti6AL4V
BAS
BMSC
DPSC

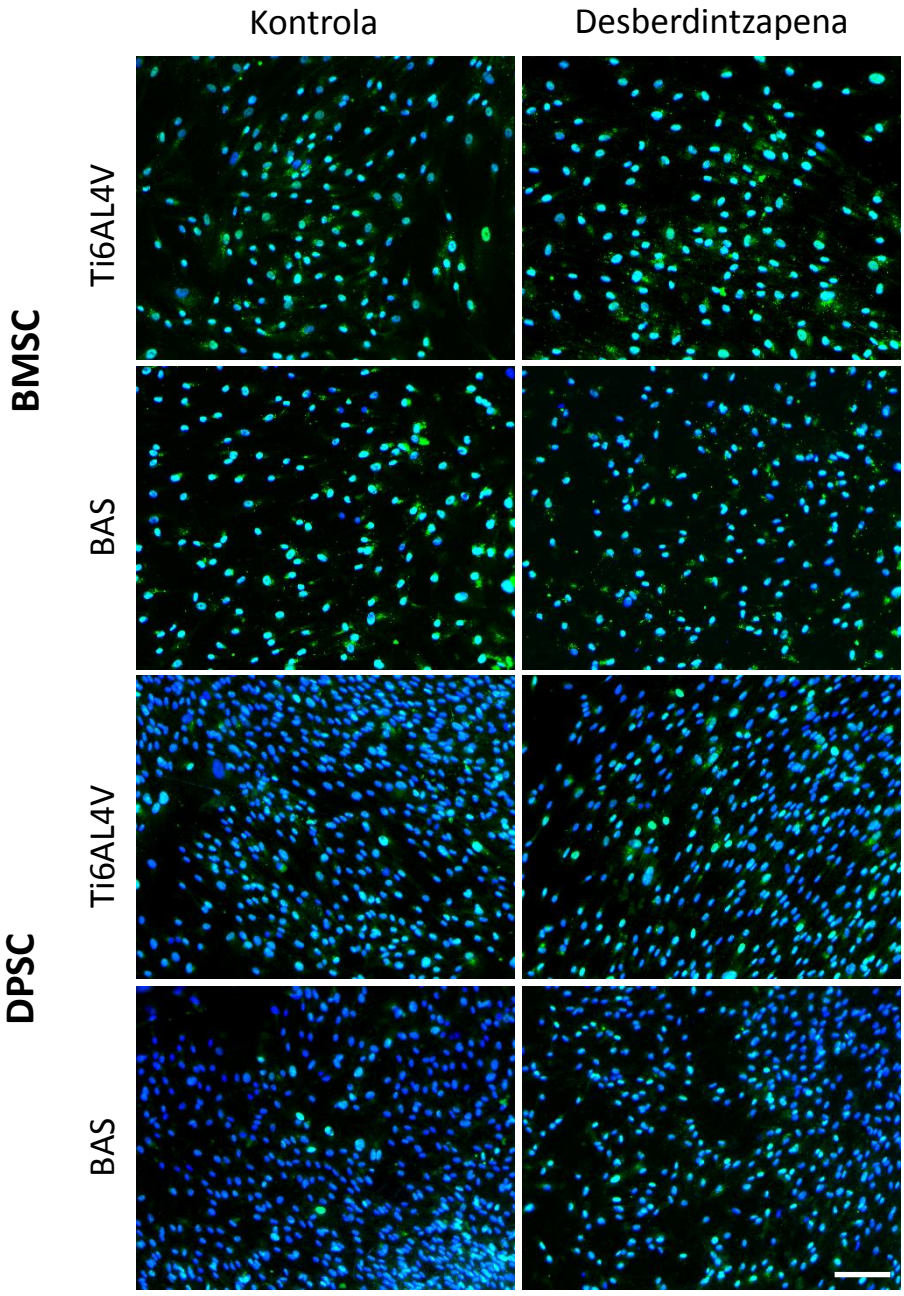
22. irudia. Kristal, Ti6AL4V eta BAS titanio azaleretan 24 eta 48 orduz hazitako gDPSC eta gBMSC-en arteko ugaritze ikerketa. Immunofluoreszentzia mikroskopio bidez ateratako gDPSC eta gBMSC-en Ki67 ugalketa markatzaile (berde) eta DAPI nukleo markatzaileen (urdin) irudiak 24 eta 48 orduren ostean (A). azalera ezberdinetan hazitako bi zelula moten ugalketa ratioaren grafika (B). Eskala barra: 100 µm. Estatistikoki esanguratsua (**p*< 0.05) denean.

Ti6AL4V eta BAS^{TM} titanio azaleren gainean desberdintzapen osteoblastiko medioaren presentzian edo gabezian hazitako gDPSC eta gBMSC-en SPARC eta Osterix desberdintzapen osteoblastiko markatzaileen immunofluorezentzia entsegua

Desberdintzapen osteoblastiko markatzaileen adierazpena ikertzeko, gDPSC eta gBSC-ak bi titanio azaleren gainean erein ziren eta desberdintzapen medioaren presentzian edo gabezian hazi ziren 14 egunez. Fijaketaren ondoren, immunofluoreszentzi entsegua egin zen Osteonektina (SPARC) eta Osterix detektatzeko. Bi zelula moten nukleoak DAPI-z tindatu ziren. 14 egun ondoren ikusi genuen bezala, bi zelula motek SPARC adierazten zuten hazkuntza medio guztietan (23. irudia). SPARC-ek zitosolean lokalizaturik zegoela ematen zuen, nukleoen inguruan kongretatua. Gainera, Ti6AL4V titanioan desberdintzapen medioarekin hazitako gBMSC-ak bakarrik erakutsi zuten nolabaiteko SPARC adierazpen handipena. Dena dela, Osterix-en adierazpena handiagoa zen gBMSC-etan gDPSC-ekin alderatuz baldintza guztietan (24. irudia). SPARC ez bezala, Osterix (transkripzo faktorea) nukleoan lokalizaturik zegoen. Nahiz eta Osterix gBMSC-etan gehiago adierazita egon, zelula hauek ez zuten ezberdintasun handirik erakutsi kontrol eta tratamendu osteoblastikoren artean, gDPSC-ek berriz, desberdintzapen osteoblastiko medioarekin hazi ondoren Osterix-en adierazpena handitu zuten.



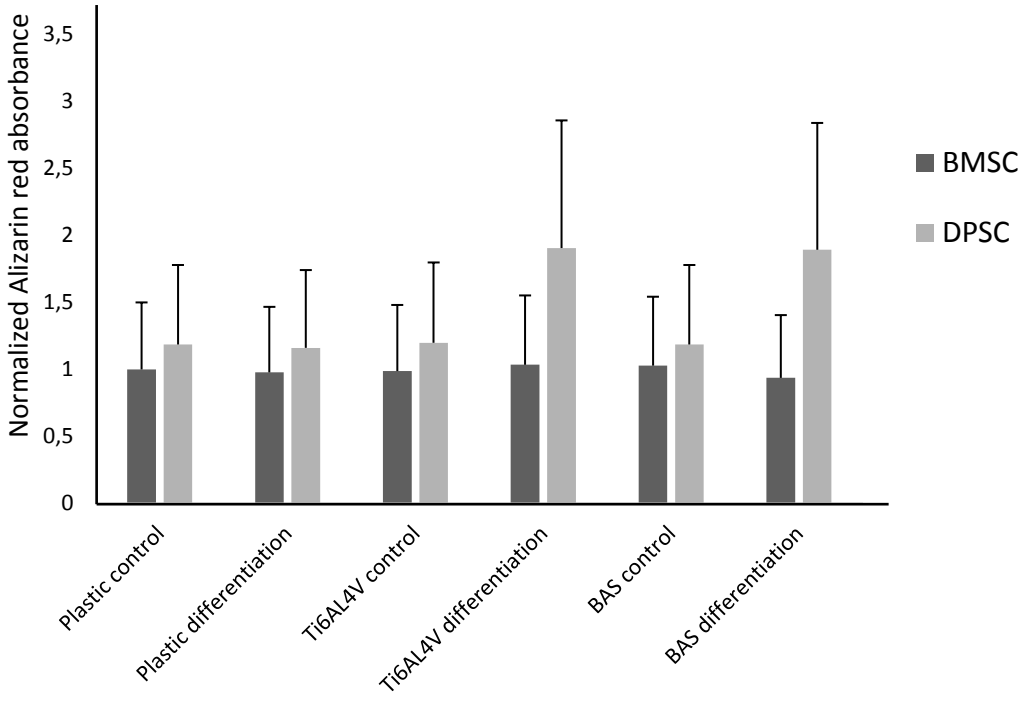
23. irudia. Ti6AL4V eta BAS^{TM} titanio azaleren gainean desberdintzapen osteoblastiko medioaren presentzian edo gabezian hazitako gDPSC eta gBMSC-en SPARC desberdintzapen osteoblastiko markatzailearen immunofluorezentzia irudiak. Bi titanio azaleretan hazi ziren gDPSC eta gBMSC-ak desberdintzapen osteoblastiko medioaren presentzian eta gabezian 14 egunez. Fijaketaren ondoren, SAPRC desberdintzapen osteoblastikoaren markatzailea (berde) eta DAPI-z zelulen nukleoak (urdin) tindatu ziren. Eskala barra: 100 µm.



24. irudia. Ti6AL4V eta BAS^{TM} titanio azaleren gainean desberdintzapen osteoblastiko medioaren presentzian edo gabezian hazitako gDPSC eta gBMSC-en Osterix desberdintzapen osteoblastiko markatzailearen immunofluorezentzia irudiak. Bi titanio azaleretan hazi ziren gDPSC eta gBMSC-ak desberdintzapen osteoblastiko medioaren presentzian eta gabezian 14 egunez. Fijaketaren ondoren, Osterix desberdintzapen osteoblastikoaren markatzailea (berde) eta DAPI-z zelulen nukleoak (urdin) tindatu ziren. Eskala barra: 100 µm.

Ti6AL4V eta BAS^{TM} titanio azaleretan hazitako gDPSC eta gBMSC-en mineralizaturiko hezur matrize gordailuen konparaketa Alizarina gorriaren bidez

Kaltzifikaturiko hezur matrize nodulu extrazelularrak, erabat desberdintzatutako zelula osteoblastikoen berrespena dira. Gordailu hauek Alizarina gorriari esker tindatu ziren, laranja(gorri kolorea lortuz. gDPSC eta gBMSC-ak plastiko, Ti6AL4V eta BAS titanioetan hazi ziren 21 egunez desberdintzapen osteoblastiko medioaren presentzian edo gabezian. Orokorrean, gDPSC-ek gBMSC-ak baina mineralizazio handiagoa erakutsi zuten baldintza guztietan, baina ez zen nahikoa izan estatistikoki esanguratsua izateko. Hala ere, mineralizazio handipenik aipagarriena desberdintzapen osteoblastiko medioarekin bi titanio azaleren gainean hazitako hDPSC-ek erakutsi zuten (25. irudia), baina errepikatua izan behar da ondorio sendoak lortzeko. Hare gehiago, bi titanio azaleretan hazitako hDPSC hauek izan ziren kontrolekiko mineralizazio handipena erakutsi zuten baldintza bakarrak. Emaitza hauek, titanio azalerek gDPSC-en desberdintzapen osteoblastikoan zuten eragin areagotzailea erakutsi zuten. Alizarina gorriz nodulu kaltzifikatuak tindatu ostean, azido azetikoz disolbatu ziren plaka irakurle batez neurtua izateko.



25. irudia. Ti6AL4V eta BAS^{TM} titanio azaleretan hazitako gDPSC eta gBMSC-en mineralizaturiko hezur matrize gordailuen Alizarina gorriaren absorbantzia grafikoa. 21 egunez hazi ondoren, hezur matrize extrazelular gordailuak Alizarin gorriz tindatu ziren, honen ostean, Alizarina azido azetikoan disolbatu zen eta plaka irakurle batez neurtu zen absorbantzia.

Plastiko, Ti6AL4V eta BAS^{TM} titanio azaleretan desberdintzapen osteoblastiko medioaren presentzian edo gabezian hazitako gDPSC eta gBMSC-en RNA sekuentziazioa

Desberdintzapen osteoblastiko prozesuan parte artzen duten gene ezberdinen eta gene bideen erregulazio positibo edo negatiboa aztertzeko, gDPSC-ak eta gBMSC-ak plastiko, Ti6AL4V eta BAS titanio diskoetan hazi ziren 14 egunez desberdintzapen osteoblastiko medioaren presentzian edo gabezian. Hazkuntzaren ondoren, zelulak desitsatsi eta RNA atera genuen Qiagen-en RNA estrakzio kit-a erabiliz eta Illumina-z sekuentziatu genuen Zürich Unibertsitateko Genomika Zentroan, lagin bakoitzeko 25.000 RNA irakurketa lortuz. Lorturiko datuak “Pathway Enrichment Analysis” bidez, “The Connectivity Map” (CMAP) eta “Biological Process Gene Ontology” (GOBP) datu baseak erabiliz analizatu zen CIC bioBuneko Genoma Analisi Plataforman.

Ezberdin adierazitako geneen ikerketa konparatiboan zentratu ginen, presentzia/ gabezia (ON/OFF) kriterioa jarraituz baldintza esperimental ezberdinen artean. 10 irakurketa gordinetan ezarri genuen gene baten adierazpen positibo (ON) muga. Baldintza ezberdinen konparaketetan atera ziren ezberdin adierazitako geneen kantitateak (2. taula).

Comparative conditions	Genes
DPSC-C-FLASK VS BMSC-C-FLASK	69
DPSC-C-FLASK VS DPSC-C-TI	7
BMSC-C-FLASK VS BMSC-C-TI	16
BMSC-C-TI VS BMSC-C-BAS	23
DPSC-C-FLASK VS DPSC-T-FLASK	34
BMSC-C-FLASK VS BMSC-T-FLASK	43
DPSC-C-FLASK VS DPSC-T-TI	17
DPSC-T-TI VS DPSC-T-BAS	1
BMSC-C-FLASK VS BMSC-T-TI	64
BMSC-T-TI VS BMSC-T-BAS	35

2. taula. Baldintza batean adierazita eta bestean adierazi gabe dauden geneen kantitateak erakusten dituen taula. Adierazitako geneen kantitate patroi ezberdinak aurkitu ziren baldintza ezberdinak alderatzean. Laburdurak: DPSC: hortz mamiko zelula amak; BMSC: hezur muineko zelula amak; C: kontrol medioa; T: desberdintzapen osteoblastiko medioa; Flask: plastiko azalera; TI: Ti6AL4V titanioa eta BAS: "Biomimetic Advanced Surface" titanioa.

Baldintza ezberdinen arteko geneen ON/OFF analisi konparaketak emaitza interesgarriak erakutsi zituen. Plastiko gainean kontrol medoarekin hazitako gBMSC-ek HOX gene anitz adierazi zituzten gDPSC-ekin konparatuta (3. taula). Gainera, desberdintzapen osteoblastikoan bereziki inplikatuta dauden SPARC, OSTERIX/SP7 eta ZBTB16 geneen adierazpena handituta aurkitu zen bi zelula motetan desberdintzapen osteoblastiko medioarekin eta/edo titanio gainean haztean (3. taula). Garrantzitsua da ZBTB16-ren kasua aipatzea, hau izan delako desberdintzapen osteoblastiko pean (tratamendu farmakologikoa eta/edo titanioa) hazitako bi zelula motetan (gDPSC eta gBMSC) adierazi den genea, osteogenesi prozesuan eduki dezakeen rol garrantzitsua iradokiz. Azkenik, neurotrofina errezeptore eta gDPSC-en "amatasun" markatzaile den NTRK3 eta erlazionaturiko GFRA2 geneak kontrol baldintzetan adierazita zeuden, baina adierazpen hau itzali egin zen titanioan desberdintzapen osteoblastiko medioarekin haztean (3. taula).

	Identifier	Gene-name	Description	DPSC-C-FLASK	BMSC-C-FLASK
DPSC-C-FLASK VS BMSC-C-FLASK	ENSG00000037965	HOXC8	homeobox C8	0	76
	ENSG00000078399	HOXA9	homeobox A9	0	66
	ENSG00000105991	HOXA1	homeobox A1	0	12
	ENSG00000105997	HOXA3	homeobox A3	0	38
	ENSG00000106004	HOXA5	homeobox A5	0	48
	ENSG00000106511	MEOX2	mesenchyme homeobox 2	0	185
	ENSG00000108511	HOXB6	homeobox B6	0	19
	ENSG00000120093	HOXB3	homeobox B3	0	129
	ENSG00000122592	HOXA7	homeobox A7	0	41
	ENSG00000123388	HOXC11	homeobox C11	0	36
	ENSG00000170370	EMX2	empty spiracles homeobox 2	0	19
	ENSG00000175879	HOXD8	homeobox D8	0	21
	ENSG00000180806	HOXC9	homeobox C9	0	22

	ENSG00000180818	HOXC10	homeobox C10	0	231
	ENSG00000197757	HOXC6	homeobox C6	0	85
	ENSG00000198353	HOXC4	homeobox C4	0	27
	ENSG00000253293	HOXA10	homeobox A10	0	116
	ENSG00000260027	HOXB7	homeobox B7	0	37
				DPSC-C-Flask	**DPSC-C-Ti**
DPSC-C-FLASK VS DPSC-C-TI	ENSG00000170374	SP7	Sp7 transcription factor	0	71
				DPSC-C-Flask	**DPSC-T-Flask**
DPSC-C-FLASK VS DPSC-T-FLASK	ENSG00000109906	ZBTB16	zinc finger and BTB domain containing 16	0	182
	ENSG00000140538	NTRK3	neurotrophic receptor tyrosine kinase 3	12	0
	ENSG00000168546	GFRA2	GDNF family receptor alpha 2	107	0
				BMSC-C-Flask	**BMSC-T-Flask**
BMSC-C-FLASK VS BMSC-T-FLASK	ENSG00000109906	ZBTB16	zinc finger and BTB domain containing 16	0	673
				DPSC-C-Flask	**DPSC-T-Ti**
DPSC-C-FLASK VS DPSC-T-TI	ENSG00000109906	ZBTB16	zinc finger and BTB domain containing 16	0	358
	ENSG00000140538	NTRK3	neurotrophic receptor tyrosine kinase 3	12	0
	ENSG00000170374	SP7	Sp7 transcription factor	0	51
				BMSC-C-Flask	**BMSC-T-Ti**
BMSC-C-FLASK VS BMSC-T-TI	ENSG00000107742	SPOCK2	SPARC (osteonectin), cwcv and kazal like domains proteoglycan 2	0	10
	ENSG00000123364	HOXC13	homeobox C13	0	11
	ENSG00000152583	SPARCL1	SPARC like 1	0	45

3. taula. Baldintza ezberdinen arteko konparaketen gene adierazpen ezberdintasunak. Laburdurak: DPSC: hortz mamiko zelula amak; BMSC: hezur muineko zelula amak; C: kontrol medioa; T: desberdintzapen osteoblastiko medioa; Flask: plastiko azalera; TI: Ti6AL4V titanioa eta BAS: “Biomimetic Advanced Surface” titanioa.

Gene adierazpen indibidualen konparaketez gain, gene bideen aberaste analisia egin genuen “The Connectivity Map” (CMAP) eta “Gene Ontology enrichment” (GO) datu baseak erabiliz. Alde batetik, CMAP-ek transkripzio adierazpen datuak erabiltzen ditu zelula fisiologia, gaixotasun eta terapiekin erlazionatzeko. Beste alde batetik, GO-k ontologia erabiltzen du gene eta beraien produktuen esanahi biologikoa lortzeko. Analisiak hiru kategoriatan banatuta daude: osagai zelularra (CC), prozesu biologikoak (BP) eta funtzio molekularra (MF). Ikerketa honetan, GOBP erabili dugu gene bidean analizatzeko (4 eta 5. taulak). CMAP zein GOBP-z eginiko analisiek baldintza esperimental ezberdinen artean aldatuta egon daitezkeen gene bide eta prozesuak erakusten dizkigute, erlazionaturiko probabilitate balioa (p) eta gene bideen gene kopuru totaletik (Size) ezberdin adierazitako geneak (Count) erakutsiz gene bide bakoitzean. Gene bide bakoitzean ezberdin adierazitako geneen kopuru minimoa 2-an ezarri genuen eta esanguratasun estatistikoa ($p < 0.05$-ean).

CMAP	ID	Size	Count	pvalueadj	Genes	Description
DPSC-C-FLASK VS BMSC-C-FLASK	ALCALAY_AML_NPMC_UP	133	8	6.98724e-09	COCH, HOXA1, HOXA10, HOXA5, HOXA7, HOXB3, HOXB6, HOXB7	Genes up-regulated in acute myeloid leukemia (AML)
	VERHAAK_AML_NPM1_MUT_VS_WT_UP	173	7	1.16885e-06	EREG, HOXA10, HOXA5, HOXA7, HOXB3, HOXB6, TNFSF10	Genes up-regulated in acute myeloid leukemia (AML)
	TAKEDA_NUP8_HOXA9_16D_UP	125	6	3.89713e-06	EREG, HOXA3, HOXA5, HOXA7, HOXB3, TNFSF10	Hematopoietic disorder
	BOQUEST_CD31PLUS_VS_CD31MINUS_UP	552	8	0.000117134	BST2, CHI3L1, COCH, CSF2RB, DOK5, HOXB7, STEAP4, TNFSF10	
	TAKEDA_NUP8_HOXA9_3D_UP	143	5	0.000184436	BST2, HOXA3, HOXA5, HOXA7, HOXB3	effects of NUP98-HOXA9 on gene transcription at 3 days after transduction UP
	TAKEDA_NUP8_HOXA9_6H_UP	66	4	0.000205999	HOXA3, HOXA5, HOXB3, HOXC6	effects of NUP98-HOXA9 on gene transcription at 6

						hours after transduction UP
	TAKEDA_NUP8_HOXA9_8D_UP	118	4	0.00178462	HOXA3, HOXA5, HOXA7, HOXB3	effects of NUP98-HOXA9 on gene transcription at 8 days after transduction UP
	TAKEDA_NUP8_HOXA9_10D_UP	147	4	0.00369089	HOXA3, HOXA5, HOXA7, HOXB3	effects of NUP98-HOXA9 on gene transcription at 10 days after transduction UP
DPSC-C-FLASK VS DPSC-T-FLASK	BASSO_GERMINAL_CENTER_CD40_UP	93	3	0.0191607	BATF, BCL2A1, HLA-DQB1	Gene up-regulated by CD40 signaling in Ramos cells
	CMV_HCMV_TIMECOURSE_12HRS_UP	25	2	0.0377068	RSAD2, TNFSF10	Genes up-regulated after infection with HCMV at 12 h
	VERHAAK_AML_NPM1_MUT_VS_WT_UP	173	3	0.040305	BCL2A1, SERPINA1, TNFSF10	Genes up-regulated in acute myeloid leukemia (AML)
	CARIES_PULP_UP	200	3	0.0462674	BCL2A1, HLA-DQB1, SERPINA1	Immune/cytokine response

4. taula. Gene bideen aberastasun analisia "The Connectivity Map"-ez (CMAP) egina. Plastiko eta titanio azaleren gainean desberdintzapen osteoblastiko medioaren presentzian edo gabezia hazitako gDPSC eta gBMSC-en gene bideen aberaspen analisi konparatiboa. Taulan agertzen ez diren konparaketak ez zuten ezarritako n= 2 eta p< 0.05 eskakizuna bete. Laburdurak: DPSC: hortz mamiko zelula amak; BMSC: hezur muineko zelula amak; C: kontrol medioa; T: desberdintzapen osteoblastiko medioa; Flask: plastiko azalera; TI: Ti6AL4V titanioa eta BAS: "Biomimetic Advanced Surface" titanioa.

GOBP	ID	Size	Count	pvalueadj	Genes	Description
DPSC-C-FLASK VS BMSC-C-FLASK	GO:0009952	104	12	1,32926E-12	HOXA1, HOXA5, HOXA7, HOXA10, HOXB3, HOXB6, HOXB7, HOXC6, HOXC8, HOXC9, HOXC10, HOXC11	anterior/posterior pattern specification
	GO:0048704	80	7	8,00122E-06	HOXA1, HOXA5, HOXA7, HOXB3, HOXB6, HOXB7, HOXC9	embryonic skeletal system morphogenesis
	GO:0009792	607	13	9,5092E-05	HOXA1, HOXA5, HOXA7, HOXB3, HOXB6, HOXB7, HOXC6, HOXC9,	embryo development

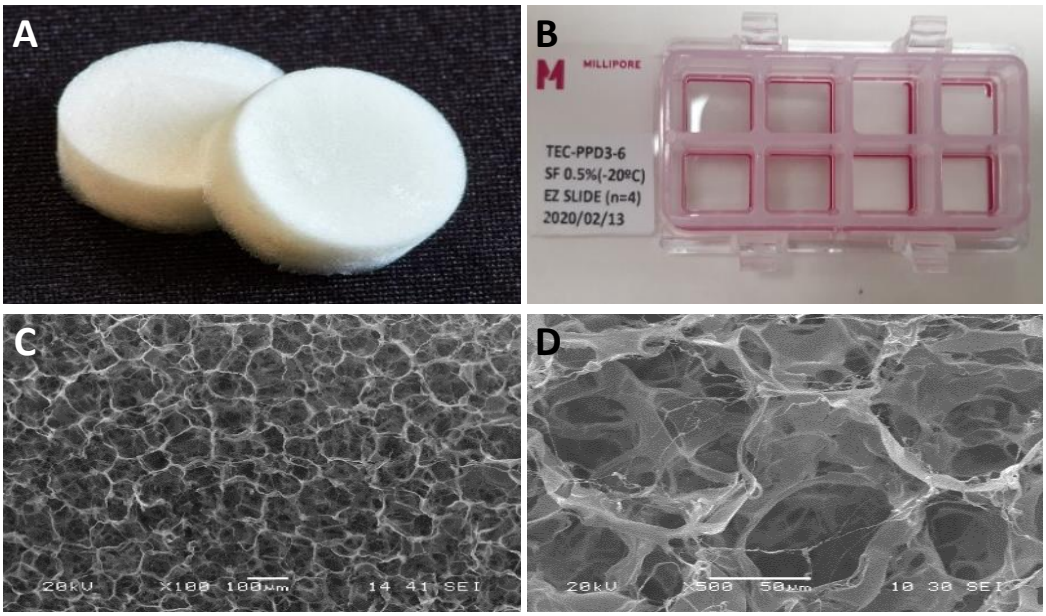
					HOXC11, MEOX2, PITX2, TBX18, HEY2	ending in birth or egg hatching
	GO:0001568	531	10	0,006077272	CHI3L1, EREG, HOXA1, HOXA3, HOXA5, HOXA7, HOXB3, MEOX2, PITX2, HEY2	blood vessel development
	GO:0065007	7094	38	0,009102425	BST2, CHI3L1, CSF2RB, CSTA, EREG, HAS1, HOXA1, HOXA3, HOXA7, HOXA9, HOXA10, HOXB3, HOXB7, HOXC4, HOXC6, HOXC8, HOXC9, HOXC10, HOXC11, HOXD8, LSP1, MEOX2, OPCML, PITX2, SIM1, ZIC1, TNFSF10, WISP3, TBX18, RASSF9, ABCC9, CNKSR2, DOK5, SUCNR1, HHIP, EBF2, NDNF, FOXP2	biological regulation
	GO:0032774	3408	26	0,01596934	EREG, HOXA1, HOXA3, HOXA5, HOXA7, HOXA9, HOXA10, HOXB3, HOXB6, HOXB7, HOXC4, HOXC6, HOXC8, HOXC9, HOXC10, HOXC11, HOXD8, MEOX2, PITX2, SIM1, ZIC1, TBX18, HEY2, EBF2, SPX, FOXP2	RNA biosynthetic process
	GO:0072358	414	8	0,016913155	CHI3L1, EREG, HOXA1, HOXA3, HOXA5, HOXA7, HOXB3, MEOX2	cardiovascular system development
	GO:0009954	30	3	0,022750337	HOXA10, HOXC10, HOXC11	proximal/distal pattern formation
DPSC-C-FLASK VS DPSC-C-TI	GO:0042755	26	2	0,023359184	LEP, TACR1	eating behavior
	GO:0046887	93	2	0,031493385	LEP, TACR1	positive regulation of hormone secretion
	GO:0050880	127	2	0,031493385	LEP, TACR1	regulation of blood vessel size
	GO:0002520	737	3	0,040817267	LEP, RSAD2, SP7	immune system development
	GO:0050867	258	2	0,04102342	HLA-DQB1, TACR1	positive regulation of cell activation
	GO:0009914	281	2	0,048691666	LEP, TACR1	hormone transport
BMSC-C-FLASK VS	GO:0010719	22	2	0,022505055	LDLRAD4, TBX5	negative regulation of

BMSC-C TI						epithelial to mesenchymal transition
BMSC-C-FLASK VS BMSC-T-FLASK	GO:0035912	5	2	0,042243127	HEY2, DLL4	dorsal aorta morphgenesis
DPSC-C-FLASK VS DPSC-T-TI	GO:0060218	13	2	0,027739491	BATF, SP7	hematopoietic stem cell differentiation

5. taula. Gene bideen aberastasun analisia "Gene Ontology Biological Process"-ez (GOBP) egina. Plastiko eta titanio azaleren gainean desberdintzapen osteoblastiko medioaren presentzian edo gabezia hazitako gDPSC eta gBMSC-en gene bideen aberaspen analisi konparatiboa. Taulan agertzen ez diren konparaketak ez zuten ezarritako n= 2 eta p< 0.05 eskakizuna bete. Laburdurak: DPSC: hortz mamiko zelula amak; BMSC: hezur muineko zelula amak; C: kontrol medioa; T: desberdintzapen osteoblastiko medioa; Flask: plastiko azalera; TI: Ti6AL4V titanioa eta BAS: "Biomimetic Advanced Surface" titanioa.

Dezelulaturiko txerri ehun adiposoa (pDAT) apar solido bezala prozesatuaren SEM irudiak

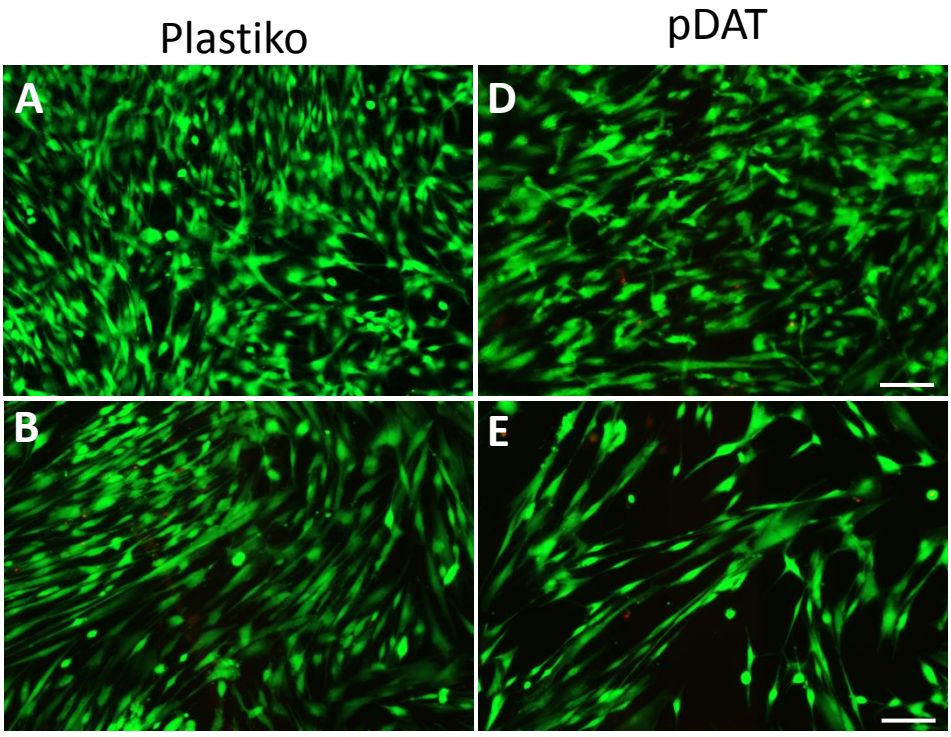
Txerrien ehun adiposoa erauzi ondoren, dezelularizazioa isopropanol eta Tritoi X-100 erabiliz gauzatu zen, garbigarri ez ionikoa. Dezelularizazio prozesua amaitzean, scaffold biologiko moduan *in vitro* zelulek hazkuntzetan erabilia izateko, apar solidoa izozte-lehortze metodoaren bidez eratu zen (26. irudia A, B). SEM irudiek interkonektibitate handiko egitura porotsua frogatu zuten. 50 eta 100 µm-en arteko poroek tamaina zutelarik (26. irudia C, D).



26. irudia. *In vitro* erabilerarako dezelularizaturiko txerri ehun adiposoaren egitura porotsua SEM irudietan. *In vitro* zelulekin konbinatuta erabiltzeko dezelularizaturiko txerri ehun adiposoaren irudi makroskopikoa (**A, B**). pDAT-ren SEM irudian 50-100 µm arteko poro tamainak erakutsiz (**C, D**).

Plastiko eta pDAT-n ereindako gDPSC-en bideragarritasunaren azterketa Kaltzeina-AM/ propidio ioduroaren bidez

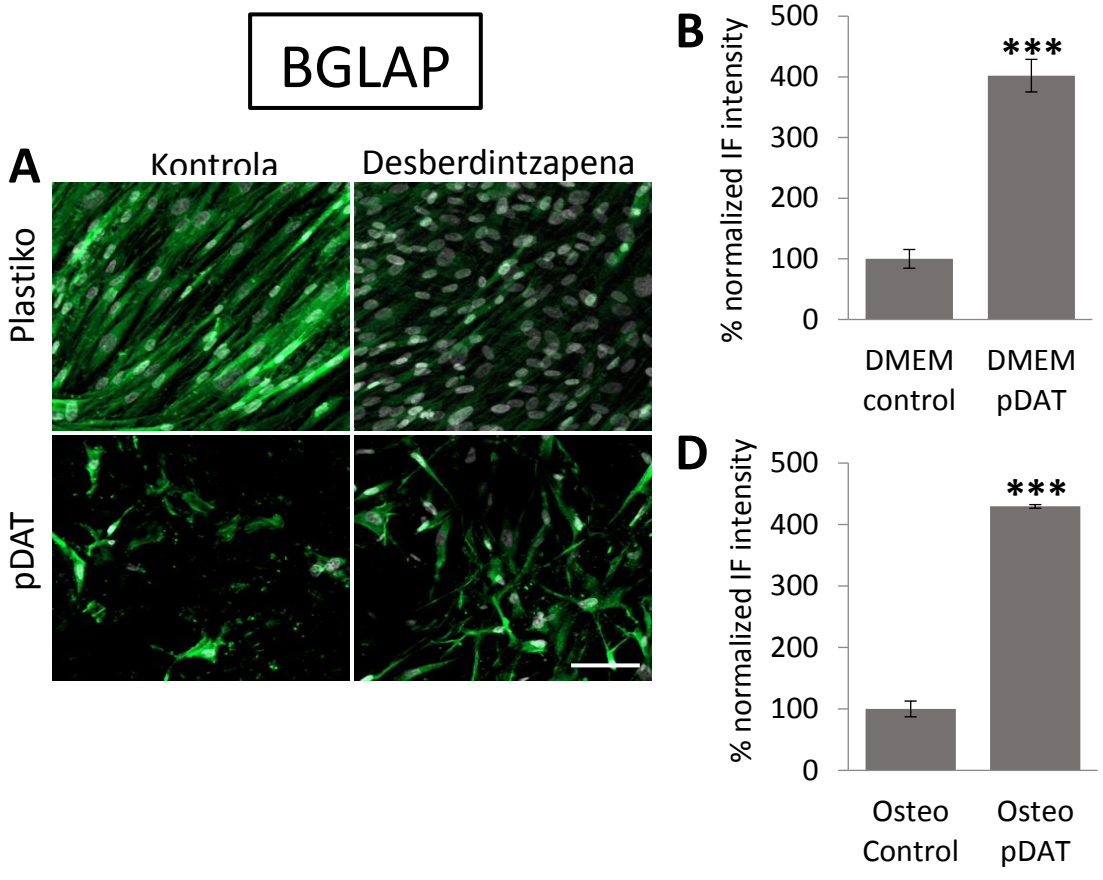
pDAT-ren eragin zitotoxikoa baztertzeko, 15.000 gDPSC zelula erein ziren bai hobitxo hutsetan (plastikoa) eta baita pDAT apar solidoetan 4 egunez. Zelulen bideragarritasun entsegua gDPSC-ak, Kaltzeina-AM (fluoreszentzia berdea) zelula bizien tindatzailearekin eta propidio ioduro (fluoreszentzi gorria) hildako zelulen tindatzailearekin haziz burutu zen. Fluoreszentzi mikroskopioarekin ateratako irudiek, gDPSC-ak pDAT-an haztean ia % 100-eko bideragarritasuna zutela frogatu zuten, zelula guztiak fluoreszentzi berdez tindaturik aurkituz. Zelula isolatuak bakarrik aurkitu ziren gorriz tindaturik, pDAT-ren zitotoxizitate eza erakutsiz (28. irudia). Bi azalera hauen arteko alderik handiena, plastikoan aurkituriko zelula kopuru handia izan zen (27. irudia A, B). Aurkitutako gDPSC zelula kopuru desberdintasun hau, plastikoan duten mono-geruza hazkuntzarengatik izan daiteke, pDAT-an duten 3 dimentsiotako hazkuntzarekin alderatuz.



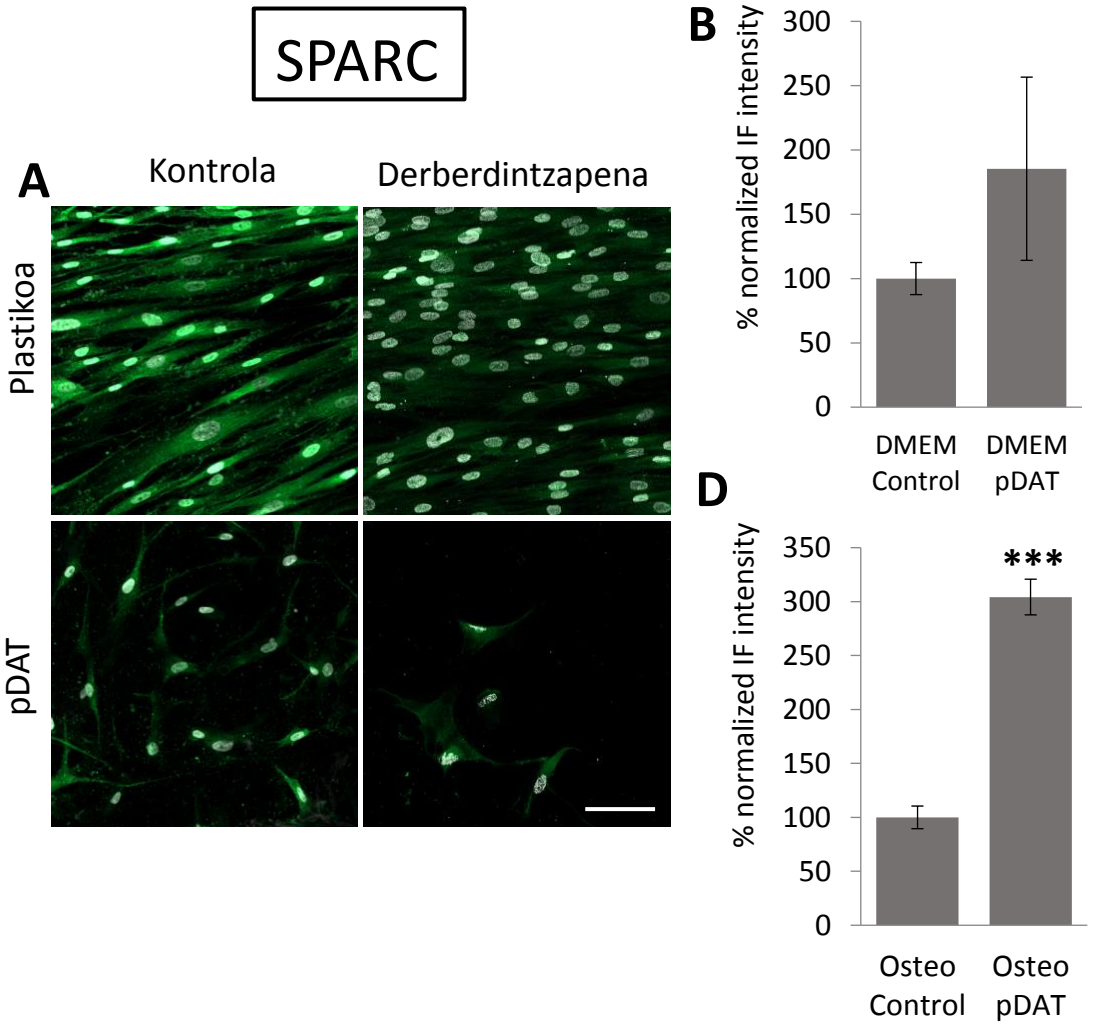
27. irudia. Plastiko eta pDAT apar solidoetan hazitako gDPSC-en Kaltzeina_AM/ propidio ioduro irudiak. gDPSC-ak 15.000 zelula/ hobitxo dentsitatean hazi ziren 4 egunez eta Kaltzeina-AM (berde) zelula bizi markatzaile eta propidio ioduro (gorri) zelula hilen markatzaileekin tindatu ziren. Plastiko gainean hazitako zelulek (**A, B**), pDAT-an hazitakoek (**D, E**) baina kopuru handiagoa erakutsi zuten, mono-geruza hazkundea dela eta. Eskala barra (A, D): 100 µm; (B, E): 50 µm.

pDAT-an hazitako gDPSC-en zelula osteoblastiko markatzaileen immunofluoreszentzia

pDAT apar solidoak 8 hobitxoko Minicell EZ-portetan integratu ziren *in vitro* erabilerarako zelula hazkundeetan. gDPSC-ak 15.000 zelula/ hobitxo dentsitatean erein ziren eta 14 egunez hazi ziren medio kontrol (DMEM) eta desberdintzapen osteoblastiko medioarekin. DAPI nukleo markatzaileak, pDAT egitura porotsuan plastikoan baino zelula gutxiago zeudela frogatu zuen, non zelulak mono-geruza atxikituetan hazi ziren (28. Irudia A). 14 eguneko hazkundearen ostean, immunofluoreszentzi entsegua burutu zen desberdintzapen osteoblastiko markatzaile diren Osteokaltzina (BGLAP) eta Osteonektina-ren (SPARC) adierazpena detektatzeko. Plastikoan hazitako gDPSC-ek bi desberdintzapen osteoblastiko markatzaile hauen adierazi zituzten. pDAT-an ereindako gDPSC-ek BGLAP eta SPARC-en intentsitate erlatiboa esanguratsuki handitua zuten bi medioekin hazita plastikoarekin alderatuz (28. irudia B; 29. irudia D).



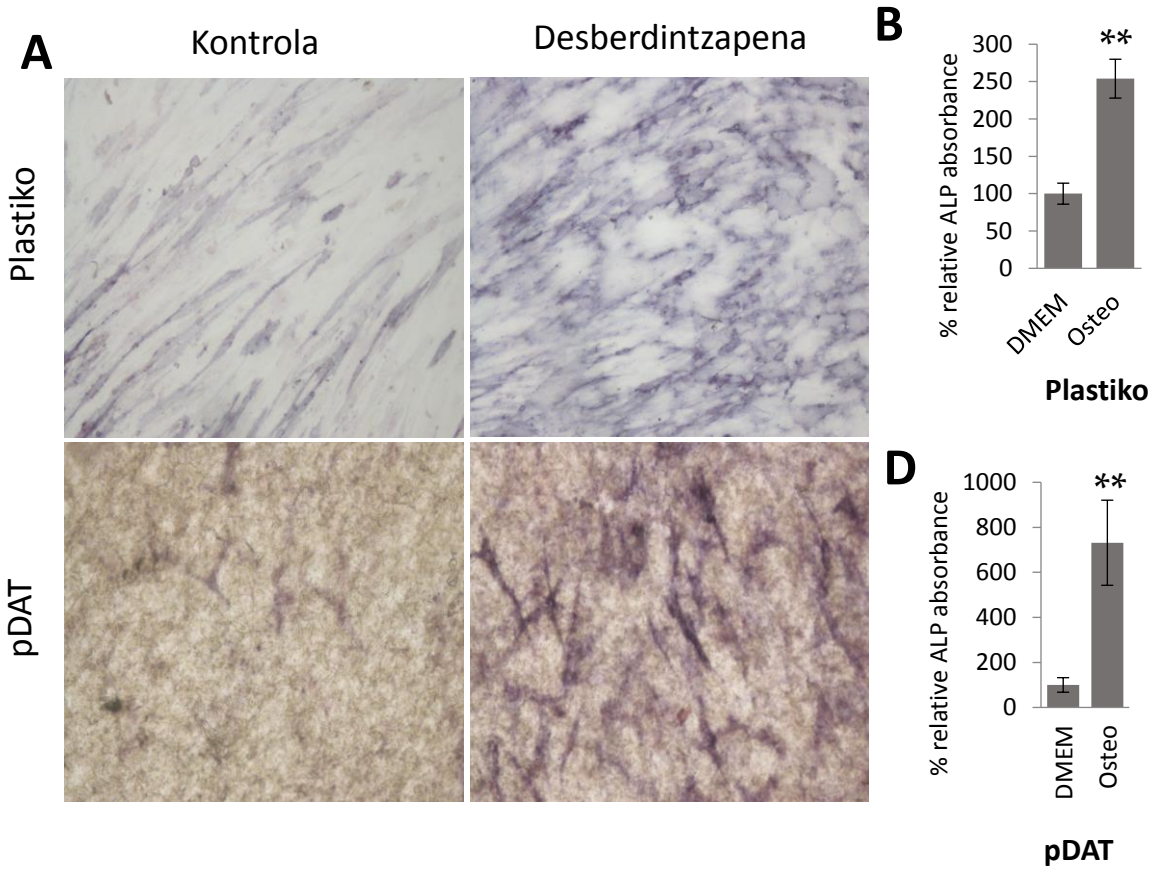
28. irudia. Plastiko eta pDAT-an desberdintzapen osteoblastikoaren presentzian edo gabezian hazitako gDPSC-en Osteokaltzina (BGLAP) immunofluoreszentzia irudiak eta intentsitate kuantifikazioa. Plastiko eta pDAT-a duten EZ-portetan 2 astez kontrol eta desberdintzapen medioekin hazitako gDPSC-en immunofluoreszentzia irudiak (**A**). ImageJ-rekin eginiko BGLAP-aren IF markaketaren kuantifikazio erlatiboa baldintza ezberdinetako zelula kopuruekiko (**B, D**). Eskala barra: 50 µm. Estatistikoki esanguratsua (***$p \leq 0.001$) denean.



29. irudia. Plastiko eta pDAT-an desberdintzapen osteoblastikoaren presentzian edo gabezian hazitako gDPSC-en Osteonektina (SPARC) immunofluoreszentzia irudiak eta intentsitate kuantifikazioa. Plastiko eta pDAT-a duten EZ-portetan 2 astez kontrol eta desberdintzapen medioekin hazitako gDPSC-en immunofluoreszentzia irudiak (**A**). ImageJ-rekin eginiko SPARC-en IF markaketaren kuantifikazio erlatiboa baldintza ezberdinetako zelula kopuruekiko (**B, D**). Eskala barra: 50 µm. Estatistikoki esanguratsua (***$p \leq 0.001$) denean.

Plastiko eta pDAT-an desberdintzapen osteoblastiko medioaren presentzian edo gabezian hazitako gDPSC-en ALP tindaketa eta kuantifikazioa

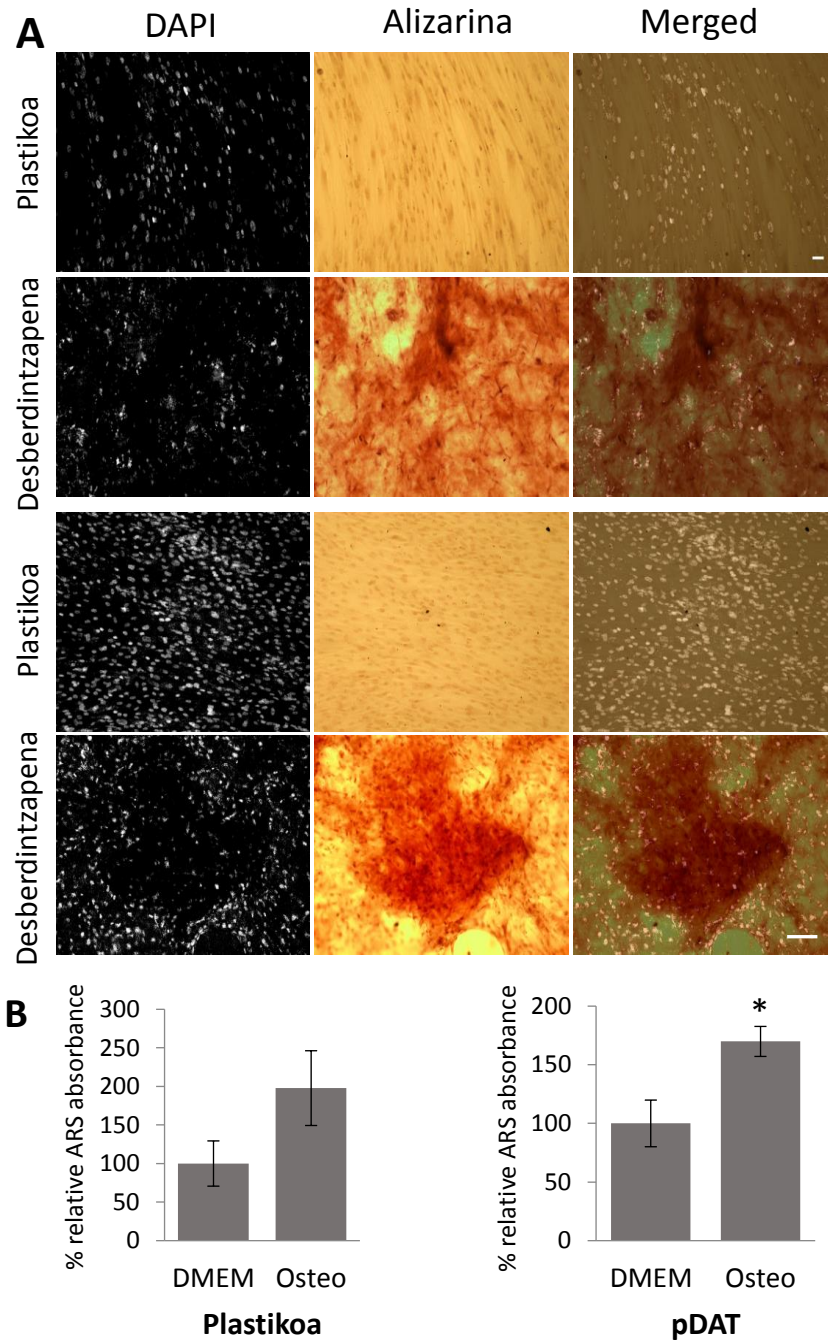
gDPSC-ak plastiko eta pDAT-an hazi ziren 14 egunez kontrol eta desberdintzapen osteoblastiko medioekin. Ondoren, mineralizatzen duten hezur zelulen ezaugarri den fosfatasa alkalino entzima tindatu zen. Irudiek erakutsi zutenez, gDPSC-ek ALP aktibitatea zuten bi azaleretan (30. irudia A). ALP kuantifikazioak, bi azaleretan desberdintzapen medioarekin hazitako zelulen markaketa handipen esanguratsua frogatu zuten (30. irudia B, D).



30. irudia. Plastiko eta pDAT-an desberdintzapen osteoblastikoaren presentzian edo gabezian hazitako gDPSC-en ALP aktibitate kuantifikazioa. gDPSC-ak 14 egunez hazi ziren plastiko eta pDAT-a zuten EZ-portetan kontrol eta desberdintzapen osteoblastiko medioarekin. ALP aktibitatea prezipitatu more/beltzen detekzioz gauzatu zen (A). ALP-ren kuantifikazioa, absorbantzia erlatiboaren ImageJ-rekin neurtuz gauzatu zen. Estatistikoki esanguratsua (**$p \leq 0.01$) demean.

Plastiko eta pDAT-an desberdintzapen osteoblastiko medioaren presentzian edo gabezian hazitako gDPSC-en Alizarin gorri tindaketa eta kuantifikazioa

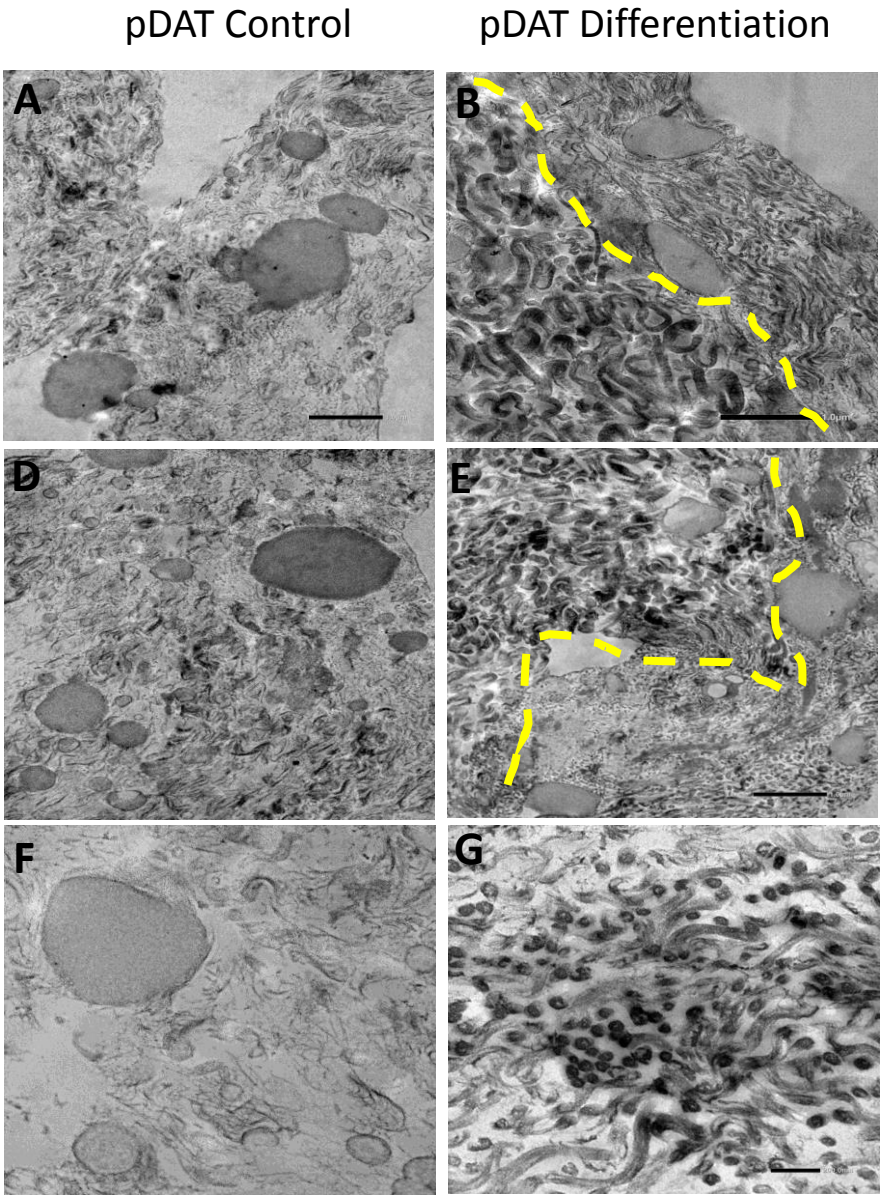
Alizarin gorria, mineralizaturiko hezur matrize gordailuak kolore laranja/gorriz tindatzen dituen kaltzio-lotzaile tindakaria da. gDPSC-ak 4 astez hazi ziren plastiko eta pDAT-an kontrol eta desberdintzapen osteoblastiko medioekin. Honen ostean, plastikozko hobitxoak zelula konfluentzia handiarekin bukatu zuten, Alizarin gorri positibo ziren zelula sakabanatuak erakutsiz (31. irudia A). Hala ere, Alizarin gorri prezipitatu dentsitaterik handienak pDAT-an ereindako gDPSC-etan aurkitu zen (31. Irudia A). neurketa semi-kuantitatiboek, bi azaleretan desberdintzapen osteoblastikoaren eraginez, tindaketa intentsitate handiagoa frogatu zen (31. irudia B). hare gehiago, eremu argiko mikroskopio irudiak DAPI immunofluoreszentzia irudiekin konbinatuz, zelulen lokalizazio erlatiboa behatu zen hezur matrize gordailuekiko. gDPSC-ak area mineralizatuen ertzetan dentsitate handiagoan kokatuta zeudela eman zuen. Gainera, nodulu kaltzifikatuekin kontaktu estuan aurkitu ziren gDPSC-ak itxura oneko nukleoak zituzten (31. irudia A).



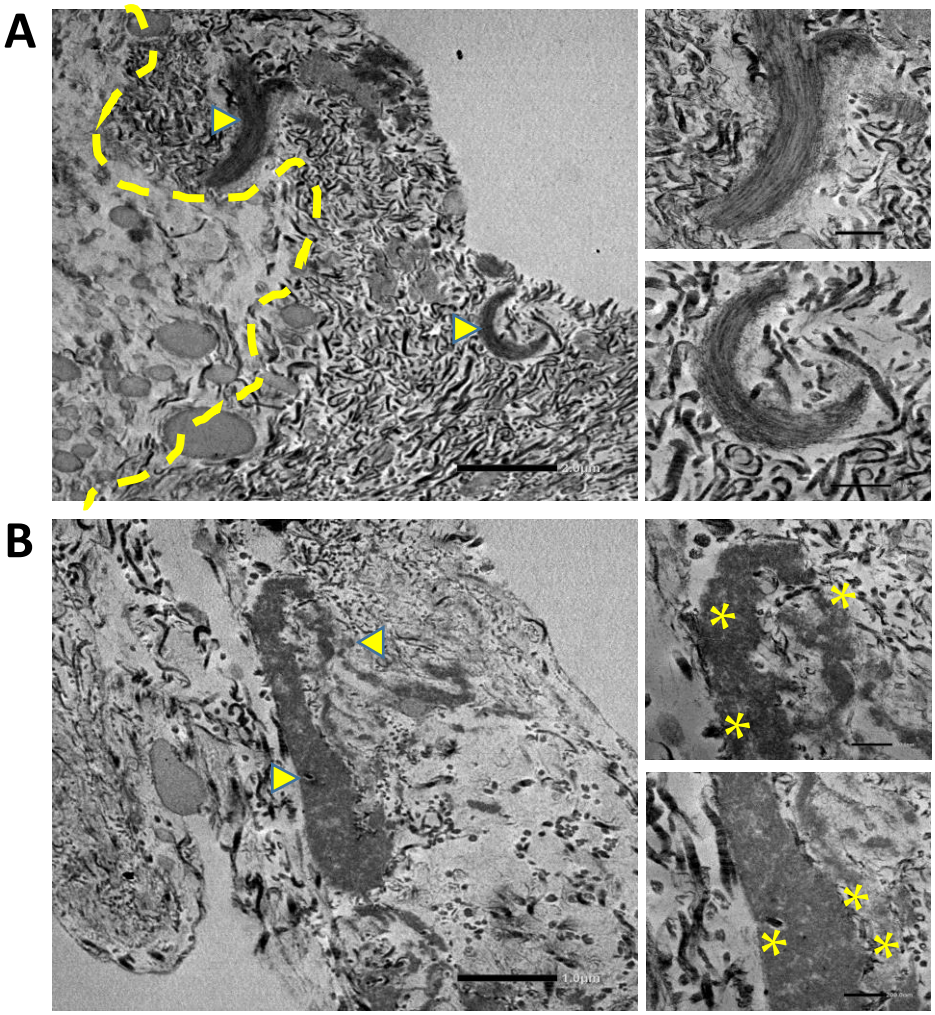
31. irudia. Plastiko eta pDAT-an desberdintzapen osteoblastikoaren presentzian edo gabezian hazitako gDPSC-en Alizarin gorri-DAPI tindaketa bikoitza eta kuantifikazioa. gDPSC-ak hobitxo hutsetan (plastikoa) eta pDAT apar solidoetan hazi ziren kontrol eta desberdintzapen osteoblastiko medioarekin 4 astez Alizarin gorri tindaketa egin baino lehen. Zelulen nukleoak DAPI-z tindatu ziren. Eremu argiko eta immunofluoreszentzia irudiak batu ziren gDPSC-en lokalizazioa aztertzeko mineralizaturiko hezur matrize noduluen inguruan (**A**). Alizarin gorriaren kuantifikazio erlatiboa neurtu zen (**B**). Eskala barra: 50 µm. Estatistikoki esanguratsua (*$p \leq 0.05$) denean.

gDPSC-ek pDAT-an haztean sortutako Sharpey-itxurako kolageno fibra sorta luzeen TEM irudiak

gDPAC-ak pDAT apar solidoetan haztean sorturiko hezur-itxurako ECM ultra-egitura aztertzeko asmoz, TEM irudiak atera ziren. Baldintza kontroletan, area handietan sakabanaturiko kolageno fibra txikiak ikusi ziren, baita ehun adiposo dezelularizazioan erabat ezabatu gabeko lipido tanta sakabanatuak ere. gDPSC-ak pDAT-an desberdintzapen osteoblastikoarekin haztean, trantsizio zorrotzak ikusi ziren kolageno mehe area eta mineralizaturiko kolageno lodi areen artean (32. irudia; marratxo horiak). Kolageno sorta lodi hauek, elektroi dentsitate handiagoa zuten eta pDAT apar solidoari loturik zeuden, zein kolageno meheago eta ez kaltzifikatuez osaturik dagoen. Apar solido barneko kolageno elektrodentsoen areatan, mintz-barneko osifikazio lekuak aurkitu ziren (33. irudia A, B; geziek seinalatua). Mineralizaturiko hezur matrizea ere aurkitu zen area elektro-dentsoetan kolageno fibrei Sharpey-gisako fibrez lotua (33. irudia B; izartxoak). Kolageno elektro-dentso area eta mintz-barneko osifikazio leku hauek ez ziren ikusiak izan zelularik erein gabeko apar solidoetan (datuak ez dira erakusten), gDPSC-ek eragindako egitura hauen *de novo* sorrera frogatuz.



32. irudia. pDAT- hazitako gDPSC-en ultra-egiturara ezaugarriak TEM irudien bidez. gDPSC-ak 4 astez hazi ziren kontrol (ezker zutabea) eta desberdintzapen osteoblastiko medioekin (eskuin zutabea). gDPSC-ek sorturiko kolageno fibra lodiago eta kaltzifikatuen hezur ECM-a eta pDAT apar solido matrizeko kolageno fibra meheen arteko trantsizio area aztertu zen (marratxo horia). Kolageno fibra elektro-dentso kantitate gehiago ikusi zen desberdintzapen osteoblastiko baldintzetan (beheko handipen altuko irudiak). Eskala barra: 1 µm (A, D eta E); 500 nm (B); 200 nm (F eta G).



33. irudia. pDAT apar solidoetan hazitako gDPSC-ek sortutako mintz-barneko osifikazio gune eta Sharpey-gisako fibrak TEM irudien bidez. pDAT apar solidoetan hazitako gDPSC-en kaltzifikaturiko kolageno sorta lodi arearen eta kolageno area ez kaltzifikatuaren arteko trantsizioa (marratxo horia). Mintz-barneko osifikazio guneak aurkitu ziren kolageno lodia zuten egitura elektro-dentsoetan. Gezi puntek seinalaturiko guneen handipenak eskuin panelean (**A**). Sharpey-moduko fibra zulatzaileen presentzia mintz barneko osifikazio guneen ertzetan (eskuin paneleko izartxoak), kolageno matrizeari ainguratzea erakutsiz. Gezi puntek seinalaturiko guneen handipenak eskuin panelean (**B**). Eskala barra: 2 µm (goiko ezkerreko irudia); 1 µm (beheko ezkerreko irudia); 500 nm (goiko eskuineko irudiak); 200 nm (beheko eskuineko irudiak).

Eztabaida

Ikerketa honen helburuetako bat, *in* vitro hezur birsorkuntzarako gDPSC-ak plasma autologo osagaiekin eta hortz inplanteen titanio biomimetikoekin konbinatzearen ebaluaketa egitea zen. Esparru honetan, ikerketa hau gDPSC-ak Ti6AL4V estandarrean eta BAS™ (Avinent implant system) titanio biomimetiko azaleretan, plasmatik eratorritako PRGF eta PRF-rekin hazteak eragin zitzakeen desberdintzapen osteoblastiko prozesu eta jariaturiko hezur matrize deposizioen handipena aztertzera bideratuta zegoen. Gronthos *et al.*-ek gDPSC-ak lehen aldiz isolatu ostean (Gronthos et al., 2000), laborategi ezberdin askok baieztatu dute gandor neuraletik eratorritako zelula ama hauen hozi geruza ezberdinetako zelula leinu ezberdinetara desberdintzeko gaitasuna (Nuti et al., 2016). Desberdintzapen gaitasun honen barruan sartuko litzateke hezur sortzaile diren zelula osteoblastikoetara desberdintzeko abilezia, hauek izanik inplantologia alorrerako zelula interesgarrienak (Giuliani et al., 2013; Tatullo, 2017).

Zelula ama mesenkimalek, hezur matrize jariatzaile diren osteoblasto eta osteozitoetara desberdintzeko prozesua gutxienez hiru pausoz osatua dago. Prozesu honetako pauso bakoitza gene markatzaile adierazpenez karakterizatua dago. Lehen pausoan, zelula ama mesenkimalek zelula osteo-kondroprogenitoreetara desberdintzeko konpromezua hartzen dute, RUNX2 transkripzio faktorearen adierazpena handituz. Gene honen adierazpenak, zelula ama mesenkimalak hezur leinuko zelula orteoprogenitoreetara desberdintzera bultzatzen ditu. Bigarren pausoan, MSC-ak osteoblasto jariatzaileetan eraldatzen dira eta BGLAP (osteokaltzina) eta SPARC (osteonektina) bezalako kaltzio lotzaile proteinen produkzioa eta jariaketa hasten dute. Bi proteina hauek Kolageno extra-zelularrarekin elkartzean, hezur matrize extra-zelularreko zati organikoa (osteoidea) sortzen dute. Honen ostean, hidroxiapatita minerala jariatzen hasten dira hezur matrize heldugabean, hau mineralizatuz. Kristalen nukleazioz ematen den mineralizazio prozesua, fosfatasa alkalinoari esker gertatzen den Ca2+ eta PO4- ioien katalisi erreakzioarengatik egiten da. Momentu honetan, MSC-ek, *OSTERIX* transkripzio faktorea bezalako erabat desberdindutako osteoblasto markatzaileak adierazten dituzte. Transkripzio faktore honen adierazpena osteozito helduetan mantentzen da, kaltzifikaturiko hezur matrize helduak osteoblastoak inguratu ostean. Osteozito heldu hauek, lehen pausoetako *SPARC* eta *Kolageno I*-en adierazpena,

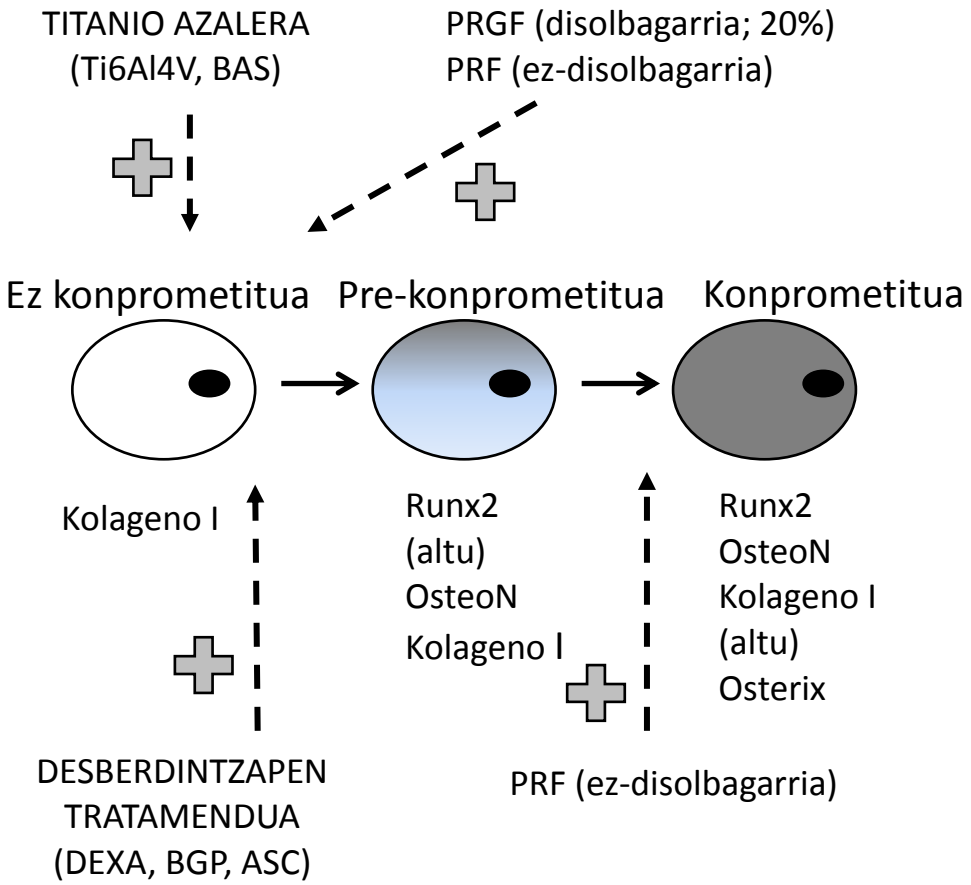
maila baxuagoan, mantentzen dute, denboran zeharreko hezur matrizearen birmoldaketarako (34. irudia).

Azken hamarkadan, erabilera klinikorako titanio materialen bio-bateragarritasunak hobekuntza garrantzitsuak jasan ditu, titaniozko hortz inplanteen konposizio eta azaleran eginiko aldaketei esker. Aldaketa hauek, titanio inplantearen inguruko MSC-en desberdintzapena eta hezur sorrera bultzatzeko helburuarekin egin ziren. Aldez aurreko ikerketek erakutsi zuten zelula ama mesenkimalen itsaste, ugaltze eta osteoblasto desberdintzapenerako gaitasuna titanio azaleren gainean haztean, desberdintzapen osteoblastiko medioarekin hazi gabe ere (Olivares-Navarrete et al., 2010). Ikerketa honen emaitzek lehen eginiko ikerketekin bat egiten dute, zelula ama mesenkimalek, gure kasuan giza emaile osasuntsuetatik eratorritako gDPSC-ak, ereindako azaleraren arabera beraien jokabidea aldatu zitekeela erakutsiz. gDPSC-ak titanio azalerara itsasteak nahikoa izan zen zelula hauek bere kabuz desberdintzapen osteoblastikoa gauzatzeko, Alizarin gorri bidez tindatu zitezkeen hezur matrize mineralizatuak sortuz. Emaitza hauek, gDPSC-ak plastiko estandarrean haztean gertatzen denaren aurkakoak dira, non β-glizerolfosfato, azido askorbiko eta dexametasonaren beharra duten desberdintzapen osteoblastikoa gauzatzeko (Langenbach and Handschel, 2013). Titanio azaleren abantaila nagusiena, zelula ama mesenkimalen desberdintzapen osteoblastiko indukzio gaitasun hau da. Bestalde, titanio azaleren makro eta mikro zimurtasunak zelulen itsaspen, ugaltze eta desberdintzapen osteoblastikoan rol garrantzitsua zuela frogatua zuten (Boyan et al., 2016b; Coelho et al., 2009). gDPSC-ek, desberdintzapen osteoblastikoa, hezur proteina morfogenetiko, hezur proteina eta baskulatura endotelialaren hazkuntza faktoreen produkzio sendoa erakutsi zuten titanio azalera porotsuan haztean (Perrotti et al., 2013). Duela gutxiko ikerketek iradoki dutenez, osteo-indukzioa areagotuta dago kimikoki aldaturiko mikro zimurtasun dun titanio azaleretan, hortz klinika aplikazioetarako abantaila garrantzitsua erakutsiz (Boyan et al., 2016b; DE Colli et al., 2018). Ikerketa honetan, *OSTERIX*-en adierazpen mailan aldaketa txiki baina esanguratsua aurkitu zen medio kontrolarekin Ti6AL4V (lehun) eta BAS (gogor) titanio azaleretan ereindako gDPSC-en konparaketan. Hala ere, gDPSC-en desberdintzapen osteoblastikoa asko areagoturik zegoen BAS titanio azaleraren gainean plaketetan

aberatsa de fibrinarekin (PRF) hazitako baldintzetan, desberdintzapen ostreoblastiko medioaren presentzian edo gabezian hazita ere. Baldintza honetan, *OSTERIX*-en mRNA adierazpen maila asko areagoturik aurkitu zen.

gDPSC-en *in vitro* hedapen eta mantentzea ere ikertua izan zen Ti6AL4V (lehun) eta BAS (gogor) titanio azaleretan. Bi azalerek, zelulen bideragarritasuna mantendu zuten, zelulen ugaltze ona baimenduz. Hau, Ki67-rako positibo ziren zelulak detektatuz egiaztatu zen baldintza basaletan. Ti6AL4V titanio azaleran hazitako gDPSC-ek mobilitate handiagoa erakutsi zuten BAS titanio azaleraren gainean hazitakoekin alderatuta, azalera lauaren ondorioz. Nahiz eta plasmatik eratorritako bi produktuek gDPSC-en ugalketa ona baimendu zuten, PRF-ak ez bezala, % 20-ko PRGF-z osaturiko medioak esanguratsuki handitu zuen zelulen hazkuntza. Emaitzen ezberdintasun hau, bi produktu hauen erabilera metodo ezberdinengatik eragina izan zitekeen. PRF mintzak plaken hobitxoetan mantendu ziren esperimentuak iraun zuen denbora osoan zehar, molekulak pixkanaka askatuz. Honen aurka, disolbagarria den PRGF-a, medio aldaketa bakoitzarekin % 20-ko kontzentrazioan berritu zen 2-3 egunero. Posiblea da, hazkunde medioari PRGF-a gehitzean eragindako hazkuntza faktoreen kontzentrazio handiak gDPSC-en ugalketa areagotu zuela esatea, eta aldi berean honek, osteoblasto helduetara desberdintzeko prozesuan eragin negatiboa zuela esatea. Bi prozesu hauek (zelula ugalketa *vs* desberdintzapena) prozesu antagonikoak direlako. PRGF-arekin hazitako gDPSC-ek, *RUNX2* eta *SPARC* bezalako osteoblasto konpromezu markatzaileen adierazpen mailak handituak zituzten. Baina nolabait, GDPSC-ek huts egin zuten osteoblastu zelula helduetara desberdintzean, *OSTERIX* transkripzio faktorearen adierazpen maila baxuak eta Alizarina gorriz tindatutako jariaturiko hezur matrize mineralizatuaren detekzioak erakutsi zuen bezala. Bestalde, PRF-z osaturiko medioarekin hazitako gDPSC-ak, fibrina mintz honek pixkanaka askaturiko hazkuntza faktore hauei esker, osteoblasto helduen desberdintzapena areagotu zuten. Garrantzitsua da aipatzea, gDPSC-al BAS titanio azaleraren gainean PRF-rekin haztean izan zela *in vitro* desberdintzapen osteoblastikorako baldintzarik eraginkorrena. Hau izan zen BAS titanio gogor azalerak Ti6AL4V titanioarekin alderatuta aukera hobea zela erakutsi zuen unea.

Metodologia hau hortz klinikara itzuli ahal izateko, *in vivo* ikerketa gehiago beharrezkoak dira. Nahiz eta ez izan giza gorputz helduan zelula ama mesenkimal iturri ugariena, inplantologia eta ebakuntza kraniomaxilofazial arloetarako, gDPSC-ak oso zelula interesgarriak dira. Istripuren batean hortzak galdu dituzten pazienteen kasuan, hau bereziki interesgarria da. Galdutako hortz piezatik lorturiko gDPSC autologoen erabilerak onura handiak ekar ditzake kalteturiko hezur gunearen birsorpena laguntzean. Kasua edozein izanik ere, BAS bezalako mikro-porodun titanio azalera biomimetikoan haztean, gDPSC-ek eraginkortasunez erantzuten dute desberdintzapen osteoblastiko protokoloei, bereziki plaketetan aberatsa den fibrina mintzarekin konbinatzean.



34. irudia. gDPSC-en desberdintzapen osteoblastiko prozesuan plasmatik eratorritako produktuen eraginen laburpena. Desberdintzapen erreaktibo farmakologikoek, hazkuntza substratuek (Ti6AL4V eta BAS) eta plasmatik eratorritako produktuekin (PRGF eta PRF) hazteak desberdintzapen osteoblastiko prozesuan duten eraginen laburpen eredu teorikoa. Zelula ama mesenkimalak egoera ez-desberdinduan hazten dira *in vitro*. Osteoblasto desberdintzapen konpromezua hartzera induzitu daitezke, non lehen

pausoan zelulek *RUNX2* transkripzio faktorearen adierazpena areagotzen duten, eta poliki-poliki geroz eta Kolageno I eta *SPARC* gehiago. Desberdintzapen osteoblastikoaren azken etapan, zelulek matrize extra-zelularra mineralizatzeko gaitasuna lortzen dute, hezur ehun heldua sortuz. Zelula/molekula mailan, *OSTERIX* transkripzio faktorearen adierazpena du ezaugarritzat, osteoblasto/osteozito helduen gene markatzailea. Erreaktibo farmakologiko, plasmatik eratorritako PRGF eta PRF, eta titanio azalerek gDPSC-en osteoblasto desberdintzapen konpromezuan eragina dutela ikusi da. Hau, *RUNX2* eta *SPARC* eta fosfatasa alkalinoaren adierazpenaren detekzioz egiaztatu da baldintza guzti hauetan. Hala ere, PRGF disolbagarriarekin hazitako gDPSC-ekez zuten osteoblasto heldu etapara eltzea lortu, seguraski ugaltze/desberdintze prozesu antagonikoen eraginez. Honen aurka, PRF mintzekin hazitako gDPSC-ek desberdintzapen osteoblastiko markatzaileen adierazpen handienak izan zituzten, bereziki BAS titanio azalera biomimetikoa eta desberdintzapen osteoblastiko medioarekin konbinatzean.

Azalduriko ikerketa honek, zelulen bideragarritasun, ugalketa eta desberdintzapen osteoblastiko gaitasun onak erakutsi zituzten titanio azaleren gainean haztean. Zelula ama mesenkimalek, hezur ehun ingeniaritzan erabiltzeko aukera onena zirela erakutsi zuten beraien itsaspen, ugaltze eta zelula ezberdinetara desberdintzeko gaitasunari esker biomaterial ezberdinen gainean haztean. Dena dela, giza gorputz helduak aurkituriko zelula ama mesenkimal iturri ezberdinengatik, ehun ingeniaritzan erabiltzeko zelularik onena zein den oraindik ez dago garbi. Hezur eta hortz birsorpen terapia eraginkorrak garatzeko, giza gorputz helduko zelula amak alderatu beharra daude aukerarik onena zein den jakiteko. Galdera honi erantzuna eman nahian, konparaketa ikerketa egin genuen gDPSC eta gBMSC-en artean, MSC mota esperantzagarri bat zeinak hezur desberdintzapen gaitasun naturala duen.

Konparaketa ikerketa honen helburua, gDPSC eta gBMSC-en zelulen arteko bideragarritasun, ugalketa eta desberdintzapen osteoblastikoa aztertzea izan zen Ti6AL4V eta BAS^{TM} gainean haztean desberdintzapen osteoblastiko medioaren presentzian edo gabezian. Emaitzen ebaluaketarako garrantzitsua da kontuan hartzea gDPSC eta gBMSC-en emaileen adina, non gDPSC emaileen adina 16-30 urte bitartean zegoen eta gBMSC emaileen adina 45 urtetan.

Ti6AL4V eta BAS titanio azaleren gainean hazitako gDPSC eta gBMSC-ek ez zuten zito-toxizitaterik erakutsi. Bi zelula mota hauek, ia % 100-eko bideragarritasuna adierazi zuten titanio azaleren gainean haztean, non isolaturiko zelularen bat bakarrik aurkitu

zen ioduro propidioarekin tindatua. Hala eta guztiz ere, zelula ugalketa esperimentuak gDPSC eta gBMSC-en arteko ezberdintasunak erakutsi zituen. 24 eta 48 orduz titanio gainean hazi ostean gDPSC-ek, gBMC-ek baino ugalketa maila handiagoa erakutsi zuten. Kristal gainean haztean, aldaketa hau 24 orduren ostean bakarrik ikusi zen, bi zelulen artean 48 orduren ondoren ugalketa maila antzekoa ikusiz. Emaitza hauek lehenago eginiko artikuluekin bat egiten dute, non emaitza berberak izan zituzten zelulak bio-material ezberdinen gainean haztean (Amid et al., 2021; Ponnaiyan and Jegadeesan, 2014).

gDPSC eta gBMSC-ek jariaturiko mineral gordailuen sorrera ere ezberdina izan zen kristal, Ti6AL4V eta BAS-ean. Alizarin gorri tindaketaren kuantifikazioak erakutsi zuenez, gDPSC-ek hezur mineral produkzio handiagoa eduki zuten gBMSC-ekin alderatuz- gainera, Ti6AL4V eta BAS titanio azaleren gainean desberdintzapen osteoblastikoarekin hazitako gDPSC-ek erakutsi zuten baldintza guztien artean hezur mineral sorrera handiena. Erlazionaturiko ikerketetan ikusi zuten bezala, gDPSC-ek gBMSC-ek baino mineralizazio handiagoa eduki zuten bi titanio azaleretan haztean (Davies et al., 2015; Mohanram et al., 2020).

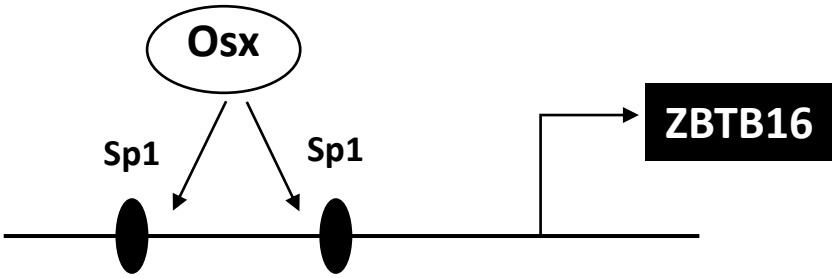
Honez gain, RNA sekuentziazioa, geneen adierazpen/ adierazpen eza kriterioa jarraituz analizatu zen. Helburu hau lortzeko, binakako analisi konparatiboak gauzatu ziren baldintza guztien artean, baldintza batean adierazita eta bestean adierazi gabe zeuden geneak begiratuz. Presentzia/ gabezia (ON/OFF) konparaketa aukeratu genuen gene set espezifikoak detektatzeko. Hauen adierazpena aktibatua egongo litzateke desberdintzapen osteogeniko baldintzetan, eta honela gDPSC eta gBMSC-en desberdintzapen osteoblastikoan inplikatuta dauden gene berri eta seinalizazio bideak identifikatu ahal izango genituen.

gDPSC eta gBMSC-ak plastikoan medio kontrolarekin haztean erakutsi zuten gene adierazpen ezberdintasun handiena gBMSC-ek adierazitako HOX gene familia izan zen. HOX geneak, zelula ama mesenkimalen ugalketaren arduradun dira, baita eskualdeetako zehaztapenak eta erlazionaturiko ehunen espezifikotasunarenak ere (Ackema and Charité, 2008). Aurreko ikerketek HOX-en adierazpen patroi

ezberdintasunak frogatu zituzten gandor neuraleko zeluletan eta barailezur helduan, aldi berean, mesodermotik eratorritako hezurrek eta mesenkimak HOX-en adierazpen positiboa zuten (Dong et al., 2014; Lee et al., 2015b; Wehrhan et al., 2011). Gene familia hau, MSC-en leku-espezifikotasunarekin erlazionaturik egon daitekeela iradoki da, kokaleku anatomikoaren arabera (Wang et al., 2009). HOX geneen adierazpen ezberdintasunak, jatorri enbrionario ezberdina duten zelula injerto autologoetan ikusi den zelulen jokabide ezberdintasunak azal ditzake (Leucht et al., 2008). Ikerketa honetan lorturiko emaitzak bateragarriak dira lehen eginiko ikerketekin, zeinak barailezurrean HOX geneen adierazpen jaitsiera ikusi zuten hezur luzeekin alderatuz (Lee et al., 2015b; Leucht et al., 2008).

Desberdintzapen osteoblastikoarekin erlazionaturiko genetan arreta berezia jarriz, desberdintzapen osteoblastikoaren markatzaile den OSTERIX/*SP7*-aren adierazpena gDPSC-etan induzituta zegoela ikusi zen titanio azaleretan desberdintzapen osteoblastiko medioaren presentzian edo gabezian hazi ondoren. gBMSC-etan adierazpen ezberdintasun hau ezin izan zen aurkitu, zelula hauek duten adierazpen basala dela eta. Emaitza hauek lehen eginiko ikerketen emaitzekin bateragarriak dira, non OSTERIX/*SP7*-ren adierazpena, bai titanio azalerengatik, bai desberdintzapen medioarengatik areagotuta zegoela ikusi zen. Honez gain, *SPARC*-ekin erlazionaturiko bi genek, *SPOCK2* eta *SPARCL1*, adierazpena isilarazita zutela erakutsi zuten gBMSC-etan baldintza kontroletan eta adierazpen positiboa zelula hauek Ti6AL4V titanio azaleran desberdintzapen osteoblastiko medioarekin haztean. Azkenik, aipatu beharreko azken desberdintzapen osteogenikoarekin erlazionaturiko genea *ZBTB16* da. *ZBTB16*, proteina-proteina elkarreragin domeinua eta DNA-ri lotzeko bederatzi Kruppel-antzeko zink hatzak dituen zink hatz-dun transkripzio faktorea da. *OSTERIX/SP7*, *ZBTB16* genearen promotoreari lotzen zaio honen transkripzioa hasteko (35. irudia). Ikerketa honetan, medio kontrolarekin haztean gDPSC eta gBMSC-ek gene hau ez zutela adierazten ikusi genuen, baina bi zelula hauek desberdintzapen osteoblastiko medioarekin haztean *ZBTB16*-ren adi erazpena nabarmenki handitu zuten. Lehen eginiko ikerketa batzuen emaitzetan gauza berbera ikusi zuten, non *ZBTB16* genea dexametasonaren eraginez adierazten zela frogatu zuten. Honengatik, *ZBTB16* zelulak desberdintzapen osteoblastiko medioarekin haztean bakarrik adierazi zen (Felthaus et

al., 2014a; Felthaus et al., 2014b; Onizuka et al., 2016). Orokorrean, gDPSC eta gBMSC-ak kultibo baldintza ezberdinetan haztean egin den RNA sekuentziazio honek *ZBTB16*-ek osteogenesiaren erregulazioan rol garrantzitsua izan dezakeela iradoki dute.



35. irudia. Osterix, ZBTB16-ren promotoreko Sp1 sekuentziari lotzen zaio.

Ezberdin adierazitako beste gene interesgarrien artean , NTRK3 eta GFRA2 geneen adierazpena zuten kontrol medioarekin plastiko gainean ereindako gDPSC-ek, eta adierazpen hau desagertu egin zen desberdintzapen medioaren eta/edo titanioen eraginez. Neurotrofinen errezeptore geneak, eta partikularki *NTRK3*, gDPSC-en "amatasun" markatzaile izan zitezkeela iradoki zenez, emaitza hauek ere lehenago eginiko ikerketekin bat egingo lukete (Luzuriaga et al., 2019b).

Azkenik, garrantzitsua da aipatzea geneen adierazpen ezberdintasun eza Ti6AL4V eta BAS titanio azaleren gainean hazitako gDPSC-etan. Adierazpen ezberdina zuten geneak gutxi ziren eta gainera patroi inkoherentea jarraitzen zuten. Emaitza honek, bi titanio azalera hauek duten gene adierazpen indukzio berbera frogatuko lukete. Horregatik ondorioztatu dugu, titanio azaleren gainean hazitako zelulen arteko gene adierazpen patroi ezberdintasunak edozein direla ere, gene adierazpen maila aldaketa arinak bilatu beharko ditugu, osteogenesiarekin erlazionaturiko gene maisuak bilatu beharrean.

Gene interesgarrien adierazpen ezberdintasunak analizatu ostean, seinalizazio bideen aberastasun analisira jo genuen, hazkuntza baldintza ezberdinetan hazitako gDPSC eta gBMSC-etan afektaturiko gene kluster eta seinalizazio bideak identifikatzeko. Honetarako, konektibitate mapak (CMAP) eta prozesu biologikoen gene ontologiaren (GOBP) bidez aztertu ziren konparaketa esperimental bakoitzean afektaturiko

seinalizazio bideak identifikatzeko. Afektaturiko seinalizazio bideak aukeratzeko, gutxienez ezberdin adierazitako bi gene eta esanguratsua $p< 0.05$ kriterioa jarraitu genuen

Datu base hauek erakutsi zuten ze prozesu biologiko eta gaixotasunekin erlazionaturik zeuden aktibaturiko gene taldeak. CMAP-ek eman zizkigun datuen arabera, gDPSC eta gBMSC-en artean ezberdintasunik handiena HOX geneetan zegoen, gene hauek leuzemia mieloide akutu eta desorden hematopoietikoa bezalako prozesu ezberdinekin erlazionaturik daude. Bestalde, GOBP-ak gene ezberdinen adierazpena, aurreko/ atzeko patroi espezifikotasuna, odol hodien garapena, sistema kardiobaskularraren garapena, odol hodien tamainaren erregulazioa, hormonen garraioa eta zelula ama hematopoietikoen desberdintzapen prozesuekin erlazionatu zituen. Aurreko/ atzeko patroi espezifikotasunarekin erlazionaturik dauden seinalizazio bideen ezberdintasun esanguratsuak eta ama zelula hematopoietikoen desberdintzapena garbi erlazionaturik egon daiteke gDPSC eta gBMSC-en jatorri enbrionario eta funtzio fisiologikoari.

Aurreko ikerketetan ikusi bezala, nahiz eta desberdintzapen osteoblastikoarekin erlazionaturiko geneen adierazpen handiagoa izan gBMSC-ek, hezur mineralaren sorrera beti handiagoa da gDPSC-etan (Davies et al., 2015; Mohanram et al., 2020). Emaitza hauek berretsita daude gure ikerketan lorturiko emaitzengatik Alizarin gorri bidezko hezur mineralaren detekzioz. Are gehiago, zelulen ugalketa maila ere handiagoa da gDPSC-etan gBMSC-etan baino. Bi zelula mota hauek isolatzeko metodologia ezberdina dela aipatzea ere garrantzitsua da. gDPSC-ak lortzeko prozedura errazagoa da eta ez ain erasokorra gBMSC-ak lortzeko metodologiarekin alderatuz. Informazio guzti honekin, hezur eta hortz ehun ingeniaritzan erabiltzeko, gDPSC-ak gBMSC-ak baino zelula ama mesenkimal iturri hobea direla iradoki dugu (Amid et al., 2021; Mohanram et al., 2020).

Lehen aipatu bezala, hortz inplanteak asko hobetu dira azken urteetan inplanteen biziraupen maila areagotuz. Biziraupen hau % 95 inguruan egongo litzateke inplantea jarri eta 10 urte igaro ondoren (Moraschini et al., 2015) eta % 88-an 20 urte

igaro ondoren (Chrcanovic et al., 2018). Hala ere, bizi-itxaropenaren handipenarekin eta herrialde garatuen biztanleriaren zahartzearengatik, denbora luzeko iraupena duten hortz inplanteen eskaria mantendu egingo dela ematen du etorkizunean.

Azken hamarkadetan zehar, hortz inplanteen osteo-integrazioak hobekuntza emaitza handiak eduki ditu, hala ere, oraindik erronka garrantzitsuak mantentzen dira. Aho inplantologiaren arazo handienetako bat, Hezur Marjinalaren Galera (MLS) da, zein inplanteen galerarekin zuzenki erlazionaturik dagoen, murtxikatze funtzioak PDL-aren gabezian hezur albeolarrari jasanarazten dion estres mekanikoarekin erlazionaturik. Murtxikatze zama guztia hezur ehun albeolarrari transferitzen zaio, hortz inplanteak bertan zuzenki ainguratuta daudelako, eta honek hezurraren birxurgapena eragin dezake. Ikerketa asko daude MLB altua periinplantitisa jasan eta inplantearen galerarekin erlazionatzen dutenak (Chrcanovic et al., 2018; Coli and Jemt, 2021; Galindo-Moreno et al., 2015). Periinplantitisa eta MLB-a murrizteko estrategiarik eraginkorrena PDL-aren berreraikuntza da, zeinek kuxin natural funtzioa betetzen duen hezur albeolar eta hortzaren artean, indar mekanikoak xurgatuz. Hala ere, PDL-aren birsorkuntza eta ingeniaritza aparteko erronka izango litzateke (Lee et al., 2020). PDL-a ordezkatzeko edozein material gogorki lotu beharko litzateke bai hezur albeolarrera bai hortz inplantera, eta aldi berean, baskularizazio handiko banda mantendu beharko luke hauen artean. Praktikan, arazo hau, bio-materialetan zelula ama osteogeniko eta baskulogenikoak ereinez bakarrik konponduko litzateke.

Zelula amen terapia bidez birsortutako PDL-aren testuinguruan, paziente berberetik eratorritako zelula amak (txertaketa autologoa) bio-materialean ereitea izango litzateke agertokirik onena, honela manipulazio egokiaren ondoren, errefuxa immunologikoa ekidinez. Aurreko ikerketetan esan bezala, hortz inplantologian erabili ahal izateko zelula amarik interesgarrienetako bat gDPSC-ak dira. Zelula haue ondo egokituta daude kriokontserbazio eta transplante autologoetarako (Ibarretxe et al., 2012b; Raik et al., 2020). gDPSC-en beste ezaugarri garrantzitsu bat, zelula mesenkimal eta zelula ez mesenkimaletara desberdintzeko dute gaitasuna da animali iturriko seruma bezalako konposatu xenogenikorik erabili beharrik gabe. Dagoeneko aztertua izan da gDPSC-ek serumik gabeko medioekin haztean duten desberdintzapen gaitasuna, zelula

baskulogeniko eta osteogenikoak lortuz. Gure taldeak orain dela gutxi argitaratu eta patentatu zuen zelula baskulogenikoak lortzeko (endotelia eta perizitoak) animali serumik gabeko metodologia (Luzuriaga et al., 2020; Pineda Martí et al., 2020). Metodo hau bio-materialetan ereindako gDPSC-ei aplika daiteke pDAT-a bezalako scaffold-ak baskularizatzeko eta honela zelula terapietan erabili ahal izateko.

Ikerketa honetan, patentaturiko dezelularizazio protokoloa (Madarieta Pardo et al., 2017) jarraiturik lortutako pDAT apar solidoak gDPSC-ekin konbinatu genituen. Ehun adiposoa, aktibitate biologikoa duten mintz itsaspen proteina iturri eskuragarria da (Yang et al., 2020). Hezur birsorkuntzaren testuinguruan, zelula osteoblastikoak eta mineralizaturiko hezur matrize ehuna lortzeko, pDAT-an ereindako gDPSC-en desberdintzapena frogatzea oso garrantzitsua zen. Ikerketa honetan, gDPSC-ak pDAT-an haztean duten desberdintzapen osteoblastiko gaitasuna aztertu zen. PDL-aren ingeniaritza testuinguruan, konpondu beharreko arazo garrantzitsu bat bat, pDAT apar solidoen hazkuntza sistemaren optimizazioa izango litzateke, material hau aldez aurretik zelula baskular eta osteoblastoetara desberdindutako gDPSC-ekin konbinatuz, zeinak galduriko PDL-aren egitura eta funtzioa imitatuko lukeen.

Ikerketa honetan lorturiko emaitzek, PDL eta hortz hezur birsorkuntzarako pDAT apar solidoak bio-material garrantzitsua izan daitekeela frogatu du. pDAT-aren formulazio honek, gDPSC-ek jariaturiko hezur matrizea esanguratsuki handitu zuen, fosfatasa alkalino eta Alizarin gorriaren ebaluaketak erakusti zuen bezala. Material honetan ereindako gDPSC-ek ez zuten bideragarritasun galerarik erakutsi baldintza esperimentaletan. gDPSC-ak dentsitate baxuetan erein ziren (15.000 zelula/hobitxo; 21.428 zelula/ zm^2) eta baita pDAT scaffoldaren bolumen baxuak (120 µl/ hobitxo) denbora luzeko ikerketetarako. Zelula dentsitate handiagoen erabilerak seguraski, baskulatura bidezko mantenugai eta oxigeno horniketa beharko luke (Nakamura et al., 2019). *In vivo* esperimentu gehiago beharrezkoak izango dira material honen potentziala ikertzeko hezur ehun eta PDL lesioen sendakuntzan.

Azkenik, pDAT-an ereindako gDPSC-ek kaltzifikaturiko kolageno fibra sorta lodiak eta mintz barneko osifikazio lekuak sortu zituzten. Kaltzifikaturiko gune hauek pDAT-ri

itsatsirik daude Sharpey fibren bidez. Emaitza guzti hauek erakutsi dutenez gDPSC-ak, edo beste ama zelula mesenkimalen bat, pDAT-rekin konbinatzean desberdintzapen osteoblastikoa induzitzeaz gain, injertoa inguruko ehun gogorrari itsasten laguntzen du. Hezur sendaketaren kasuan itsaspen honek interes berezia du, non scaffold-a eta hezur ehunaren arteko itsaspen fisikoa beharrezkoa den. *In vivo* transplantazio esperimentuak beharko diraaurkiketa hauek baieztatzeko, non zelula amen garraiobide izateaz aparte, scaffold-ak ECM bio-induktore gisa jokatuko luke, hezur ehun gogorrarekin itsaspen kaltzifikatua areagotuz eta baita hezur ehun ostalariko ama zelula mesenkimalen errekrutatze eta aktibazioa eraginez.

Ondorioak

Azken hamarkadetan, zelula ama mesenkimal ezberdinek hezur ehun ingeniaritzan erabiliak izateko potentziala frogatu dute. Beraien ugalketa ratio handia eta multi-leinu desberdintzapen gaitasunengatik, zelula hauek hautagai aproposak dira birsortze terapietarako. Gainera, inplante eta scaffold-en fabrikazio hobekuntzek, porositate, laztasun eta iraunkortasunak hobetu dituzte. Aldaketa hauek, zelula amen ugalketa eta desberdintzapenerako bio-bateragarri-tasunaren hobekuntzetarako bideratuta daude. Azkenik, plasmatik eratorritako hazkuntza faktoreak irtenbide ezin hobea dela erakutsi du terapia autologoetan erabiliak izateko zelula amen *in vitro* hazkuntza eta desberdintzapenerako. Honela, erantzun immune ez desiragarriak deuseztatuz animali jatorriko serumak ekidinez.

Jarraian datozen ondorioak atera dira lan honen emaitzetatik:

1. gDPSC-en bideragarritasuna eta ugalketa ez dira kaltetu Ti6AL4V eta BAS titanio azaleretan haztean.
2. Bi titanio azalerek eragin osteo-induktiboa erakutsi dute gDPSC-engan desberdintzapen osteoblastiko medioaren beharrik gabe.
3. Plasmatik eratorritako PRGF-ak gDPSC-en *in vitro* ugalketa areagotu du, aldi berean PRF-ak desberdintzapen osteoblastikoa eta kaltzifikaturiko hezur matrize produkzioa maximizatu du.
4. BAS titanio azalera plasmatik eratorritako PRF-arekin konbinaturik, gDPSC-en zelula hezur-sortzaileetarako desberdintzapen osteoblastikoa bultzatu du. Emaitza hauek, klinika praktika ohikoetan zabalduriko plasmatik eratorritako fibrina koaguluaren erabilerari laguntza esperimentala eman diote, mikro-porodun titanio inplante azaleren aldameneko hezur produkzioa bultzatzeko.
5. Ti6AL4V eta BAS titanio azaleretan hazitako gDPSC eta gBMSC-en ikerketa konparatiboak, gDPSC-ek ugalketa eta mineralizazio handiagoa dutela erakutsi dute gBMSC-ekin alderatuta, baina esanahi estatistikoaren faltaren ondorioz, datu esperimental gehiagoren beharra erakutsi dute ondorio sendoak ateratzeko. Isolamendu errazago eta inbasibotasun txikiagoa, ugaltze ratio altu

eta hezur mineralen deposizioek erakutsi dutenez, gDPSC-ak gBMSC-ak baino aukera hobeagoa direla erakutsi dute hezur birsortze terapietarako.

6. Dezelularizatutako txerri ehun adiposoa-k (pDAT) gDPSC-en itsaspen eta bateragarri-tasunean lagundu du.
7. pDAT-k gDPSC-en desberdintzapen osteoblastikoa lagundu du kaltzifikaturiko hezur matrize produkzioarekin, mintz barneko osifikazioa eta Sharpey fibra-antzeko atxikimendu egituren formakuntzaren bidez.
8. Ehun adiposo material gordina material iturri erlatiboki ugaria eta eskuragarria dela kontuan izanik, eta DAT zein gDPSC-ak giza emaileetatik isolatuak izan daitezkeenez, aukera paregabea ematen du terapia kliniko pertsonalizatuetan konbinaturik erabili ahal izateko hezur kaltetuen sendaketa eta hortz inplantologiarentzako.

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Dental pulp stem cells as a multifaceted tool for bioengineering and the regeneration of craniomaxillofacial tissues

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Dental pulp stem cells, or DPSC, are neural crest-derived cells with an outstanding capacity to differentiate along multiple cell lineages of interest for cell therapy. In particular, highly efficient osteo/dentinogenic differentiation of DPSC can be achieved using simple *in vitro* protocols, making these cells a very attractive and promising tool for the future treatment of dental and periodontal diseases. Among craniomaxillofacial organs, the tooth and salivary gland are two such cases in which complete regeneration by tissue engineering using DPSC appears to be possible, as research over the last decade has made substantial progress in experimental models of partial or total regeneration of both organs, by cell recombination technology. Moreover, DPSC seem to be a particularly good choice for the regeneration of nerve tissues, including injured or transected cranial nerves. In this context, the oral cavity appears to be an excellent testing ground for new regenerative therapies using DPSC. However, many issues and challenges need yet to be addressed before these cells can be employed in clinical therapy. In this review, we point out some important aspects on the biology of DPSC with regard to their use for the reconstruction of different craniomaxillofacial tissues and organs, with special emphasis on cranial bones, nerves, teeth, and salivary glands. We suggest new ideas and strategies to fully exploit the capacities of DPSC for bioengineering of the aforementioned tissues.

Keywords: DPSC, differentiation, tooth, bone, salivary gland, nerve, cell therapy

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Wnt/β-Catenin Regulates the Activity of Epiprofin/Sp6, SHH, FGF, and BMP to Coordinate the Stages of Odontogenesis

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Background: We used an *in vitro* tooth development model to investigate the effects of overactivation of the Wnt/β-catenin pathway during odontogenesis by bromoindirubin oxime reagent (BIO), a specific inhibitor of GSK-3 activity.

Results: Overactivating the Wnt/β-catenin pathway at tooth initiation upregulated and ectopically expressed the epithelial markers *Sonic Hedgehog (Shh), Epiprofin (Epfn), and Fibroblast growth factor8 (Fgf8)*, which are involved in the delimitation of odontogenic fields in the oral ectoderm.This result indicated an ectopic extension of the odontogenic potential. During tooth morphogenesis, *Fibroblast growth factor4 (Fgf4), Fibroblast growth factor10 (Fgf10), Muscle segment homeobox 1 (Msx-1), Bone Morphogenetic protein 4 (Bmp4), and Dickkopf WNT signaling pathway inhibitor 1 (Dkk1)* were overexpressed in first molars cultured with BIO. Conversely, the expression levels of *Wingless integration site 10b (Wnt-10b)* and *Shh* were reduced. Additionally, the odontoblast differentiation markers Nestin and *Epfn* showed ectopic overexpression in the dental mesenchyme of BIO-treated molars. Moreover, alkaline phosphatase activity increased in the dental mesenchyme, again suggesting aberrant, ectopic mesenchymal cell differentiation. Finally, Bmp4 downregulated *Epfn* expression during dental morphogenesis.

Conclusions: We suggest the presence of a positive feedback loop wherein *Epfn* and β*-catenin* activate each other.The balance of the expression of these two molecules is essential for proper tooth development. We propose a possible link between Wnt, Bmp, and Epfn that would critically determine the correct patterning of dental cusps and the differentiation of odontoblasts and ameloblasts.

Keywords: Wnt/β-catenin, tooth development, GSK-3, BIO-culture, Epiprofin/Sp6, odontogenesis

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n Dental Pulp Stem Cells to Neuralwww.cellphysiolbiochem.com

BDNF and NT3 Reprogram Human Ectomesenchymal Dental Pulp Stem Cells to Neurogenic and Gliogenic Neural Crest Progenitors Cultured in Serum-Free Medium

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Key Words: Serum-free culture media • Calcium imaging • Cell differentiation • Dental Pulp Stem Cells • Brain Derived Neurotrophic Factor

Abstract

Background/Aims: Human Dental Pulp Stem Cells (hDPSCs) are one of the most promising types of cells to regenerate nerve tissues. Standard DMEM+10% fetal bovine serum (FBS) culture medium allows a fast expansion of hDPSC as a surface-adherent cell monolayer. However, the use of FBS also compromises the clinical use of these protocols, and its longterm presence favors hDPSCs differentiation toward mesenchymal cell-derived lineages, at the expense of a reduced capability to generate neural cells. The objective of this work was to characterize the role of neurotrophin signaling on hDPSCs using a serum-free culture protocol, and to assess the neurogenic and gliogenic capacity of hDPSCs for future nerve tissue bioengineering and regeneration. ***Methods:*** We compared the different expression of neurotrophin receptors by RT-PCR, Q-PCR, and IF of hDPSCs cultured with different growth media in the presence or absence of serum. Moreover, we assessed the response of hDPSCs to stimulation of neurotransmitter receptors by live cell calcium imaging under these different media. Finally, we compared the osteogenic potential of hDPSCs by Alizarin red staining, and the differentiation to gliogenic/neurogenic fates by immunostaining for Schwann lineage

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ADHESION, INTEGRATION AND OSTEOGENESIS OF HUMAN DENTAL PULP STEM CELLS ON BIOMIMETIC IMPLANT SURFACES COMBINED WITH PLASMA DERIVED PRODUCTS

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Abstract

Dental implants are the usual therapy of choice in the dental clinic to replace a loss of natural teeth. Over recent decades there has been an important progress in the design and manufacturing of titanium implant surfaces with the goal of improving their osteointegration. In the present work, the aim was to evaluate the usefulness of hDPSCs (human dental pulp stem cells), in combination with autologous plasma components, for *in vitro* bone generation on biomimetic titanium dental implant materials. In this context, the combination of hDPSCs stimulated by PRGF or PRF and cultured on standard Ti6A14V and biomimetic BAS™ (Avinent Implant System) titanium surfaces were studied in order to evaluate possible enhancements in the osteoblastic differentiation process out of human mesenchymal cells, as well as bone matrix secretion on the implant surface. The results obtained in this *in vitro* model of osteogenesis suggested a combination of biomimetic rough titanium surfaces, such as BAS™, with autologous plasma-derived fibrin-clot membranes such as PRF and/or insoluble PRGF formulations, but not with an addition of water-soluble supplements of plasma-derived growth factors, to maximise osteoblastic cell differentiation, bone generation, anchorage and osteointegration of titanium-made dental implants.

Keywords: Dental pulp stem cells, titanium implants, osteoblast differentiation, platelet rich in growth factors, platelet rich fibrin, biomimetic advanced surface.

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Vasculogenesis from Human Dental Pulp Stem Cells Grown in Matrigel with Fully Defined Serum-Free Culture Media

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Abstract: The generation of vasculature is one of the most important challenges in tissue engineering and regeneration. Human dental pulp stem cells (hDPSCs) are some of the most promising stem cell types to induce vasculogenesis and angiogenesis as they not only secrete vascular endothelial growth factor (VEGF) but can also differentiate in vitro into both endotheliocytes and pericytes in serum-free culture media. Moreover, hDPSCs can generate complete blood vessels containing both endothelial and mural layers in vivo, upon transplantation into the adult brain. However, many of the serum free media employed for the growth of hDPSCs contain supplements of an undisclosed composition. This generates uncertainty as to which of its precise components are necessary and which are dispensable for the vascular differentiation of hDPSCs, and also hinders the transfer of basic research findings to clinical cell therapy. In this work, we designed and tested new endothelial differentiation media with a fully defined composition using standard basal culture media supplemented with a mixture of B27, heparin and growth factors, including VEGF-A165 at different concentrations. We also optimized an in vitro Matrigel assay to characterize both the ability of hDPSCs to differentiate to vascular cells and their capacity to generate vascular tubules in 3D cultures. The description of a fully defined serum-free culture medium for the induction of vasculogenesis using human adult stem cells highlights its potential as a relevant innovation for tissue engineering applications. In conclusion, we achieved efficient vasculogenesis starting from hDPSCs using serum-free culture media with a fully defined composition, which is applicable for human cell therapy purposes.

Keywords: stem cells; DPSCs; neovasculogenesis; endothelial cells; Matrigel; vasculature

cells

MDPI

Article

Wnt-3a Induces Epigenetic Remodeling in Human Dental Pulp Stem Cells

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Abstract: Dental pulp stem cells (DPSCs) from adult teeth show the expression of a very complete repertoire of stem pluripotency core factors and a high plasticity for cell reprogramming. Canonical WntandNotchsignalingpathwaysregulatestemnessandtheexpressionofpluripotencycorefactorsin DPSCs, and even very short-term (48 h) activations of the Wnt pathway induce a profound remodeling of DPSCs at the physiologic and metabolic levels. In this work, DPSC cultures were exposed to treatments modulating Notch and Wnt signaling, and also induced to differentiate to osteo/adipocytes. DNA methylation, histone acetylation, histone methylation, and core factor expression levels where assessed by mass spectroscopy, Western blot, and qPCR. A short-term activation of Wnt signaling by WNT-3A induced a genomic DNA demethylation, and increased histone acetylation and histone methylation in DPSCs. The efficiency of cell reprogramming methods relies on the ability to surpass the epigenetic barrier, which determines cell lineage specificity. This study brings important information about the regulation of the epigenetic barrier by Wnt signaling in DPSCs, which could contribute to the development of safer and less aggressive reprogramming methodologies with a view to cell therapy.

Keywords: dental pulp stem cells; chromatin remodeling; cell cycle; pluripotency; DNA methylation; histone acetylation; histone methylation; Notch pathway; Wnt pathway

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Nanostructured scaffolds based on bioresorbable polymers and graphene oxide induce the aligned migration and accelerate the neuronal differentiation of neural stem cells

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Abstract

Withinthe field of neural tissue engineering,there is a huge need for the development of materials that promote the adhesion,aligned migration and differentiation of stem cells into neuronal and supportive glial cells. In this study, we have fabricated bioresorbable elastomeric scaffolds combining an ordered nanopatterned topography together with a surface functionalization with graphene oxide (GO) in mild conditions. These scaffolds allowed the attachment of murine neural stem cells (NSCs) without the need of any further coating of its surface with extracellular matrix adhesion proteins. The NSCs were able to give rise to both immature neurons and supporting glial cells over the nanostructured scaffolds in vitro, promoting their aligned migration in cell clusters following the nanostructured grooves. This system has the potential to reestablish spatially oriented neural precursor cell connectivity, constituting a promising tool for future cellular therapy including nerve tissue regeneration. © 2020 Elsevier Inc. All rights reserved.

Key words: Micro- and nanopatterning; Neural stem cells; Migration; Cell differentiation; Graphene oxide; Biodegradable polymer

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Regeneration of the nervous system still remains very challenging due to its limited plasticity and poor ability to heal ments for this specific biomedical application play a pivotal role. Much progress has been made in determining the ideal features a biomaterial should have for its use as a neural replacement graft, and in understanding the interactions of growing axons within thesebiomaterials;however,theregenerationlevelsinducedbythe biomaterial usually do not match those obtained by nerve tissue autografts and the development of new and effective nerve regeneration therapies is still an urgent clinical need.[2,3]

The biomaterials for nerve tissue regeneration should be biocompatible and biodegradable, while providing structural cues that promote oriented axon regeneration and guidance signals from extracellular matrix (ECM)-like components. Additionally, they shouldalsopresentlong-termstoragecapabilityandeaseofhandling/ suturing.[4–6] One important aspect to take in consideration is that the

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