eman ta zabal zazu

UPV EHU

Department of cell biology and histology

School of medicine and nursing

University of the Basque country

Study of neurogenic functional potential of dental pulp stem cells (DPSCs) in neural regeneration

Jon Luzuriaga Gonzalez

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Thesis directors:

Dr. Gaskon Ibarretxe Bilbao

Dr. Fernando Unda Rodriguez

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Abstract

The dental pulp of adult teeth contains a very active stem cell population with a neural crest (NC) cell phenotype, termed dental pulp stem cells (DPSCs). Human DPSCs (hDPSCs) present substantial advantages compared to other stem cell types that can be found in the adult human body, such as a much higher accessibility than endogenous neural stem cells, big capacity of ex-vivo expansion, basal expression of a wide variety of neural cell markers and subsequent neural differentiation, leaving the door open to neuroregenerative autologous cell therapy. The common proliferative and maintaining culture mediums are composed of fetal bovine serum (FBS) that leads fastest growth of hDPSC as well as surface-adherent cell monolayer generation. However, the use of FBS is not compatible with the clinical employment of these protocols as far as it is the cause of allergies and immune reaction in grafts *in vivo*. Furthermore, several authors have related the presence of serum with the differentiation toward mesenchymal cell lineages, and hence, the reduction of neurogenic capacity of hDPSCs. All these inconveniences have challenged the scientific society to design and improve new innovative serum-free neural inductive protocols.

In the present work, we wanted to characterize the phenotypic properties of adult hDPSCs cultured in serum free mediums. Our results confirmed that non-engineered and non-modified adult DPSCs cultured in different serum-free mediums were able to generate neurosphere-like structures, singular structures generated by neural stem cells (NSCs), called dentospheres. Furthermore, we noticed the basal expression of early neural markers, the remain of mesenchymal stem cell being and the pluripotential character of DPSCs both in serum-free medium and serum containing mediums. However, each specific serum-free culture medium provide different unique features to hDPSCs. Thus, when hDPSCs were grown in Neurocult™ differentiation neurogenic induction media, a cell culture medium without serum routinely used for the differentiation of adult neural stem cells, we showed the neurogenic potential of hDPSCs by the expression of mature neuronal and glial markers. In addition, we also described for the first time non-engineered and non-modified hDPSCs expressing vascular endothelial cell marker CD31, as well as stem mesenchymal and neural early markers in the hDPSCs cultured in Neurocult™ proliferation maintaining media. Surprisingly, 1 month post-intracranial graft into athymic nude mice these CD31+, Nestin+ cells were located within brain blood vessels increasing their laminin expression and co-existing together with murine vascular endothelial cells.

On the other hand, hDPSCs were cultured in a commercial specific mesenchymal stem cell culture medium: StemPro MSC™ (STP). Taking advantage of the molecular and physiological profits provide by this medium, we observed an increase of NC cell and pluripotential markers expression as well as higher neurogenic potential of hDPSCs, that suggest a step back to Neural crest stem cells (NCSCs)- like phenotype.

Our results demonstrate the possibility of employment serum-free mediums to culture hDPSCs. Indeed, not only was avoid the serum associated issues, but it was achieved the activation of neural crest stem/progenitor cell character that facilitate the differentiation toward neural lineages. Moreover, unexpectedly, we are the first group that discovered an efficient way to obtain functional, they showed histo-integration and capacity to generate neovasculature *in vivo,* endothelial cells from hDPSCs in serum-free conditions.

To sum up, in this work are clarified the doubts that serum-free cultures may produce; such as, those related with cell viability and stem being switch, the neurogenic potential of hDPSCs and the integration of these cells in live systems have been widely studied. We conclude that the neurogenic potential and newly discovered endothelial potential among the other characteristics aforementioned convert hDPSCs in a real option to use as a stem cell source for neuroregenerative therapies.

Keywords: Dental Pulp Stem Cells, DPSCs, Dentosphere, Culture media, Serum-free, Calcium imaging, Cell differentiation, Neurogenic, Endothelial, Cell survival, Regenerative medicine.

Abbreviations

Aβ = Amyloid beta

AD = Alzheimer´s disease

AT-MSCs= Adipose tissue mesenchymal stem cells

bFGF= fibroblast growth factor

BME= b-mercaptoethanol

BDNF= Brain derived neurotrophic factor

BHA = Butylated hydroxyanisole

BMPxxx= Bone morphogenetic proteinxxx

BM-MSCs= Bone marrow mesenchymal stem cells

CCl4= Carbon tetrachloride

CDxxx= Cluster Differentiation

CLECs = Cord lining epithelial cells

CLMCs= Cord lining mesenchymal cells

CNS= Central Nervous System

CT1= Cardiotrophin-1

DA= Dopaminergic

DARPP = Cyclic AMP regulated phosphoprotein

DFSCs= Dental follicule stem cells

DMEM= Dubbelco´s modified eagle´s medium

DMP1= Dentin matrix protein 1

DMSO= Dimethylsulfoxide

DSPP = Dentin sialophosphoprotein

ECCs= Embryonal carcinoma cells

ECM= Extracellular matrix

EGF = Epidermal growth factor

EMSCs= Ectomesenchymal stem cells

EMT= Epithelial-mesenchymal transition

EPCs= Endothelial progenitor cells

ESC= Embryonic stem cells

FBS= Fetal Bovine Serum

GDNF = Glial cell line derived neurotrophic factor

GFAP= Glial fibrillary acidic protein

GMP= Good manufacturing practice

hDPSCs= Human DPSCs

hESCs= Human ESCs

HGF= Hepatocyte growth factor

HNF-4α = Nuclear factor 4 α

HSC= Hematopoietic stem cells

IBMX= 3-isobutyl-1-methylxanthine

ICM= Inner cell mass of the blastocyst

IDO= Indoleamine 2,3-dioxygenase

IL-xx= Interleukin-xx

ITSx= Insulin-Transferrin- Selenium-x

ISCT=International Society for Cellular Therapy

LMX1a = LIM homoeo- box transcription factor 1 α

MAP-2= Microtubule-associated protein-2

MCAO= Middle cerebral artery occlusion

mESCs= Mouse ESCs

MI = Myocardial infarction

MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyri- dine-

MSCs= Mesenchymal stem cells

NC = Neural crest

NCSCs= Neural crest stem cells

NeuN= Neuronal nuclei protein

NGF= Nerve growth factor

NTRKxx= Neurotrophic tyrosine kinase

6

NT-3= Neurotrophin 3

NSC= Neural stem cell

OSX= Osterix

OGD= Oxygen-glucose deprivation

PD= Parkinson´s disease

Pdx1= Duodenal homeobox 1

PDLSC= Periodontal ligament stem cells

PitX= Paired-like homeodomain transcription factor

PNS= Peripheral Nervous System

POSS-PCL= Polyhedral oligosilsesquioxane–poly (ε-caprolactone)

POSS-PCU= Polyhedral oligomeric silsesquioxane poly (carbonate-urea) urethane

PPSCs= Primitive pluripotent stem cells

PSCs= Pluripotent stem cells

RA = Retinoic acid

RGCs= Retinal ganglion cells

RhoA= Ras homolog gene family member A

RUNX2= Runt-related transcription factor 2

SCAP= Stem cells from apical papilla

SCI= Spinal cord injury

SCNT= Somatic cell nuclear transplantation

SFCs = Synovial fluid cells

SGZ= Dentate gyrus Subgranular zone

SHED= Stem cells from primary exfoliated deciduous teeth

Shh= Sonic hedgehog

SSEA= State-specific embryonic antigens

STP= StemPro MSC^{TM}

SUR1= Sulfonyl- urea receptor1

SVZ= Subventricular zone

TERT= Telomerase reverse transcriptase

TGF-β3=Transforming growth factor-β III

TH = Tyrosine hydroxylase

TLR x= Toll-like receptor x

TNF-α = Tumor necrosis factor-α

TON= Traumatic optic neuropathy

UCB-SCs = Umbilical cord blood stem cells

UC-MSCs= Umbilical cord mesenchymal stem cells

VMAT = Vesicular monoamine transporter

VSELs = Very small embryonic-like stem cells

WHO= World Health Organization

6-OHDA= 6-hydroxydopamine

TGF-β3=Transforming growth factor-β III

Introduction

1.Stem cells

Stem cells are classically defined as cells with ability to self-renew, generating copies of themselves, and cells with capacity to differentiate under specific physiologic or experimental conditions to mature types of cells that constitute distinct organs and tissues (Potten and Loeffler, 1990).

Stem cells were first described in 1950, when researchers isolated embryonal carcinoma cells (ECCs) from terathocarcinomas (Stevens and Little, 1954; Yu and Thomson, 2008). Few years after in 1964, Kleinsmith and Pierce discovered the ability of these cells to differentiate into all three germ layers. Given self-renewal and multi-lineage differentiation ability, the pluripotent stem cell (PSCs) concept was born, providing the first step to mouse and human embryonic stem cell (ESCs) description. All these discoveries along with propagation and study were only performed for *in vitro* models, until some researchers started searching *in vivo* models (Kahan and Ephrussi, 1970). In order to discover the *in vivo* similar ECCs model like, inner cell mass of the blastocyst (ICM) was analyzed (Martin, 1980). ICM showed to have even more differentiation potential than ECCs, increasing their use in *in vitro* models for development. This ICM is located in the blastocyst during embryonic development. After the division of the zygote when morula structure is achieved, outer layer cells differentiate to trophectoderm, which will derive in the placenta. ICM however, will create all cells of the adult body. Both human ESC (hESCs) and mouse ESC (mESCs) were obtained deriving ICM of human and mouse blastocyst, respectively. hESC are karyotypically common and remain the ability to differentiate into all three germ layers (Amit et al., 2000).

Briefly, mesenchymal stem cells (MSCs) pluripotency was described taking in consideration the ability they showed to differentiate into mesenchymal derived cell lineages, such as; osteoblasts, chondrocytes, adipocytes and myoblast. Indeed, their ability to differentiate into one germ layer was defined like multipotency. This first MSCs were obtained culturing bone marrow cells and selecting thus that adhere to the plate (Chamberlain et al., 2007; Friedenstein et al., 1976) (figure1).
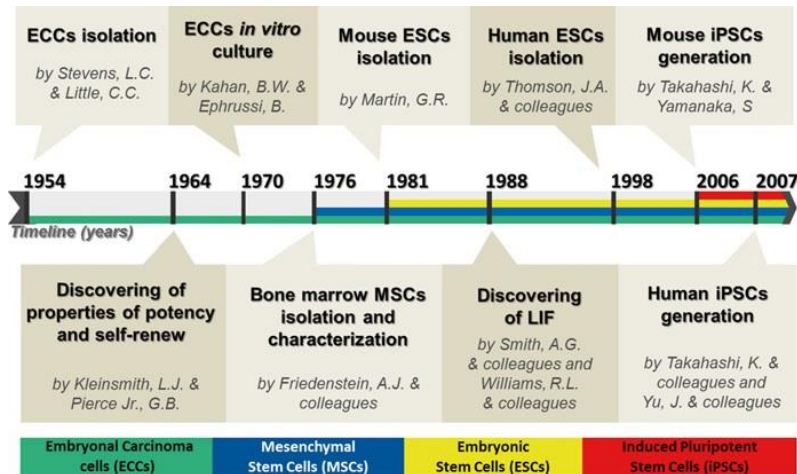
Figure 1. Schematic timeline showing the most important historical milestones in stem cell research (Oliveira et al., 2016).

The differentiation potential of stem cells is defined by the developmental stage that they are in. Taking this fact in consideration stem cells could be divided in 3 groups:

Totipotent: These cells can generate an individual, creating both a completely viable embryo and temporary support tissues (placenta and the umbilical cord). The totipotentiality of the cells lasts from just first fertilization to first cell division rounds, until blastomeric stage, 4 days after fertilization (Brook and Gardner, 1997).

Pluripotent: During the differentiation undergo by embryonic cells during first 4 days of development, blastocyst is generated. ICM within the blastocyst are the ones defined as pluripotent stem cells. They are able to differentiate into cells from 3 germ layers, and hence to generate any tissue of the organism. However, the lost the ability to form extraembryonic tissues, such as placenta (Smith, 2001).

Multipotent: The differentiation ability of multipotent stem cells is highly bounded compared with the two other stem cells described above. These cells can only differentiate into few cell types, and biological function is related with the reparation of their original tissue (Slack, 2000; Spangrude et al., 1988).
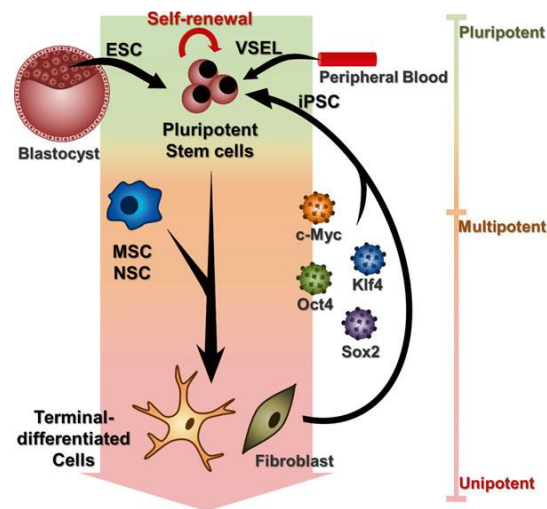
Figure 2. Differentiation potential of pluripotent stem cells and their origins (Oliveira et al., 2016).

Within stem cells, there are to kind of cells; i) Embryonic stem cells, both totipotent and pluripotent cells. ii) Adults (after-birth) stem cells, both pluripotent and multipotent stem cells (figure 2).

1.1 Embryonic stem cells (ESCs)

These pluripotent stem cells are located in the ICM of the blastocyst, and they remain the ability to differentiate to every adult tissue type. Since mouse ESCs were isolated for first in 1981, the molecular mechanisms involved in the maintenance of self-renewing and pluripotency were studied due to their potential therapeutic applications (Evans and Kaufman, 1981) . Among these mechanisms, epigenetic chromatin changes, signal pathways and transcription factor effects were described as the responsible of pluripotency (Marks and Stunnenberg, 2014; Welling and Geijsen, 2013). Furthermore, transcription factors show an important role maintaining pluripotency both *in vivo* and *in vitro* (Dunn et al., 2014; Takashima et al., 2014)*,* including Oct4 and Nanog, which seem indispensable in regulation (Boyer et al., 2005; Loh et al., 2006).

1.2 Adult stem cells

All three stem cell types can be obtained from embryos; however, only two of them are achievable from adult individual. *In vivo*, pluri- and multi- potent stem cells are present in few specific tissues, even also can be generated using *in vitro* techniques.

1.2.1 Hematopoietic stem cells (HSCs)

Hematopoietic stem cells (HSCs) are the precursors that gives raise to all red and white blood cells. The generation of enteric hematopoietic system is given by these rare cells, which have a self-renewal ability and pluripotent capacity. Blood cell production occurs during the life of organism in the bone marrow (Ng and Alexander, 2017). Taking in consideration that as over than million of blood cells must be replaced every day, and there are only thousands of stem cell population in adult organism throughout entire life, it is clear the importance of correct regulation to maintain homeostatic production and to face diseases and stress. HSC are located in bone marrow within adult mammals, and along with the hematopoietic microenvironment, they form the niche which is the modulator network to control broad aspects of hematopoiesis (Hoggatt et al., 2016). The most popular hypothesis says that primitive pluripotent stem cell (PPSCs), also known as very small embryonic-like stem cells (VSELs) CD45$^-$, are at the top of the hierarchy of stem cell in the adult bone marrow. They can give raise to long-term repopulating HSC CD45$^+$, short-term HSCs, MSCs and endothelial progenitor cells (EPCs) (Ratajczak, 2008). Adult long-term HSCs are located in a specific niche, closely associated with endosteum where they exist in a relative hypoxia condition (Morrison and Scadden, 2014; Nombela-Arrieta et al., 2013). These cells remain quiescent until cell cycle recruitment is activated by external signaling, such as; cytokine thrombopoietin, the presence of megakaryocytes or via interferon (Baldridge et al., 2010; Nakamura-Ishizu et al., 2015; Yoshihara et al., 2007). In addition, it is suggested that lineage specification of HSCs can be committed very early in the hematopoietic hierarchy in different HSCs progenitor cells. Thus, progenitors with self-renewing restricted ability may emerge directly from HSCs (Yamamoto et al., 2013). Therefore, there may coexist the idea of direct pathway from HSCs to specific mature cells and the idea of the progenitor mature cell pathway. Furthermore, new studies suggest the recruitment of long-lived progenitor populations maintains blood cell population in steady-state (Sun et al., 2014). The most primitive myeloid progenitors are known as common myeloid progenitor (CMP) and common lymphoid progenitor (CLP) to lymphoid progenitor(Iwasaki and Akashi, 2007). However, recent studies show that before to classical lymphoid/myeloid pathways, HSC undergo myelo-erythroid myelo-lymphoid committing (Arinobu et al., 2005) (figure 3).
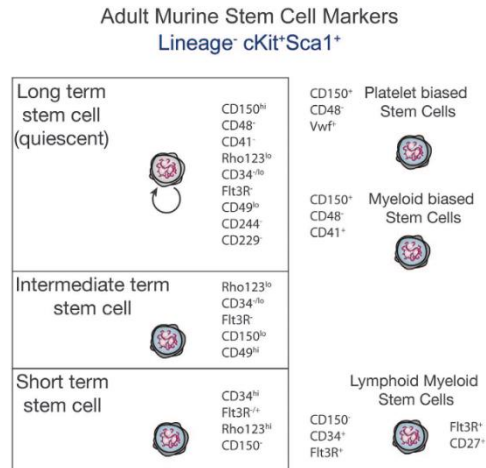
Figure 3. Immunophenotypic markers of adult murine HSCs and 'lineage-restricted' HSC populations (Ng and Alexander, 2017).

1.2.2 Very small embryonic/epiblast-like stem cells (VSELs)

It is a backup population that renews the pool of tissue committed stem cells defined as undifferentiated remain of stem cell population residing in adult tissue firstly described in 2006 (Kucia et al., 2006). In addition, they are able to circulate throughout peripheral blood in stress situations and they express pluripotency markers Oct4, Nanog and SSEA, along with the capacity to differentiate into all three germ layers (Ratajczak et al., 2012).

1.2.3 Neural stem cells (NSCs)

The multipotent nature of neural stem cells (NSC), allow these to differentiate into central nervous system (CNS) neural cells. Contrary to what was thought years ago, NSC are not only found in embryo developing, but they are located also in adult brain. During embryogenesis, after neural tube formation by neuroepithelial cells, they proliferate continuously in the ventricular zone in order to generate CNS (Merkle and Alvarez-Buylla, 2006). Furthermore, these cells are transforming to radial glial cells during embryogenesis, another NSC subtype with ability to differentiate in any neural cell type (Götz et al., 2015). Despite of at first it was thought that radial glial cells served only as a neuronal migration scaffold, several researches demonstrated high proliferation and the ability to generate neurons, therefore, they were defined as kind of stem cell (Hartfuss et al., 2001; Mo et al., 2007; Noctor et al., 2004) . While differentiated neural type are created, more radial glial cells are generated by asymmetric division to maintain stem cell population during brain development (Miyata et al., 2004). Later, these radial glial cells derive to multipotent NSCs in mammal postnatal brain (Merkle and Alvarez-Buylla, 2006; Merkle et al., 2004). The vertebrates with outstanding adult

neurogenesis even contain radial glia within the adult brain (Adolf et al., 2006; Grandel et al., 2006). Although vast neurogenesis in mammals is restricted to embryonic brain development, nowadays, it is accepted that there are two neurogenic cell-containing locations in human adult brain. New neural cell generation is observed in the dentate gyrus subgranular zone of the hippocampus (SGZ) (Gage et al., 1998) and in subventricular zone (SVZ) (Doetsch et al., 1999). SVZ shows a peculiar ranked NSCs. Radial glial cells disappear after birth, and only type B cells are remained, which will become in type C cells, identified by Mash1 expression. C cells at same time are the precursors of neuroblasts, also known as type A cells, and oligodendrocytes (Parras et al., 2004) (Figure 3). Contrary to SVZ, NSCs of the SGZ in adult brain are named as radial astrocytes, and they share both astrocytic and stem features (Kriegstein and Alvarez-Buylla, 2009). These cells function as precursors of the new neural cells in dentate gyrus (Garcia et al., 2004; Steiner et al., 2004) and new neurogenesis is related with learning and memory (Zhao et al., 2008). Radial astrocytes, also known as type I progenitors (Fukuda et al., 2003) , show an vertical elongation crossing the granular cell layer as well as horizontal little process along SGZ. Furthermore, contrary to other astrocytes, radial astrocytes show both GFAP and nestin expression (Seri et al., 2004; Steiner et al., 2006). Radial astrocytes are not able to give raise to neurons, but they are deriving in processes lacking D type cells, also referred as type II progenitors (Seri et al., 2004). In spite of both radial astrocytes and D type cells express Sox2, it is suggested that only nonradial cells are direct progenitors of new neurons and glial cells (Suh et al., 2007). After division, D cells can undergo symmetric division before differentiate into D2 type cells, which show a prominent process and express stage dependent different neural markers, such as; doublecourtin, PSA-NCAM, neun, etc..(Kriegstein and Alvarez-Buylla, 2009) (Figure 4). Very few oligodendrocytes are obtained *in vivo* in SGZ compared with SVZ. Only Mash1 overexpression is essential to redirect the neuronal fate to oligodendrocytic in NSCs of SGZ (Jessberger et al., 2008).
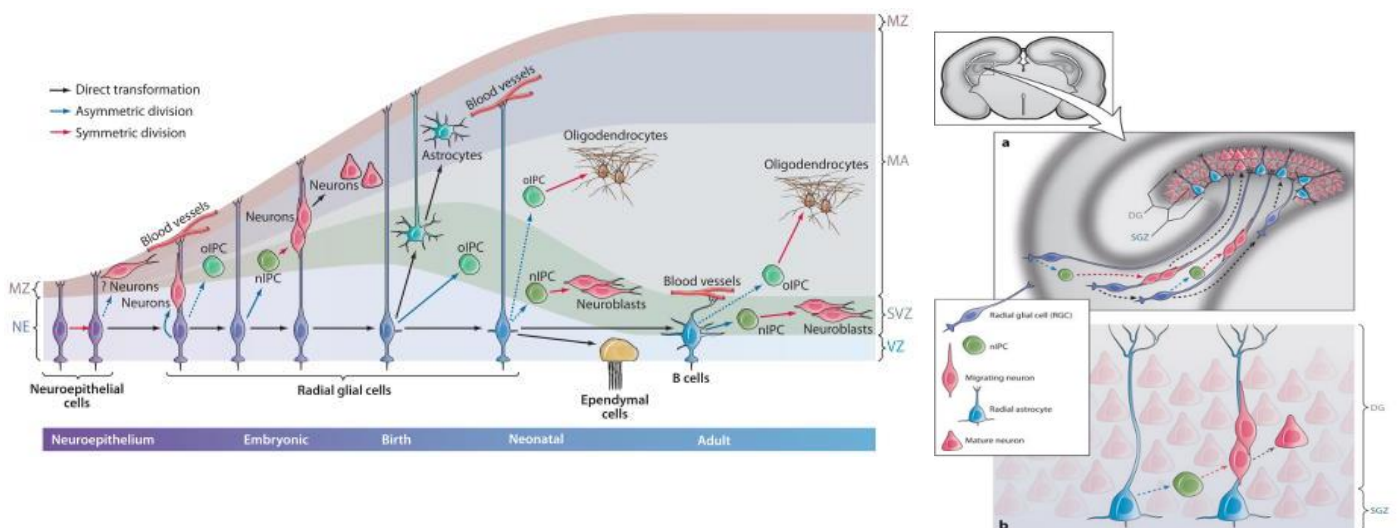
Figure 4. Neural stem cells origin and organization. (Kriegstein and Alvarez-Buylla, 2009)

1.2.4 Induced pluripotent stem cells (iPSCs)

First time cell engineering was used to achieve genetically altered stem cells, the nucleus of a differentiated cell was transferred to an enucleated mouse oocyte by somatic cell nuclear transplantation technique (SCNT) (Wakayama et al., 1998). One year before, Wilmut et al published the successful cloning of Dolly sheep transferring the nucleus of differentiated cell to an enucleated unfertilized egg (Wilmut et al., 1997). However, there was an ethical issue related with human cells. In fact, this method could outcome in individual cloning that is a broadly discussed and controversial topic still nowadays.

Taking in consideration ethical limitations showed by this technique, the aim became to develop reprogrammed pluripotent stem cells from differentiated somatic cells. Thus, the objective was the obtaining of cells with self-renewal and pluripotency properties similar to ESCs. iPSC concept was for first introduced by Takahashi and Yamanaka when they achieved the reprogramming of mouse fibroblasts. The reprogramming was given by retroviral transduction of Oct4, Sox2, Klf4 and c-Myc, also known as Yamanaka factors (Takahashi and Yamanaka, 2006). It was the same group in charge of reprogramming human cells using the same method (Takahashi et al., 2007). In an effort to refine reprogramming procedure some changes were done in the methodology, among others; Nanog and Lin28 were used instead of Klf4 and c-Myc (Yu et al., 2007) and a combination of six factors to increase the efficiency in human fibroblast iPSCs (Liao et al., 2008). Several improvements were obtained such as; c-Myc seems to be dispensable in pluripotency induction (Wernig et al., 2008) and the combination of these factors with proteins, RNA and peptides to avoid viral infection related issues (Malik and Rao, 2013; O'Malley et al., 2009).

Within adult stem cell type cells one of the most used cells are MSCs. Due to their ease of obtaining as well as the amount of them, these are the preferably selected adult stem cell to approach *in vivo* and *in vitro* tissue regeneration assays.

1.2.5 Mesenchymal stem cells (MSCs)

Since Caplan popularized the term mesenchymal stem cell in 1990s, some researchers avoid to refer to cells with stem features in this way (Caplan, 1991). However, in 2000 in an International Society for Cellular Therapy (ISCT) workshop, the terminology was accepted and the acronym was coined (Horwitz and Keating, 2000). ISCT published a position statement to

settle the bases of the criteria to accurately describe and avoid misunderstood about MSCs. In agreement with this criteria MSC are primitive non-hematopoietic stromal cells able to differentiate into mesenchymal derived tissues with tissue regeneration function. They are located in bone, cartilage, muscle, ligament, tendon, adipose tissue (Chamberlain et al., 2007), in perivascular area (Crisan et al., 2008) and they could be also isolated from placenta, menstrual blood, umbilical cord and dental pulps in adult organism (Du et al., 2016; Ma et al., 2014; Portmann-Lanz et al., 2006; Tirino et al., 2011).

The most recognize immune-phenotype MSCs has been identified as positive for CD13, CD44, CD105, CD73 and CD90, while negative for hematopoietic markers CD34, CD45 and CD14.

Following these features, Mesenchymal and Tissue Stem Cell Committee (MTSCC) of ISCT proposed minimal set to define MSC (Dominici et al., 2006):

1. MSCs have to be plastic-adherent when maintained under standard culture conditions.

2. MSCs must have the ability for osteogenic, adipogenic, and chondrogenic differentiation.

3. MSCs must express CD73, CD90, and CD105 (Table 1).

4. MSC must lack expression of the hematopoietic lineage markers c-kit, CD14, CD11b, CD34, CD45, CD19, CD79α,and human leukocyte antigen (HLA)-DR.

It is important to highlight the immune-phenotype difference depending on MSC tissue origin, as it is describe in table 1.

The highlights of MSCs are the self-renewal ability and pluripotent differentiation capacity. MSCs have the ability to differentiate into mesenchymal cells, such as adipocytes, fibroblasts, osteocytes and chondrocytes. In addition, MSCs have the capacity to become in other non-mesenchymal lineage specific cell types, undergoing the process called cross-differentiation (Hass et al., 2011; Ullah et al., 2015).

Apart from pluripotency, also their clinical application could be very interesting due to their homeostasis maintenance as well as their contribution in processes as aging, tissue damage and inflammatory tissue (Le Blanc et al., 2003; Sordi et al., 2005).

	BM-MSCs	AT-MSCs	UC-MSCs
Positive	CD13, CD44, CD73, CD90, CD105, CD166, STRO-1	CD9, CD13, CD29, CD44, CD54, CD73, CD90, CD105, CD106, CD146, CD166, HLA I, STRO-1 CD11b,	CD29, CD44, CD90, CD73, CD105
Negative	CD14, CD34, CD45	CD11b, CD14, CD19, CD31, CD34, CD45, CD79a, CD133, CD144, HLA-DR	CD31 CD34, CD45, CD117,HLA-DR

Table1. BM-MSCs, AT-MSCs and UC-MSCs immune-phenotype.

1.2.5.1 Bone marrow mesenchymal stem cells (BM-MSCs)

Bone marrow not only contains hematopoietic stem cells, but also non-hematopoietic stem cells, such as bone marrow mesenchymal stem cells (BM-MSCs) and endothelial progenitor cells (Anjos-Afonso and Bonnet, 2007). BM-MSCs showed natural immune function, inhibition of local inflammatory responses and sepsis (McLean et al., 2011; Wang et al., 2015; Yang et al., 2013). It is known that CD44, CD73, CD90, CD105 and CD166 are positively marked, while CD14, CD34, CD45 are negatively expressed in these cells (table 1) (De Ugarte et al., 2003; Pittenger et al., 1999).

Although their differentiation ability is limited in comparison with ESC, BM-MSCs have showed an incredible differentiation capacity *in vitro*. Indeed, they are able to differentiate into all three lineages cell type; ectoderm, mesoderm and endoderm (Ullah et al., 2015; Zhang et al., 2018a).
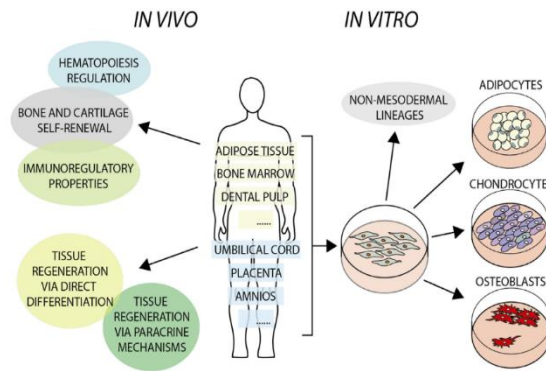
Figure. 5. Mesenchymal stem cell sources. (Guasti et al., 2018)

Ectoderm: Under well-known neural induction medium consisting of growth factor cocktails, some groups have identified BM-MSC ectodermal lineages cell type differentiation despite of mesenchymal being of BM-MSCs. Rat and human BM-MSC were differentiated into neuronal cells in 2000 by containing simple induction media consisting in DMEM/2% dimethylsulfoxide (DMSO)/200 µM butylated hydroxyanisole (BHA) (Woodbury et al., 2000). Several groups follow this way in order to achieve more effective differentiation protocol. Both mouse and rat BM-MSCs were differentiated in neural cells by bFGF, FGF-8 and BDNF cocktails for 7 days, sequentially (Jiang et al., 2002). Even specific neuronal cell types are achieved modifying primary culture conditions. Thus, human BM-MSCs reprogramming with LMX1a, which is described as important piece in dopaminergic differentiation both *in vivo* and *in vitro,* resulted in dopaminergic differentiation (Barzilay et al., 2009; Wilkins et al., 2009). Regarding to dopaminergic induction, new investigation show the importance of cell passage number, the more passages have a cell, less dopaminergic differentiation and lower spiking and bursting frequencies shows it (Shall et al., 2018). As a counterpart of dopaminergic differentiation, also cholinergic neurons were obtained following a 1 mM b-mercaptoethanol (BME) and nerve growth factor (NGF) (100 ng/mL) consisting of 2 step induction protocol (Naghdi et al., 2009).

Mesoderm: Taking in consideration the mesenchymal origin of BM-MSCs, it is not a surprise the ability of BM-MSCs to derive in mesodermal lineages cells. Several projects have confirmed the *in vitro* differentiation into adipocytes, osteocytes and chondrocytes using several differentiation cocktails. First stablished chondrocyte differentiation protocol was given for BM-MSCs (Mackay et al., 1998). After that, the differentiation was confirmed by some other research, while differentiation was analyzed by measuring oil droplet generation, mineralization and type collagen II secretion respectively (Gronthos et al., 1994; Muruganandan et al., 2009; Ranera et al., 2013; Wagner et al., 2005; Zhang et al., 2011). Combining the *in vitro*

differentiation capacity and new gene recombination technologies, more healer BM-MSCs have been obtained in order to use them in regenerative therapies. In this case BMP-7 overexpression in BM-MSCs lead to a better fracture healing ratio than wild type BM-MSCs (Yan et al., 2018). Apart from these three cell types, also cardiomyocytes were derived from BM-MSCs. 9 years after the first attempt in rat BM-MSCs using 5-azacytidine, where multinucleated myotubes were obtained (Wakitani et al., 1995), the same protocol was used in human BM-MSCs to achieve myocyte-related genes, β-myocin heavy chain, α-cardiac actin, desmin and calcium-potassium-induced calcium channels expressing differentiated cells (Xu et al., 2004). Moreover, the differentiation into endothelial cells have been seen in BM-MSCs, where the down-regulation of lncRNA MEG3 seems to be a key as well as the effect of biphasic calcium phosphate ceramic (Chen et al., 2018; Sun et al., 2018; Urbich and Dimmeler, 2004).

Endoderm: Although hepatocytes were thought to derived only from endodermal progenitor cells, MSCs demonstrated an unexpected hepatogenic differentiation ability. Exposing BM-MSC to specific 2 step protocol consisting in; a week of a differentiation step where basal medium was supplemented with bFGF, EGF and nicotinamide, following by maturation step where medium was supplemented with oncostatin M, dexamethasone and ITS+ (insulin, transferrin, selenium) premix, they are able to become into hepatic cells. After the treatment they expressed liver specific transcription marker albumin, α-fetoprotein, nuclear factor 4 α (HNF-4α) as well as liver cells characteristic functions such as; albumin production, glycogen storage, urea secretion, uptake of low-density lipoprotein, and phenobarbital-inducible cytochrome P450 production (Lee et al., 2004; Stock et al., 2014). Even non-human BM-MSCs hepatocytic differentiation is demonstrated in different species; from mouse to Tibetan mastiff (Xu et al., 2017b; Zhang et al., 2018a). Furthermore, the use of coating substrates to improve the proliferation, hepatic differentiation and hepatocyte-specific functions during the culture along with to ease the engraftment *in vivo* as scaffolding, has become an aim in last years. Coating plates with both liver extracellular matrix (ECM) and matrigel showed a significant improvement of differentiation and viability in BM-MSCs (Wang et al., 2018).

Like happens with hepatocytes, pancreocyte differentiation ability of BM-MSCs was unexpected. However, there are some elegant works where BM-MSCs are successfully differentiated to insulin producing β-cells both *in vitro* and *in vivo*. The ability to differentiate these cells *in vitro* by specific inductive media, is going beyond than only morphological change but they are able to correct the streptozotocin-induced hyperglycemic condition (Gabr et al., 2013, 2014; Tang et al., 2012). It is also demonstrated the capacity of these cells to differentiate into endocrine pancreatic lineage cells *in vivo* in the presence of paracrine factors, extracellular vesicle-mimetic nanovesicles, etc..(Bhonde et al., 2014; Oh et al., 2015; Phadnis et al., 2011).

1.2.5.2 Adipose tissue mesenchymal stem cells (AT-MSCs)

The adipose tissue has been classified as energy storing tissue composed by different cells. Within adipose tissue could be find adipocytes, endothelial cells, hematopoietic cells, fibroblasts and adipose tissue mesenchymal stem cells (AT-MSCs), all of them irrigated by an intermingling vasculature (Eto et al., 2009). Although AT-MSCs are similar to BM-MSCs in morphology and phenotype, there are important differences in cell harvest and cell yield (Markarian et al., 2014). In addition, it is demonstrated that transcriptome is different depending on MSC tissue origin. Thus, proliferation related genes are upregulated in AT-MSCs compared with BM-MSCs, which match with the fact of AT-MSC multiplied faster to up to 20 passages compared to BM-MSCs (Wagner et al., 2005) . AT-MSCs can be isolated from the patient himself with minimal donor morbidity, being useful to "autologous" cellular therapies. These cells are present in subcutaneous adipose tissue in both inguinal and abdominal areas (Hu et al., 2014; Maharlooei et al., 2011; Zografou et al., 2013). The AT-MSCs denomination requires specific immune-phenotype and differentiation capacity criteria. Commonly described as fibroblastic-like large nucleus containing cells, their immune-phenotype have been defined as positive to CD9, CD13, CD29, CD44, CD54, CD73 (SH3), CD90, CD105 (SH2), CD106, CD146, CD166, HLA I, STRO-1 and negative to CD11b, CD14, CD19, CD31, CD34, CD45, CD79a, CD133, CD144, HLA-DR (table 1) (Chen et al., 2013; Schäffler and Büchler, 2007; Zuk et al., 2002).

Like happens with BM-MSCs, AT-MSCs are able to differentiate into all three lineage cells (ectodermic, mesodermic and endodermic) apart from expected mesenchymal lineage cells (Gadelkarim et al., 2018; Schäffler and Büchler, 2007; Ullah et al., 2015).

Ectoderm: Neural differentiation of AT-MSCs was first reported by Safford, who confirmed neural differentiation by nestin and neuronal nuclei protein (NeuN) expression (Safford et al., 2002). Years later, several groups followed differentiating neural cells from AT-MSCs. Different inductive protocols were used for this aim, among others; simple neurobasal medium (Dave et al., 2018), neural induction media containing FGF2, EGF, BMP-9, retinoic acid and heparin to obtain cholinergic and dopaminergic neurons (Marei et al., 2018), olfactory ensheathing cells (OECs) or Schwann cells (SCs) conditioned medium (CM) (Lo Furno et al., 2018), and even one group achieve NSCs combining neurobasal media with EGF and bFGF (Petersen et al., 2018). In addition, new approach of neuro-differentiation based in the most common neuro-inductive material, graphene oxide, and in the one of the most important neuronal migration, proliferation and function regulators, calcium, also have demonstrated their

important role in the differentiation of AT-MSC to neuronal lineages (Feng et al., 2018; Goudarzi et al., 2018).

Mesoderm: Transcriptional and molecular events responsible of lineage-specific mesodermal differentiation into osteocytes (Lian et al., 2004; Zhao et al., 2018), chondrocytes (Otto and Rao, 2004), adipocytes (Rosen, 2002) and myocytes (Brand-Saberi, 2005) are already studied and well-known. AT-MSCs derived adipocytes develop both white and brown adipocyte mature features, such as; lipolytic capacity under catecholamine stimulation, anti-lipolytic activity mediated by α_2-adrenoceptors, and the secretion of typical adipokines, such as adiponectin and leptin. (Dicker et al., 2005; Kilroy et al., 2018; Rashnonejad et al., 2018). Could be interesting the use of preadipocytes more than the use of mature adipocytes, thanks to their smaller size mediated quicker revascularization ability, *in vivo* differentiation effectiveness and low oxygen consumption (Heimburg et al., 2005) . New sources of osteogenic progenitor cells are needed since bone tissue engineering requires large amounts of osteocytes. It is described the similar ability for osteogenic differentiation showed by BM-MSCs and AT-MSCs, even if the age of donor is increase (Shi et al., 2005). However, it is also published that AT-MSCs have inferior potential for osteogenesis and inferior ability in the treatment of partial growth arrest in murine model than MSCs have (Hui et al., 2005; Im et al., 2005). Both human and mouse AT-MSCs can acquire typical osteogenic differentiation hallmarks, such as mineralized extracellular matrix production, osteoblast-associated proteins osteocalcin and alkaline phosphatase expression, responsiveness to fluid shear stress and the expression of mechanosensitive genes (osteopontin, collagen type Iα1, and COX-2) after mechanical loading (Ardeshirylajimi et al., 2014; Jia et al., 2018; Knippenberg et al., 2005). Several signaling pathways have been identified as regulators of osteodifferentiation including TGFB/BMP, Wnt/B-Catenin, Notch, Fibroblast growth factor and Hedgehog. BMP is in particular an important key of osteogenesis, and the most important elements are BMP2 and 7. Treating AT-MSCs with recombinant BMP2 and BMP7 stimulates osteogenic and adipogenic differentiation, respectively (Dragoo et al., 2003; Knippenberg et al., 2006). Thus, genetically modified AT-MSCs overexpressing BMP2 were successfully used to heal femoral defects (Peterson et al., 2005). BMP can activate differentiation either activating BMPR I and II receptors or activating both Runt-Related Protein 2 (Runx-2) and osterix (OSX), which are considered the master regulation genes for bone formation (Heldin et al., 1997; Lian et al., 2006). Furthermore, other elements, such as Tbx3 (Lee et al., 2007b) and FOXO1 (Zhao et al., 2018) have showed an important role in osteogenic differentiation of AT-MSCs. Apart from specific differentiation factors, extracellular matrix substitutes and three-dimensional environment scaffolds are critical for a successful differentiation. Hence, chitosan particle-agglomerated scaffolds, fibrin scaffolds, α-tricalcium phosphate scaffolds as well as gum tragacanth hydrogel have been reported as suitable

scaffoldings (B Malafaya et al., 2005; Haeri et al., 2016; Hattori et al., 2006). There are many protocols to obtain chondrogenic differentiation; from the simplest one in which there are not factor added (Wagner et al., 2005), to the standard protocol for chondrogenesis combines DMEM with ITS+, linoleic acid, selenious acid, pyruvate, ascorbate 2-phosphate, dexamethasone and transforming growth factor-β III (TGF-βIII). It is known that pre-induction stage of chondrogenic differentiation carry MSCs express type I and II collagens. The expression of chondrogenic genes and adhesion molecules depends on the presence of TGF-β family (TGF-β1, TGF-β2and TGF-β3) soluble factors. The maturation of chondrocyte is achieved once they express Sox9, L-Sox5 and Sox6 chondrogenic transcription factors (Chimal-Monroy and Díaz de León, 1999; Ikeda et al., 2004). Furthermore, apart from TGF-β1, which in humans interacts with Wnt/β- catenin pathways to inhibit osteogenic differentiation to support chondrogenesis (Zhou, 2011) , other growth factors such as, insulin like growth factor-I (IGF-I) and BMP-2 are known to induce the chondrogenic differentiation of AT-MSCs (An et al., 2010; Wei et al., 2006). Commonly used polyhedral oligomeric silsesquioxane poly (carbonate-urea) urethane (POSS-PCU) and polyhedral oligosilsesquioxane–poly (ε-caprolactone) (POSS-PCL) cyto-compatible novel bioscaffolds, are effective in chondrogenic differentiation (Griffin et al., 2017; New et al., 2017).

Many investigations support AT-MSCs differentiation toward cardiomyocytes. When AT-MSC are cultured under specific conditions, they are able to achieve cardiomyocytes-like phenotype with pacemaker activity and specific cardiac marker expression (Planat-Bénard et al., 2004). Moreover, other researchers have showed how the engraftment of AT-MSCs in murine myocardial injury models they are able to differentiate in cardiomyocytes, promote angiogenesis and even either improve or heal injured areas (Miyahara et al., 2006; Strem et al., 2005). Even these data were obtained from murine models some new works supported also differentiation into functional contractile cardiomyocytes from human origin (Choi et al., 2010) and emphasized in the important role of ascorbic acid in generation of AT-MSCs derived cardiomyocytes through MEK/ERK signaling pathway (Liu et al., 2018b).

Unexpectedly, as happens with BM-MSCs, AT-MSCs have demonstrated the ability to differentiate into endothelial cells, which show proangiogenic potential, post-ischemic neovascularization and vessel-like structure formation as well as secretion of endothelial cell-related elements, such as VEGF, hepatocyte growth factor (HGF), placental growth factor, FGF-2, TGF-β , and angiopoietin-1 (Bekhite et al., 2014; Cao et al., 2005; Rehman et al., 2004).

Endoderm: First human AT-MSCs were differentiated into pancreatic endocrine phenotype using the differentiation factors activin-A, exendin-4, HGF, and pentagastrin. These cells were able to express endocrine pancreatic hormones insulin, glucagon and somatostatin along with

pancreatic developmental transcription factors Pax-6, Ipf-1, and Ngn-3 (Timper et al., 2006). Despite new investigations shedded light to the AT-MSCs differentiation ability comparing with BM-MSC, it was not clear what was the process underwent by these cells (Gabr et al., 2014). Several groups manipulate AT-MSCs genetically, with the purpose to provide new strategies to understand pancreatic endocrine differentiation mechanism. Thus, has been demonstrated how the combined effect of Pdx1 and Shh overexpression in rat AT-MSCs insulin producing cells, can increase insulin production along with the expression of MafA, Nkx2.2, Nkx6.1, Ngn3, insulin, and *Isl1*. At the same time, these cells exhibited the ability to normalize blood glucose values in diabetic rats (Hashemi Tabar et al., 2018). In the same way, another investigation point to the activation of Wnt pathway during differentiation (Wang et al., 2017b). The use of 3D scaffolding will probably be the future in autologous diabetes mellitus I cell therapies. Combining both 3D scaffolding PVA and platelet-rich plasma (PRP) the differentiation protocol of AT-MSCs derived insulin producing cells have been optimized (Enderami et al., 2018).

AT-MSCs cultured with HGF, oncostatin M (OSM), and dimethyl sulfoxide derived in hepatocyte-like phenotype expressing albumin and α-fetoprotein with the ability to avoid low-density lipoprotein increase and produce urea (Seo et al., 2005). Murine models demonstrated the viability of AT-MSCs hepatocytic differentiation assays resulting in functional periportal and pericentral hepatocyte. They even had the ability to restore the liver function after carbon tetrachloride (CCl_4)-induced acute fulminant liver failure (Winkler et al., 2015; Xu et al., 2015a). As model counterpart, there are several works, where totally functional and histo-compatible hepatocyte-like cells are obtained from human AT-MSCs both *in vitro* and *in vivo* using different induction protocols (Aurich et al., 2009; Lee et al., 2012; Wang et al., 2014).

1.2.5.3 Umbilical cord stem cells (UC-MSCs)

The development of human umbilical cord starts in the 5th week of embryogenesis (Ellis, 1989). In addition to common adult tissues (adipose tissue, bone marrow), MSCs can be obtained from birth associated tissues including placenta, amnion and umbilical cord (figure 5). Umbilical cord stem cells (UC-MSCs) are a high accessible and ethically acceptable novel cell source. These cells can be non-invasively obtained after birth, and they seem to be more primitive than the others MSCs located in adult tissues, ergo, they have intermediate features between adult and embryonic stem cells (El Omar et al., 2014). Taking in consideration last investigations about UC-MSCs, their immune-phenotype has been standardized as positive for CD29, CD10, CD13, CD58, CD59, CD61, CD44, CD90, CD73, CD106, CD166, CD325, CD56, CD51 and CD49(b-e) and negative for CD31, CD3, CD11b, CD14, CD19, CD31, CD33, CD34, CD38, CD40, CD49a,CD34, CD45, CD117, CD133, CD86, CD80, CD71, CD56, CD53,

CD50 and HLA-DR. Some data are contradictory depending on the report; CD54, CD105, CD106, CD117, CD144 and CD146. The contradiction of CD105 is specially unexpected due to its importance in mesenchymal stem cell identification. However, in most studies CD105 is expressed, only few studies have shown that either it is not expressed or the expression is decreasing along with the passage is increasing (table 1) (Batsali et al., 2013; Can and Karahuseyinoglu, 2007; El Omar et al., 2014; Seo et al., 2009). The investigations carried out after their description in 1656 have demonstrated the big amount of these cells, the high proliferation capacity and the safety to use them in cell therapies (Fong et al., 2011; Friedman et al., 2007). After animal disease model experiments support the use of human UC-MSCs in clinical trials some clear conclusions are taken. UC-MSCs used in cardiac, skin, musculoskeletal, ophthalmologic, pulmonary, endocrine, liver, hematologic and neurologic diseases showed anti-apoptotic, immunomodulatory, anti-fibrotic and angiogenic properties along to stem cell related benefits. Furthermore, they did not show infusion-related toxicity, which make these cells attractive tool for cell therapies. However, all works mentioned the need to refining nomenclature in researches to discern between different cell types of umbilical cord (Arutyunyan et al., 2016; Can et al., 2017).

Hence, it is important to highlight that UC-MSCs can be isolated from different parts of umbilical cord, such as Wharton´s jelly, cord lining and umbilical cord blood. Both cord lining and umbilical cord blood contains more than one cell type, only few of them are MSCs (Maria et al., 2010).

1.2.5.3.1 Wharton´s jelly

Umbilical cord tissue used to be medical waste until 1991, when fibroblast-like cells where discovered in this tissue (McElreavey et al., 1991). Years later, these cells were taken as MSCs due to their immune-phenotype and their differentiation ability (Wang et al., 2004). Two arteries and one vein, all of them surrounded by mucoid connective tissue known as Wharton´s jelly, compose the umbilical cord (figure 6). This gelatinous connective tissue is protecting blood vessels from clumping providing them flexibility, and it is also covered by an amnion derived epithelium, which is named cord lining. It is described that Wharton´s jelly is composed by glycosaminoglycans, specially hyaluronic acid and chondroitin sulfate. In absence of elastic fibers, collagen fibers are the main fibrillary component. Regarding to cell-composition, it is fill by mesenchyme derived cells, such as fibroblasts, myofibroblasts, smooth muscle cells and mesenchymal stem cells (Arutyunyan et al., 2016). MSCs can be isolated from perivascular, intervascular and subamnion zones within Wharton's jelly (Maria et al., 2010).
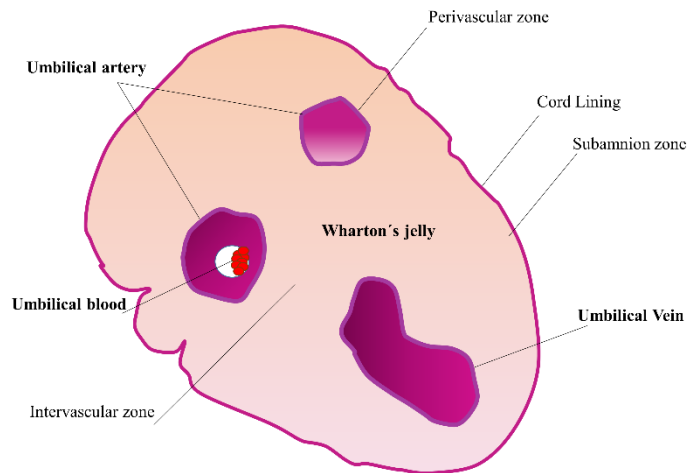
Figure 6. UC-MSC. Modified from (Ding et al., 2015)

1.2.5.3.2 Cord lining

Two kind of cells can be isolated from this area, cord lining mesenchymal cells (CLMCs) and cord lining epithelial cells (CLECs). CLMCs are achieved from the subamnion region dissecting out Wharton´s jelly and have been used for burn and diabetic wound healing. On the other hand, CLECs have been used both to skin cosmetic trials, ocular disorders, insulin dependent diabetes, liver failure, wound regeneration and for treating persistent corneal epithelial defects(Lim and Phan, 2014; Saleh and Reza, 2017).

1.2.5.3.3 Umbilical cord blood stem cells (UCB-SCs)

Umbilical cord blood stem cells (UCB-SCs) are first isolated fetal stem cells. UCB-SCs were declared source of hematopoietic stem and progenitor cell in 1974 (Ali and Al-Mulla, 2012; Knudtzon, 1974). Nowadays, umbilical cord blood containing different stem cell populations are defined. Like it is described in figure 7, there are 3 kind of stem cell within UCB-SCs.
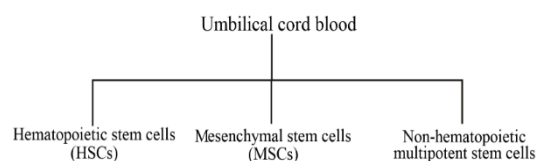
Figure 7. UCB-SCs types (Ali and Al-Mulla, 2012)**.**

<u>Hematopoietic stem cells</u>: As it is described above (HSCs, 1.2.1 chapter), all blood cells are created from these multipotent stem cells. HSCs located in umbilical cord blood contain different commitment stages of HSCs. In comparison with any other MSCs, their main characteristic is the expression of hematopoietic origin surface markers; CD133, CD34 and CD45 (McGuckin et al., 2003).

<u>Mesenchymal stem cells</u>: These cells are the mesenchymal part of stem cellular components in umbilical cord blood, also known as umbilical cord mesenchymal stem cells (UC-MSCs). As the others MSCs, UC-MSCs showed the ability to differentiate into mesodermal lineage cells as well as into non-mesodermal lineages (da Silva Meirelles et al., 2006). They have a high proliferation capacity (Bieback et al., 2004).

<u>Multipotent non-hematopoietic stem cells</u>: Their presence is minimum and are negative to CD45, hematopoietic marker (McGuckin et al., 2005; Zhao et al., 2006). They have displayed embryonic pluripotential related OCT4, SOX2 and NANOG markers, as well as human embryonic stem cell specific surface markers state-specific embryonic antigens, SSEA-3 and SSEA-4, among others (Inamdar et al., 2009). These cells have shown the ability to differentiate into three germ layers cell types. Multipotent non-hematopoietic stem cells have been differentiate into ectodermal lineage neural cells (Zangiacomi et al., 2008) , hepatic and pancreatic endodermic lineage cells (Denner et al., 2007; McGuckin et al., 2005), and endothelial cells from mesodermal lineage(Ma et al., 2006). The differentiation potential and accessibility, become UC-MSCs in a promising source of stem cell source for research and even to cell therapy applications (Ali and Al-Mulla, 2012).

Ectoderm: UC-MSCs have the ability to differentiate into neurons, astrocytes and other glial cells, even it is demonstrated that they can rescue a stroke in a rat model increasing neurotrophic factors and β1-integrin (Ding et al., 2007; Liu et al., 2010). Differentiation protocols have been optimized to achieve specific neuronal cells; expressing dopaminergic specific markers, such as tyrosine hydroxylase (TH), dopamine, cyclic AMP regulated phosphoprotein (DARPP) 32, paired-like homeodomain transcription factor (PitX) 3 and vesicular monoamine transporter (VMAT) 2 with 65% of differentiation efficiency (Datta et al., 2011; Fong et al., 2011; Mitchell et al., 2003). Also oligodendrocytes and astrocyte differentiation were achieved (Leite et al., 2014; Mitchell et al., 2003).

There also few works that differentiate UCB-MSCs into neural lineages. The combination of telomerase reverse transcriptase (TERT) and brain derived neurotrophic factor (BDNF) improved UCB-MSC viability, proliferation and neural differentiation (Zhao et al., 2014). In other case, Cardiotrophin-1 (CT1) stimulates differentiation of UCB-MSCs and their survival through PI3/AKT pathway (Peng et al., 2017).

Mesoderm: In spite of UC-MSCs derived adipocytes have shown to create small lipid vacuoles, they also have the capacity to maintain multipotency for longer periods compared with BM-MSCs (Fong et al., 2012; Mennan et al., 2013). Some investigations focus their efforts in elucidate which mechanism interfere in adipogenic differentiation. BMP-9 show to be a key in adipogenic, chondrogenic and osteogenic differentiation (Shu et al., 2018). Like happens with other MSCs, UC-MSCs can be cultured in different scaffoldings, however, never substrate stiffness was taken as important element in differentiation. Different lineage commitment can be undergo depending on the rigidity of the material. UC-MSCs show to tended to differentiate into adipocytes in soft matrix, however, cells cultured in high-stiffness undergo osteoblastic differentiation (Xu et al., 2017a).

Osteogenic differentiation was less effective in UC-MSCs than other MSCs (Ishige et al., 2009), however MSCs from other part of the umbilical cord showed higher osteogenic potential. Wharton jelly´s MSCs had higher osteogenic potential than the other UC-MSCs (Mennan et al., 2013).

Regardless of the place of the umbilical cord they were isolated from, all the cells revealed the same chondrogenic potential, increasing in 3 the potential showed by BM-MSCs (Mennan et al., 2013).

Endoderm: Human UC-MSCs are able to express hepatic markers and to differentiate both *in vivo* and *in vitro*. When UC-MSCs were transplanted into chemically injured liver rat model they differentiated into hepatocytes and even they successfully engrafted into injured liver (Campard et al., 2008; Lin et al., 2010).

Regarding to insulin producing cells, effectively differentiated human UC-MSCs *in vitro* could recovery normal glycemic values after portal vein injection both in rats and mice (Tsai et al., 2012; Wang et al., 2011).

1.2.5.4 Dental pulp stem cells (DPSCs)

Like happens with other tissues, also oral cavity contains MSCs population in adult organisms (Huang et al., 2009). Gronthos et al. described in 2000 the existence of unique stem

cell population very similar to BM-MSCs derived from dental pulp tissue (Gronthos et al., 2000). Due to their ability to self-renewal and capacity to differentiate into neurogenic and mesenchymal lineage cell, they were named as dental pulp stem cells (DPSCs) (Gronthos et al., 2002). The tooth structure consists of an outer enamel layer (created by ameloblasts), following by dentin layer (created by odontoblasts), which surrounds a dental pulp located into tooth cavity (Shi and Gronthos, 2003). Dental pulp is a soft connective tissue containing nerves, blood vessels, mesenchymal tissues and mesenchymal cells (mesenchymal stem cells, fibroblasts, immune cells). This has an important role in primary and secondary tooth development and protection in front of insults and injuries, such as caries. DPSCs have shown high-proliferative capacity and more important, they are accessible without invasive surgical procedures (Gardin et al., 2016) (figure 8)(figure 9, red). Nowadays, there are not standard immune-phenotype to define DPSCs. However, it is well defined the expression of three surface stem mesenchymal markers CD73, CD90, CD105 required by ISCT to consider DPSCs mesenchymal stem cells. In addition, several works have compiled the expression of CD27, CD29, CD44, CD146, CD166, CD271 and STRO-1. In the contrary, DPSCs do not express CD34, CD45 (hematopoietic markers), CD14 (monocyte or macrophage marker), CD19 (B cell marker) and HLA-DR (human leukocyte antigen) surface molecules (Anitua et al., 2018; Gronthos et al., 2002; Kawashima, 2012). Moreover, DPSCs express Oct-4, Nanog and Sox2 stem pluripotential markers as well as neural stem cell markers (Nestin, Vimentin) and mesenchymal (Vimentin, Collagen I) markers. In addition, DPSCs express other cranial neural crest cell-related neural markers such as glial fibrillary acidic protein (GFAP), β-III tubulin and microtubule-associated protein-2 (MAP-2) (Feng et al., 2013; Ibarretxe et al., 2012; Király et al., 2009).
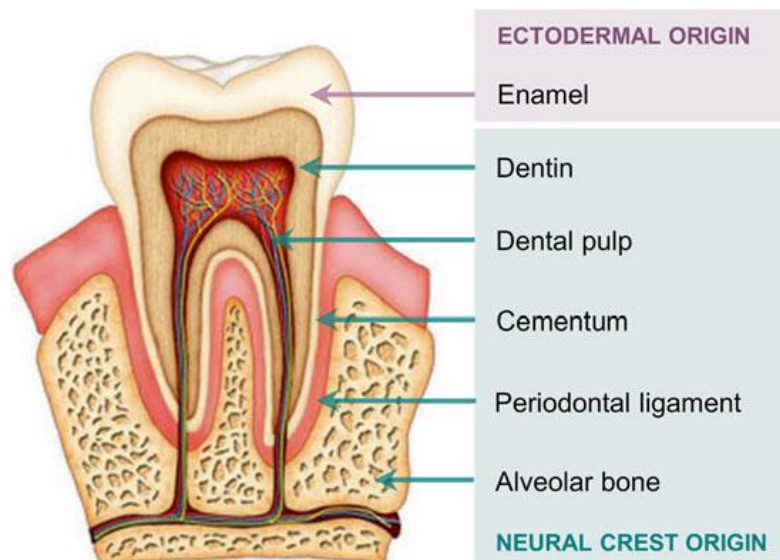

Figure 8. Tooth longitudinal section (Gardin et al., 2016)**.**

1.2.5.4.1Neural crest origin and ectomesenchymal being

The neural crest (NC) is a transient structure composite by a population of a pluripotent cells located at the lateral edge of neural tube in vertebrate embryos. Neural crest stem cells (NCSCs) have the capacity to migrate throughout the body to generate several cells type achieving diverse array of tissues, such as skin melanocytes, endocrine cells in the adrenal and thyroid glands, peripheral nervous system (PNS) and most craniofacial structures such as bones, tongue, craniofacial nerves and muscles (Shyamala et al., 2015; Vega-Lopez et al., 2017). In fact, craniofacial structures are composed by special NCSCs. During neural tube creation, the edges of neuroectoderm edges join after neural fold. Once, tubular shape is achieved neural crest stem cells undergo epithelial-mesenchymal transition (EMT), becoming ectomesenchymal stem cells (EMSCs). So, they are already EMSCs, when NCSCs migrate to create craniofacial structures, which create craniofacial connective tissues including dental pulp and periodontal ligament during embryonic development (figure 9) (Ibarretxe et al., 2012). DPSCs and the others dental stem cells described above, have been constituted as NCSCs source in adult tissue due to their neural crest origin and neural crest marker remain (Chai et al., 2000; Janebodin et al., 2011).
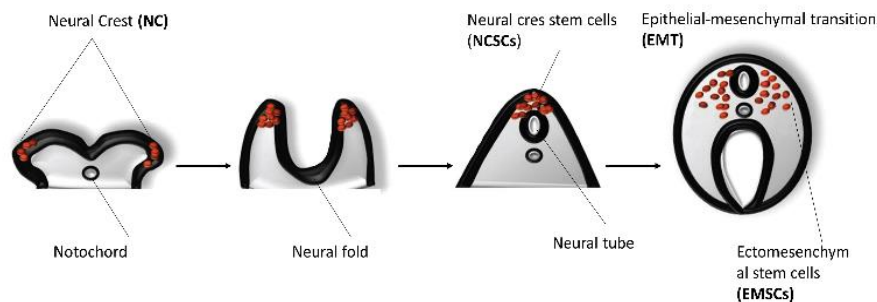

Figure 9. Neural crest cells origin (Ibarretxe et al., 2012)**.**

1.2.5.4.2Other dental stem cells

Although DPSCs are the most used cells for research purposes, it must be remarked that not only DPSCs have been identified among dental stem cells. There exist dental follicle stem cells (DFSCs) and stem cells from apical papilla (SCAP) in developing teeth tissues and there
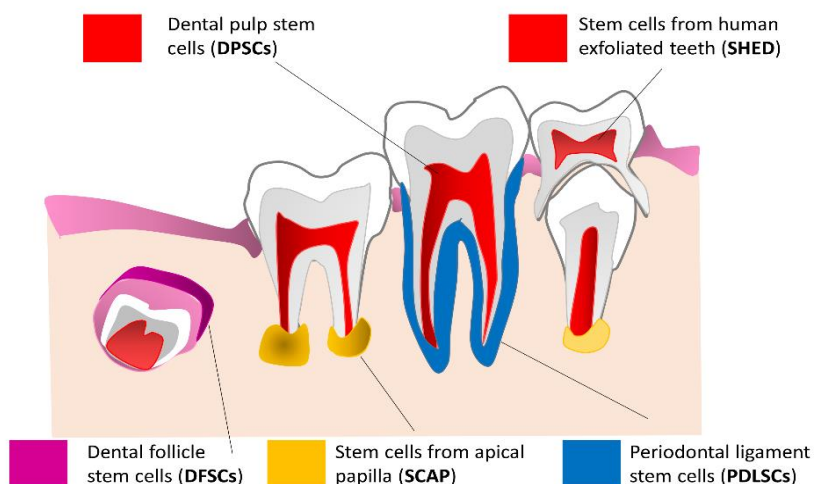
are periodontal ligament stem cells (PDLSCs) and stem cells from primary exfoliated deciduous teeth (SHED) in adult teeth (Ibarretxe et al., 2012; Sharpe, 2016) (figure 9).

DFSCs: These cells are isolated from the ectomesenchymal embryonic tissue that cover tooth germ in tooth development. In adults, DFSCs can be found in pre-erupting wisdom teeth follicle (figure 10, purple) (Morsczeck et al., 2005). Despite of the specific ability of DFSCs to differentiate into periodontal tissue related cells, they are also able to differentiate into other cell lineages *in vitro* (Honda et al., 2010; Morsczeck et al., 2017).

SCAP: This high proliferative ectomesenchymal stem cell (EMSC) population is located surrounding apices of root sheath of developing teeth (figure 10, yellow). Although these cells are currently present in development of teeth morphogenesis, SCAP is also present in pre-erupting wisdom teeth in adults (Bakopoulou et al., 2011). SCAP have shown differentiation capability, not only to common mesenchymal lineage cells, but also show neurogenic and angiogenic potential in specific conditions (Nada and El Backly, 2018).

PDLSCs: In 2004, Seo et al. obtain MSCs with similar proliferation, clonogenic differentiation capability and basal expression of pluripotential markers to adult DPSCs from perivascular space of periosteum, these cells were named periodontal ligament stem cells (PDLSCs)(Liu et al., 2018a; Seo et al., 2004; Trubiani et al., 2010) (figure 10, blue).

SHED: As happens with DPSCs, also primary human exfoliated deciduous teeth are an EMSC population. SHED high proliferative rate, basal neural marker expression, expression of pluripotency markers and even the capacity to survive and integrate in host brain tissue (Kerkis et al., 2006; Miura et al., 2003)(figure 10, red). In addition, SHED have shown a remarkable differentiation ability to mesenchymal lineages (Bento et al., 2013; Sakai et al., 2010) as well as neurogenic lineages (Brar and Toor, 2012; Zhang et al., 2016b). Taking in consideration such properties population could be used as EMSCs source, however, their availability is limited in

Dental pulp stem cells (**DPSCs**)

Stem cells from human exfoliated teeth (**SHED**)

Dental follicle stem cells (**DFSCs**)

Stem cells from apical papilla (**SCAP**)

Periodontal ligament stem cells (**PDLSCs**)

adult human being and their collected material amount is reduced due to root resorption during exfoliation.

Figure 10. Dental stem cell types. Modified from (Sharpe, 2016)**.**

In order to elucidate if DPSCs are actually a good source of stem cell to use in regenerative cell therapies these cells have been compared with other studied stem cell sources. In one of the first works where DPSCs were compared with BM-MSCs, similar differentiation capabilities and neural and glial specific marker expression were discovered in both cell types. However, they showed DPSCs were more active and developed metabolically than BM-MSCs, concluding DPSCs remain better neural and epithelial stem cell features (Karaöz et al., 2011). Later, DPSCs were compared with other accessible MSCs sources. In this work the morphology, proliferation and differentiation capability of UC-MSCs, menstrual blood cells and DPSCs were faced. Although UC-MSCs were higher proliferative stem source, DPSCs demonstrated lower cellular senescence as well as higher osteogenic capabilities than the other two stem cell types (Ren et al., 2016). These results are correlated with the results of the next investigation, where the upregulation of growth factor activity, receptor activity and signal transduction in DPSCs were demonstrated. In contrast, cell proliferation, immune response and angiogenesis related genes were overexpressed in UC-MSCs. In conclusion, UC-MSCs higher proliferation was correlated with the overexpression of cell proliferation genes and DPSCs lower senescence was correlated with the increase of growth factor activity genes (Kang et al., 2016). In other research, the differentiation capability was compared between DPSCs, SHEDs, BM-MSCs and synovial fluid cells (SFCs), all of them MSCs. Whereas BM-MSCs and SFCs showed higher osteogenic and chondrogenic capacity, DPSCs and SHEDs had higher neurogenic ability. In the same work SHEDs demonstrated higher proliferation rates compared DPSCs and BM-MSCs (Isobe et al., 2016). As was expected due to developmental origin of SHEDs, they showed more stem cell markers than DPSCs. In addition, SHEDs had higher osteogenic capabilities than DPSCs, but less than BM-MSCs, which also express less stem cell markers (Aghajani et al., 2016). iPSCs, are main stem cell source to study neurogenetic syndromes. There are in literature multiple research focused on the efficiency and safety of DPSCs reprogramming into iPSCs. It is a fact that DPSCs intrinsically express stem cell markers even before to be induce, hence, some authors hypothesize these cells could be more efficient source of creating iPSC than cells traditionally used for it (Pisal et al., 2018; Yan et al., 2010).

1.2.5.4.3 Serum-free cultures and dentosphere formation

Most differentiation treatments consists of fetal serum (bovine or calf) addition in order to provide cells proliferation and monolayer adherent growth. The use of serum, committed DPSCs to osteo/odontoblastic lineages, following their intrinsic path (Pisciotta et al., 2012; Yu et al., 2010). Furthermore, xenogeneic elements could cause immune reactivity and even rejection of transplanted cells in cell therapies (Gregory et al., 2006; Horwitz et al., 2002). Since MSCs were defined as cell-based medicinal products (CBMPs), DPSCs need to be prepared under hard culture measurements in order to achieve good manufacturing practice (GMP) required quality standards, as described in EU Regulation 2003/94/EC (Pacini, 2014). Serum free medium culture protocols has been studied with the purpose of avoid these problems. Al contrary of what showed mDPSCs (Zainal Ariffin et al., 2013) there is no evidence of spontaneous osteo/odontoblastic differentiation of human DPSCs (hDPSC). Serum-free based studies demonstrated the viability of hDPSCs in this condition as well as the maintenance of stem characteristics (Hirata et al., 2010). As we said above the NC origin of hDPSCs grants to these cells some special features.

Although *in vitro* formation of neurospheres is a singular neural stem/progenitor cell characteristic (Pineda et al., 2013), it is not a surprise, due to their EMSCs origin, the capacity of hDPSCs to form neurospheres like structures in serum-free conditions, called dentospheres. There is a controversy about delicate regulation of dentosphere differentiation ability in literature, which is essential to control the role and capacity of DPSCs derived dentospheres. Some works describe osteogenic pre-disposition achieved by dentosphere-like DPSCs in serum-free conditions (Bakopoulou et al., 2017; Bonnamain et al., 2013), while other works defend the increase of pluripotential markers in dentospheres increase when DPSCs are cultured in serum-free medium for several days (Hirata et al., 2010; Lee et al., 2017; Xiao and Tsutsui, 2013). Not all the authors are agree on the timing of dentosphere formation during neural differentiation, Gervois et al. showed dentosphere formation in the initial differentiation phases, while Karbanova et al. demonstrated this formation during late phase. However, both of them as well as other several studies suggested a direct link between the differentiation into neural lineages, even dopaminergic neuron-like cells were obtained, and the expression of numerous neural markers in dentospheres (Chun et al., 2016; Gervois et al., 2015a; Karbanová et al., 2011; Osathanon et al., 2014).

1.2.5.4.4Neuroprotective and immunomodulative properties of DPSCs

As important as pluripotent differentiation capability is the expression and secretion potential of nerve growth factor (NGF), brain- derived neurotrophic factor (BDNF), glial cell line derived neurotrophic factor (GDNF), neurotrophin 3 (NT-3), vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) showed by DPSCs (Mead et al., 2014; Sakai et al., 2012). It is both *in vivo* and *in vitro* demonstrated the higher expression of these factors by DPSCs compared with BM-MSCs and AT-MSCs (Caseiro et al., 2016). These results support the idea of that DPSCs exhibit bigger neuroprotective characteristic to face nervous system related affections. It has been described in the literature the importance of these factors. For example, some groups have identified the role of trophic factors, i) in the reduction of neurodegeneration in the early stages of neural apoptosis and sensory neuron survival (Nosrat et al., 2001) or ii) in the promotion of axon regeneration in spite of axonal growth inhibitor (Arthur et al., 2009; Kolar et al., 2017) in spinal cord injury (SCI) model. In the same way but in ischemic injury of astrocyte model, DPSCs provide both direct and indirect cytoprotection (Song et al., 2017).

Beyond of trophic factor secretion, it has been also documented the importance of immunomodulatory and anti-inflammatory factors in DPSCs-based tissue regeneration assays. The expression of interleukin-8 (IL-8), interleukin-6 (IL-6), and TGF- β via Toll-like receptor (TLR) 4 as well as TGF-β, HGF and indoleamine 2,3-dioxygenase (IDO) by DPSCs has been reported (Bianco et al., 2016; Özdemir et al., 2016; Tomic et al., 2011). TGF-β, HGF and IDO are able to suppress both the activation of T cells and the proliferation of peripheral blood mononuclear cells, even allogeneic immune response was inhibited (Kwack et al., 2017; Sugita et al., 2015). In addition, IL-8 preserves axon integrity in SCI crush injury (He et al., 2013). The co-culture of DPSCs and T cells resulted in human leukocyte antigen-G, vascular adhesion molecule-1, intracellular adhesion molecule-1, IL-6, TGF-β, HGF, and IL-10 secretion. Moreover, proinflammatory IL-2, IL-6 receptor, IL-12, IL-17A and tumor necrosis factor-α (TNF-α) cytokines were downregulated (Demircan et al., 2011), as well as the proliferation rate of T cells was inhibited in 90% (Pierdomenico et al., 2005). In another research, intraperitoneally injected DPSCs showed a remarkable modulation effect increasing $CD4^+CD25^+FoxP3^+$ regulatory T cells (Hong et al., 2017). In conclusion, these immunomodulatory effects could be a key to create safe and effective allogenic and autogenic stem cell therapies.

1.2.5.4.5 Differentiation of DPSCs

Due to their EMSC condition, it is not unexpected the relative ease to differentiate DPSCs into specialized connective tissue cells, such as adipocytes, chondroblasts and osteo/odontoblasts (Gronthos et al., 2002; Kawashima, 2012). However, as already is described above, the EMSC origin of DPSCs provide them to become easily into at least two of the three germ layer lineage cells, though, they can differentiate into cells of three germ-layers.
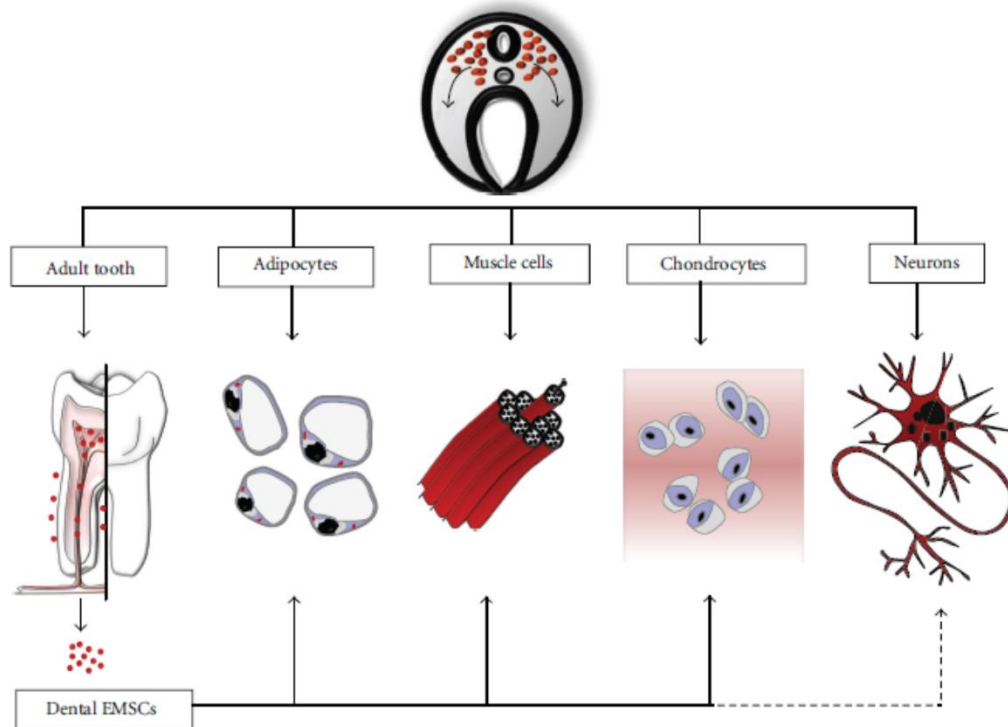

Figure 11. DPSCs derived mesodermic and ectodermic cell types (Ibarretxe et al., 2012)

<u>Ectoderm:</u> Two of the most important advantage of DPSCs compared with other MSCs are the neural crest origin and the neuron-like characteristics that facilitate *in vitro* induction of DPSCs into functional neurons. In the last years have been developed many neural differentiation protocols. The use of growth factors, neurotrophins and some other small molecules, such as fibroblast growth factor (bFGF), epidermal growth factor (EGF), NGF, BDNF, GDNF, sonic hedgehog , NT-3, retinoic acid (RA), forskolin and heparin have been necessary in these differentiation inductive mediums. Also, B27, ITSx , non-essential amino acids, and N2 culture supplements have been commonly used in these protocols (Arthur et al., 2008; Chang et al., 2014; Gervois et al., 2015a; Kanafi et al., 2014; Király et al., 2011; Osathanon et al., 2014; Xiao and Tsutsui, 2013; Zhang et al., 2017) (figure 11 and 12).

The most of the works above mentioned and the works described in paragraph 1.3.4.3, have in common the creation of dentospheres as an essential step in neural differentiation

protocols. However, some groups have been able to through along this step. Using endogenous environmental factors and multistep inductive protocols they achieved specialized motor and dopaminergic sub-lineages (Chang et al., 2014; Gnanasegaran et al., 2018; Kanafi et al., 2014). Even spiral ganglion neuron-like cells were obtained after BDNF, NT-3 and GDNF treatment of DPSCs (Gonmanee et al., 2018).

Leaving aside typically used neural differentiation confirmation hallmarks, such as NeuN, MAP-2, DCX and neural adhesion molecules, few studies used electrophysiological assay in order to deepen in functional neural differentiation of DPSCs. However, it is not totally clear the genuine neuron differentiation from DPSCs as far as no action potential burst has been described, although voltage-gated sodium and potassium functional channel expression have been demonstrated (Arthur et al., 2008; Gervois et al., 2015a; Király et al., 2009).

Several investigations support the idea of either glial or neuronal precursor differentiation. Oligodendrocytic differentiation of mDPSCs was used to show the clonal heterogeneity into dental pulp, where only nestin positive clones were able to undergo differentiation (Young et al., 2016). Genetically modified human DPSCs turn into oligodendrocyte progenitor cells after *Olig2* gene overexpression (Askari et al., 2014). In addition, Martens et al. were able to differentiate DPSCs into functional Schwann cells, which could generate myelin sheath around axons in a 3-dimensional collagen type I hydrogel neural tissue construct (Martens et al., 2014).

Mesoderm: The differentiation toward osteoblasts, adipocytes and chondroblasts is one of the criterion required by ISCT to consider DPSCs as MSCs (Dominici et al., 2006).

The osteogenic differentiation has been quite well documented in several researches. Like occurs with other differentiation protocols, also osteodifferentiation needs some components commonly repeated in almost all receipts. Dexamethasone, L-ascorbic acid and β-glycerol phosphate are key elements to achieve alkaline phosphatase, collagen type I, osteocalcin, osteonectin, osteopontin, osterix and runt-related transcription factor 2 (RUNX2) expressing DPSCs derived osteoblast-like cells (Ajlan et al., 2015; Atari et al., 2012a; Bhuptani and Patravale, 2016; Goto et al., 2016; Riccio et al., 2010). Indeed, RUNX2 is one of the most important osteo/odontoblastic differentiation regulator. It activates osteoblast gene expression and mineralization in the early stages of osteoblast differentiation (Vimalraj et al., 2015; Xu et al., 2015b). RUNX2 is also responsible of odontoblastic potential through dentin sialophosphoprotein (DSPP) activation, which encodes both dentin sialoprotein and phosphoprotein (Han et al., 2014). DSPP and dentin matrix protein 1 (DMP1) upregulated expression along with alkaline phosphatase activity increasing are the hallmarks of ondotoblastic differentiation (Abuarqoub et al., 2015; Paduano et al., 2016).

There are evidence of adipogenic differentiation of DPSCs by insulin, dexamethasone and 3-isobutyl-1-methylxanthine (IBMX) containing inductive medium. Both O staining of lipid droplets and the expression of peroxisome proliferator–activated receptor γ, glucose transporter type 4, fatty acid binding protein 4 and lipoprotein lipase adipogenic markers are necessaries to confirm differentiation (Grottkau et al., 2010; Lee et al., 2015; Zhang et al., 2006).

Chondroblastic differentiation is given by inductive medium composed of ITSx, dexamethasone, L-ascorbic acid, L-proline , sodium pyruvate and TGF-β3 (Hilkens et al., 2013; Jang et al., 2016; Nemeth et al., 2014). TGF-β protein superfamily plays an key role in chondrogenesis main phases, being the controller of differentiation/de-differentiation activities in the cartilage (Dexheimer et al., 2016).

DPSCs are able to differentiate into less common cell types, such as cardiomyocytes (Ferro et al., 2012) and smooth muscle (Song et al., 2016). Endothelial cells were obtained culturing DPSCs at least for 30 days in a-MEM culture medium with 20% (d'Aquino et al., 2007). Endothelial-like cells were also achieved by Marchionni et al. using DMEM with 2% FBS and 50 ng/ml VEGF for 7 days (Marchionni et al., 2009) (figure 11 and 12).

DIFFERENTIATED DPSCs

Osteoblast Adipocyte Chondroblast Neuron

Odontoblast Cardiomyocite Endothelial cell

Hepatocyte Pancreatic cell Smooth muscle
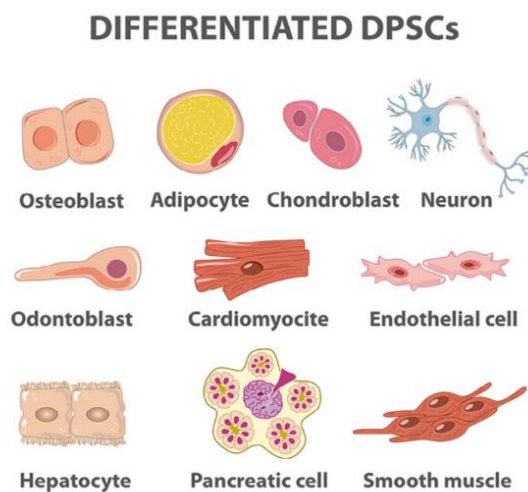
Figure 12. DPSCs derived three germ-layer cell types (Anitua et al., 2018)

<u>**Endoderm:**</u> Contrary to what was thought years ago, not only ectoderm and mesoderm lineage cells could be obtained from hDPSCs. New researches have shed light into pluripotential ability of DPSCs. It is confirmed in the last five years, the huge plasticity of these cells, even achieving endodermal lineage cell differentiation (figure 12). Hepatic differentiation of human DPSCs was successfully demonstrated by the expression of several hepato-specific markers. All these

investigations were carried on using serum-free or low serum (1%-2%) concentrations combined with at least two of the next elements; HGF, ITSx, dexamethasone and oncostatin M (Chen et al., 2016; Ferro et al., 2012; Han et al., 2017; Ishkitiev et al., 2012) . Also pancreatic cell lineage differentiation has also been reported from human DPSCs. Achieved cells were able to produce insulin, glucagon, somatostatin and pancreatic polypeptide as well as the expression of pancreatic markers were enhanced. Ishkitiev et al. showed that CD117+ cells were the most capable cells to undergo pancreatic differentiation. Moreover, Yagi Mendoza et al. figured out the PI3K/AKT and WNT pathways key role in pancreatic cell differentiation, while Carnevale and his group determined that the differentiation began at day 7 (Carnevale et al., 2013; Ishkitiev et al., 2013; Yagi Mendoza et al., 2018).

1.2.5.4.6 DPSC based regeneration therapies

Apart of differentiation capability it is must be taking in consideration the immunomodulatory, angiogenic and neuroprotective properties of DPSCs (Luo et al., 2018). All these features make DPSCs the ideal source to use in regeneration therapies, specifically in nerve tissue regeneration due to NC origin shared with PNS cells. Furthermore, vasculogenesis may be essential in neural tissue regeneration, providing nutrients and oxygen to injured tissue
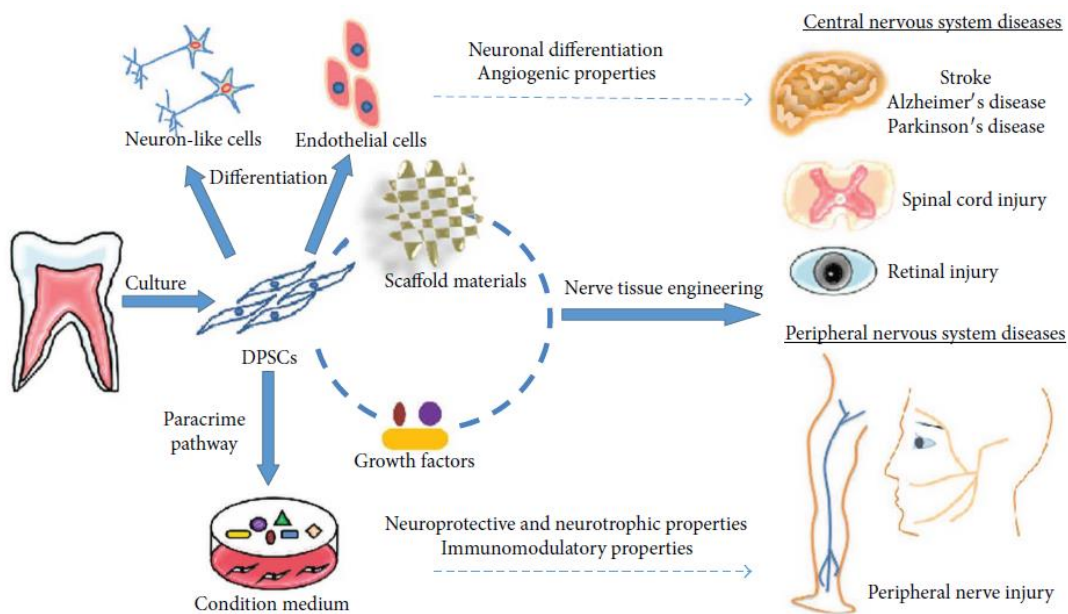
area (figure 13).

Figure 13. DPSCs and scaffold constructs to application in nervous system diseases (Luo et al., 2018)**.**

1.2.5.4.6.1Vasculogenesis and angiogenesis by DPSCs

Vasculogenesis and angiogenesis are essential processes necessary to create the vascular network during embryonic development. Although these two terms often are used like synonymous, actually, vasculogenesis is the formation of new blood vessel *de novo* while angiogenesis is the formation of new blood vessels from existing vessels. Both of them are very necessary to maintain normal blood supply needed to maintain normal function of organs and tissues (Patan, 2004). Some angiogenic trophic factors are the responsible of creation of new blood vessels (Carmeliet, 2000). Like the most of MSCs, it is described the capacity of DPSCs to secrete these angiogenic factors; colony-stimulating factor, interleukin-8, angiogenin, endothelin-1, angiopoie tin-1, and insulin-like growth factor binding protein-3 (Bronckaers et al., 2013; Ratajczak et al., 2016). In combination with secretion of VEGF, PDGF, bFGF, and NGF stimulatory growth factors (Mead et al., 2014; Tran-Hung et al., 2008), DPSCs can promote tubulogenesis, proliferation and survival of endothelial cells (Tran-Hung et al., 2006). Furthermore, Bronckaers et al. discovered that migration and vasculogenesis were activated by PI3K/AKT and MEK/ERK pathway of endothelial cells. Taking together the endothelial differentiation capability and angiogenic and stimulatory factor expression DPSCs could promote therapeutic angiogenesis (Psaltis et al., 2008). Even in 1999 Takahashi et al. began to use stem cells and EPCs to stimulate vasculogenesis in order to treat ischemic disease (Takahashi et al., 1999). Then, in 2008, rat DPSCs were transplanted into myocardial infarction (MI) model. The formation and function of new blood vessels were improved and the infarct size reduced by presence of DPSCs (Gandia et al., 2008). Three years later, focal cerebral ischemia rat model was injected with porcine cd31$^-$/cd146$^-$ DPSCs, resulting in vasculogenesis induction and ameliorated ischemic brain injury after middle cerebral artery occlusion (MCAO) (Sugiyama et al., 2011). One of the few studies focused in human DPSCs in rodent ischemia model suggested the paracrine dependent effect of DPSCs in post-stroke recovery, given importance to non-neural replacement therapies (Leong et al., 2012). Also the combination of DPSCs with HUVECs, showed the formation of microvessel-like formation, which suggests that DPSCs could act as perivascular cells for *in vivo* angiogenesis (Nam et al., 2017). Recently, the use of new 3D print top technique has facilitated the creation of vascularized dentin/pulp structure (Hilkens et al., 2017).

1.2.5.4.6.2Neural regeneration by DPSCs

Neuroregeneration therapies have ever been a priority research due the social, economic and dependency burden suffered by both patients and their close people. The use of NSCs should be crucial in nerve tissue regeneration therapies, however, the low incidence of these

cells in adult organism as well as obtaining difficulties force researchers to find new stem cell sources. Taking in consideration the large amount and relatively easy access to them, MSCs have been selected as ideal source to fill the hole left by NSCs (Kerkis et al., 2006; Kim et al., 2012). Nevertheless, not all MSCs are optimal for these purpose, in fact, DPSCs have the needed properties, such as, embryonic pluripotent and neural stem cell markers, NC origin, neural differentiation ability and they can be harvested from the own patient without harmful intervention. Furthermore, although DPSCs are less abundantly available compared with other MSCs, they present higher proliferation rates (Gronthos et al., 2000). Moreover, using own cells of the patients to use in autologous therapy, it possible to avoid immune reactivity, which could be a fatal event in neural tissue. Several investigations have been carried on in both central nervous system (CNS) and peripheral nervous system (PNS). Some of them are based in cell-cell substitution, but overall, these researches are secretome-based cell therapies.

1.2.5.4.6.2.1 Central nervous system (CNS)

Central nervous system is the organ that integrate environmental signals and controls and coordinates the response for them. It is composed by brain and spinal cord. Stem cell treatment as novel CNS disease therapy is already a reality (Tatullo et al., 2015; Varga and Gerber, 2014). The low precursor cell population (Mead et al., 2017), expression of myelin-associated growth inhibitory factors (Geoffroy and Zheng, 2014) and the intrinsic formation of glial scar (Stichel and Müller, 1998), make the repair and regeneration capacity of CNS especially weak in front of several insults.

-Neurodegenerative diseases: Alzheimer´s (AD) and Parkinson´s disease (PD) are the first and second commonest age-related neurodegenerative diseases. Both of them have been extensively studied and new DPSC-based cell therapies have been published in the last years (Apel et al., 2009). AD is neurodegenerative pathology caused by intracellular neurofibrillary tangles, neuronal loss and generation of insoluble peptide deposits. These physiological changes lead to well-known symptomatology; that comprehend memory loss, cognitive deficits and linguistic disorders, among others (Huang and Mucke, 2012). Both *in vivo* and *in vitro* AD models have been used to demonstrated the benefit in behavioral and physiological deficits of use DPSCs based cell therapies (Apel et al., 2009; Shin et al., 2014). Moreover, other AD disease models have been tested. Thus, okadaic acid induced AD was repaired by DPSCs promoted neuronal regeneration, microtubule stability protection and maintaining cytoskeletal structure (Wang et al., 2017a). Two researches pointed that DPSCs demonstrated high secretion of VEGF, fractalkine, RANTES, and fms-related tyrosine kinase 3, seem to be important in the reduction

of amyloid beta (Aβ) peptide-induced cytotoxicity and apoptosis (Ahmed et al., 2016; Mita et al., 2015).

DPSCs have been broadly used to dopaminergic (DA) neuron obtaining *in vitro* (Chang et al., 2014; Gnanasegaran et al., 2018; Kanafi et al., 2014). Parkinson´s disease (PD) progressive neurodegeration results in loss of DA neurons of nigrostriatal pathway. The loss of DA neurons causes bradykinesia, resting tremor, muscle rigidity and postural instability in patients (Dauer and Przedborski, 2003). Like it is described with other CNS affections, DPSCs showed anti-inflammatory activity is a key in damaged tissue recovery. Thus, intrathecal transplantation of DPSCs into 1-methyl-4-phenyl-1,2,3,6-tetrahydropyri- dine- (MPTP) induced old-aged PD mouse model achieved behavioral deficits recovery and DA function restoring by upregulation of IL2, IL4, and TNF-β anti-inflammatory factors, while the secretion of pro-inflammatory factors IL-1α, IL- 1β, IL6, IL8, and TNF-α were reduced (Gnanasegaran et al., 2017a). Also *in vitro*, MPTP associated deficits were reduced by neuroimmunomodulatory activity of DPSCs (Gnanasegaran et al., 2017b). In addition, a different PD model deficits, in this case a model created by 6-hydroxydopamine (6-OHDA) both *in vitro* (Chun et al., 2016; Nosrat et al., 2004) and *in vivo* (Fujii et al., 2015), were solved by DPSCs promoting of DA neurons survival.

-Stroke: It is defined as blood supply interruption or reduction of the brain, which deprives brain tissue and could cause either brain death, long-term disability, even the death (Sughrue et al., 2004). The interest in this affection is huge since it is the second leading cause of preventable death in worldwide and the fourth leading cause of lost productivity in 2012 (Kuklina et al., 2012). Damages undergone by brain during long interval of starving could be irreversible. Moreover, sadly there are few effective restorer therapies (Hossmann, 2006). Nowadays, the use of stem cells in this therapies seems optimistic due to the special properties showed by these cells (Lemmens and Steinberg, 2013). In regard to the use of DPSCs, some *in vivo* works demonstrated the therapeutic effectiveness of DPSCs injection into focal ischemic areas of MCAO in rats, either by cell-cell DA neurons substitution (Yang et al., 2009) or by trophic secretion (Sugiyama et al., 2011). Recently published research work shows how the intravenous administration of combination of rat DPSCs and BDNF in MCAO model rats can lead neurological function more effectively compared with either BDNF or DPSCs injection alone (Zhang et al., 2018b). Apart from neuronal replacement, song et al. showed the importance of secreted anti-inflammatory and trophic factors, using both DPSCs and conditioned medium of DPSCs to human astrocyte cytoprotection in model of oxygen-glucose deprivation (OGD) ischemia in *in vitro* (Song et al., 2015).

-*SCI:* SCI can cause partial or complete loss of sensory and motor control of the body depending on injury severity and location. World health organization (WHO) estimates that between 250 000 and 500 000 people suffer this affection every year, in 2018. SCI can divided in two independent physiopathologic intervals. The first comprehend nerve and vascular tissue destruction, and the second one, which causes neuro-inflammatory responses such as, excitotoxicity, apoptosis, oxidative stress, glial scar formation) (Jiang et al., 2014). NC origin and ectomesenchymal properties have made DPSCs very popular in SCI therapies. DPSCs have shown the ability to differentiate in both neuron and oligodendrocyte-like cells that provide a new source of cell to avoid initial primary tissue disruption (Yamamoto et al., 2014a, 2014b). In the same way, DPSCs can attack inflammatory secondary injury, reducing progressive hemorrhagic necrosis related with IL-1β, ras homolog gene family member A (RhoA), and expressing sulfonyl-urea receptor1 (SUR1) that leads axonal regeneration by neurotrophic factors production (Nosrat et al., 2001; Yang et al., 2017). Combining DPSCs with chitosan, SCI derived cell apoptosis was inhibited and motor functional recovered (Zhang et al., 2016a).

-*Retinal Injury:* Photoreceptors, bipolar cells and retinal ganglion cells (RGCs) create the retina (Ramsden et al., 2013). Several affections can cause retinal injuries, such as, traumatic optic neuropathy (TON) and degenerative RGCs damaging glaucoma (Munemasa and Kitaoka, 2015). The low production of neurotrophic growth factors and axonal growth inhibitory molecules avoid retinal regeneration (Berry et al., 2008). To face it, use of DPSCs could provide the necessary factors to active patients own retinal neural survive and DPSCs neural differentiation toward both RGCs-like (Roozafzoon et al., 2015) and photoreceptors (Bray et al., 2014) *in vivo*. Indeed, Mead et al. intravitreally transplanted DPSCs after optic nerve injury in a rat model caused NGF, BDNF, and NT-3 related axonal recovery along with RGCs neuroprotection were reported (Mead et al., 2013). Also, glaucoma was attenuated per 35 days after DPSCs intravitreal transplantation by preventing RGCs death (Mead et al., 2016).

1.2.5.4.6.2.2 Peripheral nervous system (PNS)

PNS is divided into the cranial nerves, from III to XII, and the nerves supplying upper and lower limbs. They work as the link between CNS and limbs and organs (Catala and Kubis, 2013). Peripheral nerve injury and diseases often outcome in physical disability and neuropathic pain. Treatments that contemplate end-nerve suturing, tissue grafts and growth-stimulatory therapies are current clinical treatments (Battiston et al., 2005; Matsuyama et al., 2000; Pfister et al., 2011). Autologous nerve grafting is considered the gold standard for peripheral nerve long gap deficits (Tamaki et al., 2016). Autografting related disadvantages, donor nerve availability and morphometric mistakes can be solved combining nerve tissue engineering and stem cell

therapies (Pereira et al., 2014). Sasaki et al. achieved injured facial nerve regeneration and functional recovery using DPSCs (Sasaki et al., 2011). Another two similar researches showed regeneration of sciatic nerve after treatment with collagen conduits filled with Schwann-like cells derived from DPSCs (Martens et al., 2014; Sanen et al., 2017). Even genetically transformed DPSCs could act as functional oligodendrocytes *in vitro* and promoted peripheral nerve repair in a mouse model (Askari et al., 2015).

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Methodology

UPV EHU

Alizarin Red assay

To test osteogenic potential after three weeks of culture, extracellular calcified bone matrix deposits were stained using Alizarin Red. Briefly, hDPSCs were fixed for 30 minutes with 10 % formaline (#F7503, Sigma, St. Louis, MO, USA), rinsed and stained using 2 g/100 ml Alizarine Red S (#400480250, Fisher Scientific, Hampton, Nou Hampshire, USA), pH 4.3 for 45 minutes. After three PBS rinses of 5 min, Alizarin Red absorbance was measured at 450 nm using a Synergy HT Multi-Mode Microplate Reader (Biotek,Winooski, Vermont, USA).

Animals and cell graft

Consanguine c57bl6 litters from Nesti-GFP mice and Athymic Swiss[nu/nu] were used as hosts for murine and human *in vivo* graft purposes. Nestin-GFP neurospheres or DPSCs dentospheres in the active growth phase were disaggregated, washed and collected in Neurocult serum-free media. Two microliters containing 100,000 cells were injected (0.5 µL/min) unilaterally at the following coordinates (from bregma): $AP = -1.9$, $L = -1.2$, and $DV = -2$ and -2.1. The cell transplantations were performed using a small animal stereotaxic apparatus (Kopf model 900) with a 10 µl Hamilton syringe and a 33 G needle (Hamilton, Bonaduz, Switzerland). All surgical procedures were performed under anesthesia with ketamine (75 mg/kg, Imalgen; Merial,Lyon, France) and medetomidine (1 mg/kg, Domitor; Pfizer, Paris, France). After the surgery, paracetamol (1.64 mg/mLDoliprane; Sanofi, France) was administered in the drinking water for 1 week. Animals were provided with food and water ad libitum and housed in a colony isolator maintained at a constant temperature of 19–22 °C and humidity (40–50%) on a 12:12 h light/dark cycle. The animal experiments were performed in compliance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) and were approved by the competent authority (Administración Foral de Bizkaia).

Conventional PCR and quantitative real-time PCR (qPCR)

cDNA (20 ng/µl) was obtained by reverse transcription of total extracted RNA using iScript cDNA Kit (BioRad, 1708890), using the following reagents: iScript reverse Transcriptase (1µl), 5x iScript Reaction Mix (4ul) and Nuclease Free water (variable), obtaining a final volume of 20 µl. To perform comparative gene expression assays, a cDNA volume of 10 µl was used as a template for conventional PCR using My TaqTM Red Mix (Bioline, BIO-25043), at a 4 ng/µl concentration. The molecular length of the amplification products were checked by electrophoresis in a 2 % agarose gel. Quantitative Real-Time PCR experiments were performed in CFX96 touch real-time Detection System (BioRad) with Power SYBR® Green PCR Master Mix 2x (4367659, Applied Biosystems [TM]). Experiments were carried out using 0.5

µl Primer and 3 ng/µl sample concentration. The molecular weights of the amplification products were checked by electrophoresis in a 2 % agarose gel. All reactions were performed in triplicate and the relative expression of each gene was calculated using the standard $2^{-\Delta\Delta Ct}$ method. Primer pairs used were obtained through either the Primer-Blast method (Primer Bank) or other publications.

Flow cytometry

Half-million DPSCs were enzymatically disaggregated and then incubated in PBS 0.15% bovine serum-albumin (BSA) solution with CD90-FITC 1:50 (Biolegend, San Diego, California, USA), CD105-PE 1:50 (eBioscience,Waltham, Massachusetts, USA), CD73-APC 1:50 (eBioscience,Waltham, Massachusetts, USA), CD45-APC 1:50 (Biolegend, San Diego, California, USA) or IgG2a κ Isotype control (Biolegend, San Diego, California,USA) for 40 minutes on ice. Then, DPSCs were washed with PBS 0.15% BSA after staining, then cells were resuspended in 300 µl of PBS 0.15% BSA and analyzed using a FACS Beckman Coulter Gallios (Beckman Coulter Life Sciences, Indianapolis, United States). Flowing Software 2.5 (University of Turku, Finland) was used for data analysis.

hDPSC isolation and culture

Primary hDPSCs were isolated from human third molars from healthy donors between 15 and 40 years of age, who gave their informed consent. Teeth were fractured mechanically, and the dental pulp enzymatically digested by 3 mg/ml collagenase and 4 mg/ml dispase (ThermoFisher Scientific, Waltham, Massachusetts, USA) for 1h at 37 ℃. After centrifugation at 15000 rpm for 5 minutes, cells were mechanically dissociated by 18G needles (BD Microlance). Then DPSCs were grown in different culture mediums depending on our interests.

DMEM: Then, DPSCs were cultured in Dulbecco's modified Eagle´s medium (DMEM, Lonza, Basel, Switzerland) supplemented with 10% of inactivated FBS (Hyclone, GE Healthcare Life Sciences, Logan. UT, USA), 2 mM L-glutamine (Sigma, St. Louis, MO, USA) and penicillin 100 U/ml + streptomycin antibiotics 150 µg/ml (Gibco, Karlsruhe, Germany). Culture media were renewed every 2-3 days. Using this protocol, hDPSCs were maintained in optimal conditions even up to 3 months, with a total accumulation of 6 passages as maximum. It is the optimal medium to cell proliferation and maintainance.

StemPro MSC SFM™ : Once the DMEM+FBS 10% culture was in confluence hDPSCs were subcultured in well defined culture medium named STP for one week, which was composed of StemPro MSC SFM basal medium (Gibco, Karlsruhe, Germany) supplemented with StemPro MSC SFM supplement (Gibco, Karlsruhe, Germany) at 9:1 ratio and in presence of antibiotics penicillin 100 U/ml and streptomycin 150 µg/ml (Gibco, Karlsruhe, Germany).

StemPro MSC SFM™ + DBDNF/NT3: After 1 week of culture, the STP medium was replaced with STP+NTP medium, consisting in the same formulation described above with the addition of 500 ng/ml BDNF (Peprotech, London, UK) and 20 ng/ml NT-3 (Peprotech, London, UK). Cells are grown in this medium for 7 days at maximum.

Neurocult™ proliferation: DPSCs were cultured in Neurocult™ proliferation medium after enzimatical disossiation for 7 days. The dentospheres here achieved had the capacity to provide more self-renewing cells in each passage. Human Neurocult medium composed of Human Neurocult NS-A basal medium (cat# 05750, Stem Cell Technologies, Vancouver, Canada) with Neurocult proliferation supplement (cat# 05753, Stem Cell Technologies, Vancouver, Canada) at 9:1 ratio, and supplemented with Heparin solution 2 µg/ml (cat# 07980, Stem Cell Technologies, Vancouver, Canada), b27 without VitA, EGF 20 ng/ml and FGFb 10 ng/ml (Peprotech, London, UK) as previously described in presence of antibiotics penicillin 100 U/ml and streptomycin 150 µg/ml (15140-122, Gibco).

hDPSCs differentiation protocols

The initial seeding for all conditions was 15000 cell/cm^2 using non-coated or coated cover slips.

Osteogenic differentiation: These cells were first cultured in DMEM+10% FBS, STP, STP+NTP. Then, culture media were rinsed and changed to DMEM+10% FBS, 20 µM β-glycerol-phosphate (Sigma, G9422, St. Louis, MO, USA), 10 nM dexamethasone (D4902, Sigma, St. Louis, MO, USA), and 52 nM ascorbic acid (#100468, Merck, Darmstadt, Germany) for three weeks.

Neurogenic differentiation: Neurogenic induction was achieved using Neurocult™ medium with differentiation supplement at 9:1 ratio (Stem Cell Technologies, Vancouver, Canada), Heparin at 2 µg/ml (Stem Cell Technologies, Vancouver, Canada), EGF at 20 ng/ml, FGFb at 10 ng/ml (Peprotech, London, UK), 2% of B27 supplement (Thermofisher, Waltham, Massachusetts, USA) and a mixture of antibiotics penicillin/streptomycin at 100 U/ml and 150 µg/ml respectively (Gibco, Karlsruhe, Germany). DPSCs were grown for at least 7 days in laminin coated coverslips. These cells were not grown in other medium before this.

Endotelial differentiation: Our results demonstrated that DPSCs cultured in human Neurocult medium composed of Human Neurocult NS-A basal medium (cat# 05750, Stem Cell Technologies, Vancouver, Canada) with Neurocult proliferation supplement (cat# 05753, Stem Cell Technologies, Vancouver, Canada) at 9:1 ratio, and supplemented with Heparin solution 2 µg/ml (cat# 07980, Stem Cell Technologies, Vancouver, Canada), b27 without VitA, EGF 20

ng/ml and FGFb 10 ng/ml (Peprotech, London, UK) as previously described in presence of antibiotics penicillin 100 U/ml and streptomycin 150 µg/ml (15140-122, Gibco) could be differentiated into endothelial cells. These cells were not grown in other medium before this.

Immunostaining of brain sections and cell culture

Animals were deeply anesthetized with Avertin 2.5 % and transcardially perfused with a 4 % paraformaldehyde solution in 0.1 M sodium phosphate, pH 7.2 and processed. After post-fixation overnight at 48ºC, the brains were embedded in agarose and then cut into 20µm sections using a VT 1200S vibratome (Leica, France). In order to detect grafted genetically unmodified human DPSCs on mice brain, specific antibodies targeted to human Nestin (MAB1259, 1:200 R&D systems)[49], and human CD31 (BBA7, 1:200 R&D systems) were used. Immunostaining of brain vasculature was developed using CD31 (550247, 1:300 BD Pharmingen), laminin (L9393, 1:200 Sigma, St. Louis, MO), and VEGF (ABS82-AF647, 1:200 Sigma, St. Louis, MO) antibodies.

After a variable days of cell culture, they were fixed by incubation with 4 % PFA for 10 minutes at room temperature and permeabilized by incubation in 0.1 % Triton X-100. They were then incubated overnight at 4 ºC with primary antibodies at the following dilutions: Glial Fibrillary Acidic Protein (GFAP) (MAB3402, 1:400; Millipore, Lake Placid, NY), Nestin (NES, 1:200 Aves Labs), S100ß (Z0311, 1:1000, Dako, Glostrup, Denmark), NeuN (EPR12763, 1:200; Abcam, Cambridge, UK), Doublecortin (DCX) (sc-8066, 1:200; Santa Cruz, Dallas, TX, USA), CD31 (550247, 1:300 BD Pharmingen, San Jose, CA , USA), Anti-STAT3 (phospho Y705) antibody (1:500, ab76315, Abcam), anti-human-CD31 (1:200, F8402, Sigma St. Louis, MO), VEGF (ABS82-AF647, 1:200 Sigma, St. Louis, MO) and laminin (L9393, 1:200 Sigma, St. Louis, MO). For both tissue sections and cell culture, secondary antibodies conjugated to Alexa 488, 568 and 647 Donkey anti-mouse, anti-rabbit or anti-goat were incubated for 2 h and 30 min respectively at room temperature. Preparations were counterstained with DAPI and images were captured using a Leica SP8 confocal microscope at 40X magnification.

Live cell calcium imaging

hDPSCs were grown on glass coverslips and allowed to grow and make contacts for 7 days. In order to register cell calcium imaging, 5 µM fura-2/AM or 2 µM fluo-4/AM (Invitrogen, Waltham, Massachusetts, USA) were incubated for 60 minutes at 37 ºC. Immediately, coverslips were placed in a flow chamber (Warner Instruments) with constant perfusion of Hank`s balanced salt solution (HBSS, Invitrogen, Waltham, Massachusetts, USA) supplemented with 10 mM Hepes (2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid) at

room temperature. Then, cells were alternately excited at 340 and 380 nm for fura-2 imaging, recording the emitted light above 510 nm. The F340/380 ratio was used as index of cytoplasmic $[Ca^{2+}]$. For fluo-4 imaging, single excitation at 488 nm and emission at 520 nm was used and data were plotted as $\Delta F/F$, where $\Delta F = F-F_0$. F is the fluorescence intensity of agonist-containing solution and F_0 is the fluorescence intensity of control solution. Recordings were performed using a C10600-10B Hamamatsu camera installed on an Olympus IX71 microscope. Data analysis was performed using manually selected regions of interest (ROI), including background subtraction using Fiji software. Results are based on recordings from hDPSCs of 3-4 patients cultured in different culture mediums.

NSCs culture

For NSC cultures isolated from Nestin-GFP mice, dissected hippocampi were removed with ice-cooled PBS-sucrose and either plated in 6 well-plates or T25 low attachment flasks with Neurocult complete medium (StemCell) that was supplemented with heparin (2 mg/ml), EGF (20 ng/ml; Invitrogen) and FGF2 (10 ng/ml; Invitrogen). Cells were maintained at standard conditions in a humidified 37 Cº incubator containing 5% CO_2. Neurosphere cultures were then passaged every 7 days by enzymatic disaggregation with Accutase (Sigma, St. Louis, MO).

RNA extraction and retrotranscription

Cell pellets from the different cultures were washed with PBS, trypsinized (T1426, Sigma) and centrifugated at 1500 rpm for 5 min. Then, they were immediately frozen at -80 ºC until use. RNA extraction was performed following RNeasy mini kit instructions (Qiagen, Hilden, Germany). RNA concentration was determined using Nanodrop Synergy HT (Biotek, Winooski, Vermont, USA) and purity was calculated by 260/280nm absorbance ratio. RNA retrotranscription to synthetize cDNA was performed starting from 1000 ng of the total extracted RNA using iScript cDNA Kit (1708890, Biorad, Hercules, California) following manufacturer instructions.

Statistical analyses

All results are presented as mean ± standard error, also referred as SEM. Statistical comparisons were performed employing Mann Whitney U test, Student's t-test, Kruskal Wallis followed by Dunn´s post hoc test or a one-way ANOVA followed by Bonferroni and Scheffe post-hoc tests. Statistical tests were performed by IBM SPSS Statistics v.22. Statistical significance was considered to be *p ≤ 0.05, ** p ≤ 0.01, ***p ≤ 0.001. Comparisons between

multiple groups were made using The number of independent experiments is shown in the respective section.

Western blot

Cells were counted and resuspended in a ratio of 20,000 cells /µl of RIPA lysis buffer (R0278, Sigma, St. Louis, MO) supplemented with protease (11873580001; Roche) and phosphatase inhibitors (78420; Thermo Scientific) to ensure the same cellular concentration for the different type of cells and culture media. From this, thirty micrograms of protein was diluted in RIPA buffer supplemented with LDS Sample Buffer (NP0007; Invitrogen by Life technologies). Then, samples were heated for 5 min at 95Cº and loaded into NuPAGE 4–12% Bis-Tris gels (NP0322BOX; Novex by Life Technologies). For electrophoresis, NuPAGE running buffer (NP001; Novex by Life Technologies) was used. Protein transfer was performed using nitrocellulose membranes (LC2000; Invitrogen by Life Technologies) and NuPAGE Transfer Buffer (NP0006-1; Invitrogen by Life Technologies) along with XCell Sure Lock Electrophoresis machine (NP0007, Novex, Life Technologies). Phosphorylated-ERK and total ERK antibodies (both 1:1000, #4370 and #4695 respectively, Cell Signaling Technologies) and phospho-Stat3 and Stat3 (both 1:1000, #9145 and #9132 respectively, Cell Signaling Technologies) were used to detect the angiogenic signaling pathways and Ponceau staining (P7170-1L, Sigma St. Louis, MO) were used as loading control to detect protein in the charged lanes.

Hypothesis

The angiogenic, immunomodulatory, anti-inflammatory, neuroprotective and pluripotent differentiation characteristics make the use of MSCs based therapies in the ideal cell source for neuroregeneration assays. However, FBS containing proliferative and differentiation mediums do not complete the demands GMP requirements to use these cells in human cell neuroregenerative therapies. In addition, the most of MSCs show two inconvenience to use them in neuroregenerative cell therapies. 1)do not keep close relationship with ectoderm germ layer, the origin of neural cells, which means a necessity to undergo a lineage trans-differentiation to achieve neurogenic fate, and 2) often is needed an invasive intervention to acquire them.

Thus, our hypothesis defend that, NC origin and ectomesenchymal properties of DPSCs would provide more effective and safe neurogenic differentiation protocol in serum-free culture mediums. Altogether, with the high accessibility, we think that DPSCs are the best option to autologous neuroregenerative cell therapies.

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Objectives

The main objective of this work has been the design of serum-free, safe, effective and transplantation permissive, neural differentiation protocols with the aim to assess the neurogenic potential of DPSCs without use neither transgenic nor epigenetic modifications.

Specific objectives

1. Ectomesenchymal characterization of DPSCs cultured in serum-free STP, specific stem mesenchymal culture medium.

2. Comparison of the main morphological and proliferative features between DPSCs cultured in DMEM + FBS 10% and serum-free STP.

3. Test the functionality of the main expressed neurotransmitter and voltage dependent calcium channels in both DMEM + FBS 10% and serum-free STP.

4. Creation of new neurodifferentiation protocol using newly discovered properties promoted by serum-free STP.

5. Assessment of the neuro/glio-genic potential of DPSCs when they are pre-cultured in different mediums.

6. Evaluation of both ectomesenchymal, endothelial and neuro/glio-genic potential in Neurocult proliferation™, specific proliferation medium for NSCs.

7. Evaluation of both ectomesenchymal, endothelial and neuro/glio-genic potential in Neurocult differentiation ™, specific differentiation medium for NSCs.

8. Evaluation of *in vivo* histo-integration, neuro/glio-genic and vasculogenesis potential of hDPSCs in mice brain engraftment.

Results and Discussion

Chapter I: *Dental pulp stem cells as a multifaceted tool for bioengineering and the regeneration of craniomaxillofacial tissues.* **(Review).**

Chapter II: *Human dental pulp stem cells grown in neurogenic media differentiate into endothelial cells and promote neovasculogenesis in the mouse brain.* **(Research article).**

Chapter III: *BDNF and NT3 reprogram human ectomesenchymal Dental Pulp Stem Cells to neurogenic and gliogenic Neural Crest progenitors cultured with serum free medium.* **(Research article).**

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General Discussion

Neural tissue related affections embrace from neurodegenerative diseases, such as AD and PD, to stroke and traumatic injuries. Every year, between 250 000 and 500 000 people suffer SCI around the world according to World Health Organization (WHO). The high incidence, the lack of effective treatment, the invalidating being and derived economic and social burden of the aforementioned diseases (Lopez, 2011) , create a primordial necessity to develop and design new neuroregenerative therapies.

Last years, several works have described the use of stem cells in innovative regeneration strategies of disrupted and degenerated nerve tissue. The use of genuine NSCs as precursor of newly generated neural cells should be the best way to nerve fiber reconstruction (Steward et al., 2013). However, the poor amount of NSCs present in adult human body and the high difficulty to access to NSCs location niches, turn these cells in useless for realistic autologous cell therapies.

MSCs have been described as the one of the most available and well-known stem cells in adult human body. They can be isolated from umbilical cord, bone marrow, adipose tissue and dental structures, among others (El Omar et al., 2014). Despite of existing SCI reparation effective therapies with conventional mesenchymal stem cells (Vaquero et al., 2018), the importance of DPSCs in neuroregeneration has been increasing since few years ago. The NC origin shared with PNS, the ectomesenchymal features, as well as low invasive extraction procedure, draw DPSCs as very compatible source to innovative regeneration approaches (Aurrekoetxea et al., 2015).

The culture of human DPSCs has gone hand in hand with FBS supplementation, that is composed of not stablished nutrients, factors and elements of animal origin. The use of serum in cultures of human DPSCs provides high proliferation rates and allows long duration cultures. However, the cell culture supply with 10-20% FBS is also related with of mesenchymal stem cell phenotype promotion. Furthermore, previous works describe the tendency of human DPSCs to spontaneously differentiation into osteo/odonto-blasts after expose them to relatively high FBS concentrations for long periods (Pisciotta et al., 2012; Yu et al., 2010). In addition, several side effects such as allergies and immune reactions are related with the use of xenogenic serum (Gregory et al., 2006; Horwitz et al., 2002). These serum related issues, make FBS useless for human neuroregeneration therapies.

This research work has focused on design, development and test of serum-free commercial culture mediums in order to obtain cells that could be used in human neuroregenerative cell therapies.

We use in our hDPSCs cultures Neurocult™ and StemPro MSC™ (STP) serum free culture mediums instead of serum containing common used DMEM+ FBS 10-20%. Both Neurocult™ and STP are genuinely employed in specific cell type cultures. Thus, Neurocult™ is currently used in NSCs and progenitor culture *in vitro,* while STP is especially formulated for human mesenchymal stem cell expansion.

The use of serum-free media is not yet broadly expanded to cell cultures, isolation or differentiation of hDPSCs. However, the few works published using serum-free mediums shed some light in innovative culture and differentiation protocols for hDPSCs. Thus, according to the results we obtained in our separate works where Neurocult™ and STP were used as culture mediums (chapter II and chapter III, results and discussion), Hirata et al. firstly mentioned the maintenance of hDPSC in serum-free media (Hirata et al., 2010). We showed that also proliferation ratio was decreased in both Neurocult™ and STP, likely due to replace of FBS. In addition, our group carried out the characterization of phenotypic change of hDPSCs into 3D-like spheroid structures. These non-adherent spheroids-like structures, termed dentospheres, are pretty similar to that spheroids, called neurospheres, generated by NSCs and NCSCs *in vitro* (Fournier et al., 2016; Lee et al., 2007a; Reynolds and Weiss, 1992b). Although the generation of spheroid –like structures in serum-free conditions by hDPSCs totally agrees with the publication of Bonnamain et al. where similar results were described, the efficiency of dentosphere generation seems to be higher in our both works. We achieved almost 100% of dentosphere creation efficiency compared with low 30% ratio described by Bonnamain et al. (Bonnamain et al., 2013).

Even if we have demonstrated in each work (chapter II and chapter III, results and discussion) that depending on culture conditions cell growth and some marker expression can be changed, analysed hDPSCs demonstrated almost indentical expression of mesenchymal and neural stem/neural crest stem markers. All three analyzed proliferation culture mediums; Neurocult™ proliferation, STP and DMEM + FBS 10%, unexpectedly expressed almost similar mesenchymal stem surface markers CD73, CD90 and CD105 (data not shown to neurocult proliferation). In addition, Nestin and GFAP were presented in similar values in analysed hDPSCs cultured in all culture mediums, even in cultures of NSCs. All these markers together comprehend the common marker pattern showed by NC-derived mesenchymal stem cells. There is described the presence of NC progenitors located in perivascular niches within dental pulp (Janebodin et al., 2011; Kaukua et al., 2014). NCSCs can be commited to either to neural lineage forming neuronal and glial cells of PNS or to ectomesenchymal lineage to form special connective tissue that will generate the most of maxillofacial structures in embryonic development (Aurrekoetxea et al., 2015).

Once we achieved NC-derived EMSCs dentospheres in Neurocult™, we realized the potential applications of these cells in neuroregenerative therapies. The employment of STP, specialized human mesenchymal stem cell culture medium, seemed to be logical for this purpose. We did not only improve our NCSCs obtaining efficiency using STP, but also achieved more pure NCSCs rather than already committed neural or endothelial progenitors discovered using Neurocult™.

Despite of the similarity of the dentospheres created by hDPSCs cultured in Neurocult proliferation and STP, these two protocols provide different special characteristic to hDPSCs. On the one hand, surprisingly, we discovered the overexpression of mRNA levels of *NTRK2* and *NTRK3* when hDPSCs were grown in STP. These two receptors belong to Tyrosine Kinase activity receptors and are involved in neural stem cell physiological and cellular activities, such as differentiation and neuronal survival among others (Bibel and Barde, 2000; Oliveira et al., 2013). To our knowledge, we are one of the first groups demonstrating the expression of these receptors in hDPSCs. 7 days after exposure of BDNF and NT3, respective ligands of NRTK2/TrkB and NRTK3/TrkC, to that cells cultured preliminarily in STP, the role of NTRK2 and NTRK3 in homeostasis and differentiation of hDPSCs was proposed. The physiological and molecular modifications; proliferation increase, common stem pluripotency core factors *NANOG*, *SOX2* and *OCT4a* overexpression and NC *HNK1* and $p75^{NTR}$ progenitor markers overexpression promoted by exogenous neurotrophins, made us to suggest a step back in DPSCs lineage, reprogramming them toward genuine NCSCs.

On the other hand, hDPSCs grown in Neurocult™ proliferation did not show aforementioned NCSCs-like modifications apart from markers they expressed basally. However, they were able to express VEGF and CD31 endothelial markers in proliferation conditions either in suspension or in laminin promoted adhesion, suggesting some type of differentiation or selection.

Likely, phenotypic similarity with NCSCs is also present in hDPSCs grown in DMEM+ FBS 10%, but according to Xiao and Tsutsui the dentospheres could be composed by different mature and progenitor cells at first. However, the hermetic structure of dentosphere that avoids the needed oxygenation, along with the starving caused by serum-free condition promote the death of committed mature cells while time goes by. In fact, neural progenitors and stem cells can survive, and even hDPSCs stemness is enhanced in hypoxic conditions (Xiao and Tsutsui, 2013).

One of the aims seeked in neuroregeneration therapies is the restoration of neuronal cells lost after a tissue injurie or pathology. The multiple connections created by large quantity of neuronal cells showed by human adult nervous systems turn the neurogeneration in a tough

challenge for researchers. In overall, we have demonstrated with those works, the capacity of hDPCSs to differentiate into neuronal and glial cells after grown in serum-free conditions. Depending on culture medium, the differentiation can be indirect or direct.

Like it is commented above, when hDPSCs were seeded in BDNF and NT-3 containing STP medium for 7 days it seemed to be a dedifferentiation procces, were NCSCs-like cells were achieved. In order to confirm the functional reprogramming of ectomesenchymal hDPSCs into early NC precursors we tested the capacity of these cells to derive into neural cells. It is described that differentiation fate can be switch depending on serum absence or presence. In fact, serum presence enhance the ectomesenchymal being of NCSCs, as we concluded with our hDPSCs grown in DMEM+FBS 10% (Lee et al., 2007a). The hDPSCs pre-exposed to FBS and then seeded in both DMEM+ FBS 10% and in STP were still remaining mesenchymal being, once BDNF and NT-3 addited, this characteristic was changed and NC and pluripotency markers were overexpressed, that are related with stemness increase (Uribe-Etxebarria et al., 2017). The genuine characteristics of both NC early precursors and ectomesenchymal cells, provide them the ability to differentiate into different type of cells .Thus, we saw the loss of capacity of BDNF/NT-3 treated hDPSCs to give rise osteoblasts or mineralized bone matrix in osteoinductive medium, however, hDPSCs exposed to DMEM+FBS 10% for long period seemed to be preconditioned to osteogenic fate. Moreover, not only mesenchymal comitting was decrease in neurotrophin treated hDPSCs, but they significantly increase the neurogenic potential once they were cultured in Neurocult™ differentiation neuroinductive medium. Compared with the hDPSCs of the same donor grown in DMEM+FBS 10%, neuronal (NeuN, DCX) and glial (p75^{NTR}, S100β) marker expression were five times increased each. Supported by these results, we can say that we firstly design a culture protocol able to increase very functional NCSCs population from not genetically or epigenetically modified hDPSCs. This could be described as an indirect neurogenic differentiation, given the need for several steps to differentiate hDPSCs into neural lineage cells (figure 1).
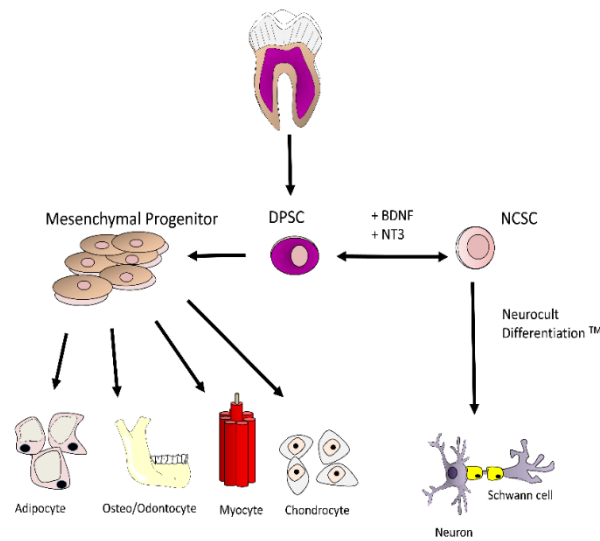
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Figure 1. Graphical abstract of "BDNF and NT3 reprogram human ectomesenchymal Dental Pulp Stem Cells to neurogenic and gliogenic Neural Crest progenitors cultured with serum free medium" (chapter III).

In the case of hDPSCs cultured in Neurocult^{TM} differentiation medium, that is a specific variant of Neurocult^{TM} proliferation medium to differentiate NSCs into mature cells, showed almost the same neurogenic capability shown by NSCs. After 1 week of culture of hDPSCs in neurocult differention serum-free medium, supplemented with B27 with vitA, hDPSCs began to express neuronal markers NeuN and DCX. In the same way, these cells expressed GFAP and S100b glial markers. There were not statistical significance difference with the marker expression values showed by NSCs. These results suggest direct neurogenic potential of hDPSCs cultured in Neurocult^{TM} differentiation (figure 2).

It is widely accepted the role of substitutive neuronal cells in neuroregenerative therapies, however, some neurological affections are not directly derived of neuronal cells loss, but they are caused by irrigation problems. Thus, since Takahashi et al. employed EPCs and stem cells for first time to vasculogenesis stimulation in ischemic disease (Takahashi et al., 1999), the importance of angiogenesis and vasculogenesis have been taken importance in some neural afections, such as ischemia, and MI, among others (Tatullo et al., 2015). In addition to already confirmed neurogenic potential, we wanted to asses the endothelial differentiation potential of hDPSCs. Despite of the existance of previous works that achieve endothelial vascular cells either they were cultured in 10% serum presence in the case of hDPSCs, or the serum-free protocol was optimized for non-human cells (d'Aquino et al., 2007; Weiss et al., 1990). We have been the first group able to derive non-genetically modified endothelial vascular cells from hDPSCs in serum free conditions. Although we achieved neurogenic cells

after neural induction using Neurocult™ differentiation medium, we realized that we could achieve VEGF and CD31, endotelial markers, expressing hDPSCs using Neurocult™ proliferation supplemented with B27 without vitA, EGF and bFGF. We also reported both the activation of STAT3 and ERK in Neurocult™ proliferation medium and the out standing role of them in endothelial differentiation. Our results totally correlate with the results of other groups, that support the importance of ERK in angiogenesis (Xu et al., 2008) and the relation of phosphor-STAT3 with VEFG overexpression (Niu et al., 2002), and endothelial cell activation (Chen et al., 2008). Furthermore, Bento et al. related the differentiation of SHED toward endothelial cells by ERK activation with the VEGF dependant VEGFR2 enhancement (Bento et al., 2013). Taking in consideration all the results obatined we concluded that we were able to give rise endothelial cells from hDPSCs (figure 2).
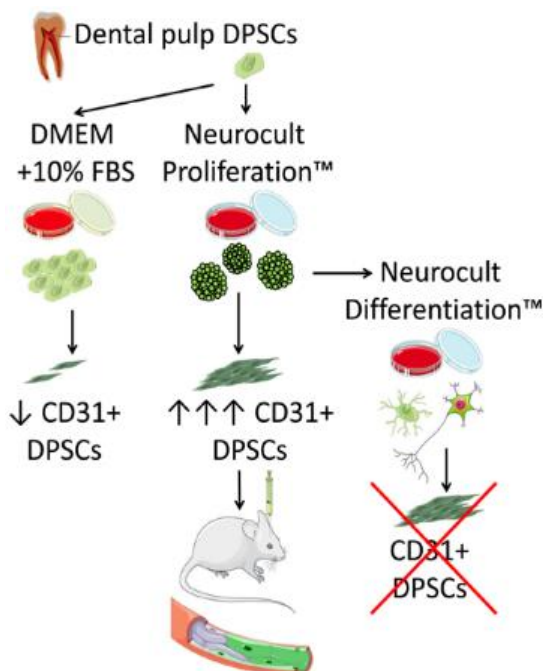

Figure 2. Graphical abstract of "human dental pulp stem cells grown in neurogenic media differentiate into endothelial cells and promote neovasculogenesis in the mouse brain" (chapter II).

Like is commented several times during this work, there are a large amount of works in the literature were is given more weight to neuroprotective activity than to the cell-cell neuronal restoration (Ratajczak et al., 2016; Song et al., 2017). In accordance to Mead et al. we also characterized the expression of *NGF*, *BDNF* and *NT-3* (Mead et al., 2014).

All the results obtained *in vitro* seems to be very hopeful to translation of these protocols to cell therapies in human. However, the employment of these cells in *in vivo* models could be totally change the approach. In fact, although hDPSCs were able to express neuronal and glial markers *in vitro* when were grown in Neurocult™ differentiation, they were previously

grown in Neurocult™ proliferation to expand, we could not find any neural marker after intracranial graft of hDPSCs *in vivo*. Probably due to specific immune system that eliminated all the neuro-differentiated hDPSCs (data not shown). On the other hand, after one month of post-intracranial graft into athymic nude mice we found CD31$^+$ Nestin$^+$ VEGF$^+$ hDPSCs. Nestin (Suzuki et al., 2010), VEGF (Ferrara et al., 2003) and laminin (Malinda et al., 2008) increased expression are taken as angiogenesis hallmark. Moreover, laminin is 50% decreased in ageing related issues (Gavazzi et al., 1995) and it can downregulate TGF-β in epithelial cells (Streuli et al., 1993), that is involved neurogenic niche quiescence during ageing (Pineda et al., 2013). In addition, the capacity to depolymerization of amyloid αβ fibrils, the laminin degradation in cerebral ischemia and laminin variation during CNS injuries, make us to realize about the importance of laminin. Accordingly, hDPSCs disaggregated from dentospheres and then grafted into the brain of immunosuppressed mice, could be advantageous in rejuvenating therapies and neurodegenerative illnes (Bronfman et al., 1996; Fukuda et al., 2004; Ji and Tsirka, 2012).

In the case of hDPSCs grown in STP, we needed to asses their histointegration capacity, as far as this is the ability of cells to adapt to new enviroment. The creation of new functional interactions and the ability to exchange information with hostage tissue is key to a correct function and good engrafment of foreign cells (Guerzon et al., 2011; Muschler et al., 2004). The aforementioned NCSC character of hDPSC is given by the addition of BDNF and NT-3, common elements released in the nervous system. More than the engrafment of already NCSCs-like reprogrammed hDPSCs, would be better to engraft hDPSCs genuinely grown in STP. Thus, we could elude the use of exogenous neurotrophins, activating overexpressed *NTRK2* and *NTRK3* by endogenous neurotrophins. Neurotransmitters are the main elements used in the intercelullar communications in nerve tissue. For example both GABA and glutamate are determinant in the creation of synaptic connectiosn during development (Ben-Ari, 2001; Egorov and Draguhn, 2013). The characterization of the possible responses given by hDPSCs to different neurotransmitters was essential to know the capacity of these cells to interact with hostage tissue and, therefore, to coordinately communicate to provide a common function of the tissue. Suprinsingly, we found that except to *P2XR7,* a purinergic receptor tested by ATPɣS agonist, more expressed in STP conditions, both DMEM+ FBS 10% and STP expressed similarly different subunits of ionotropic neurotransmitter receptors. Moreover, the stimulation of hDPSCs by certain agonists were clearly demonstrated by microfluorescence calcium assays. Apart from neurotransmitter receptors hDPSCs of two culture conditions expressed also several groups of Connexins (Cx26, Cx43) and Pannexin (Pnx1) subunits, that are responsibles of Ca^{+2} propagation, a special characteristic of neural tissue during development (Bruzzone et al., 2003; Harrison et al., 2007; Nadarajah et al., 1997; Ray et al., 2005). We also confirmed the presence of functional Bay-K-sensitive L-type voltage dependent calcium channels, that were described

for first in hDPSCs by Ellis et al (Ellis et al., 2014). All these elements are necessary to good integration in nervous tissue, that make gDPSCs ideal to regeneration therapies.

To sum up, all these results here gathered not only suggest the good viability of human DPSCs in different serum-free mediums, but also means that these mediums can promote physiological, molecular and functional changes in the cells that could be interesting to use in neuroregeneration assays. Furtheremore, the clear neurogenic and endothelial differentiation potential, neuroprotective cues expression along with the integration and neovacularization of human DPSCs in the mouse brain are the needed requirements (Luo et al., 2018) to open the window of a new generation of tissue regeneration cell therapies, apart from nervous system related tissues also, even in pathologies of non-nerve tissues .

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Conclusions

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The use of innovative new serum-free culture mediums has resulted in the discovery of new physiological, molecular and behavioral features in hDPSCs that clearly suggest the high capacity of these cells to differentiate into neurogenic lineage cells. The neuroprotective and vasculogenic properties also remark the importance of DPSCs in neural regeneration cell therapies.

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

1. The culture of hDPSCs in serum-free STP, does **not affect** the **ectomesenchymal characteristic** expression compared with those grown in DMEM + FBS 10%. They remain expressing **neural stem**, **mesenchymal** and **pluripotent** markers in STP.

2. While hDPSCs cultured in DMEM + FBS 10% grow in **monolayer**, hDPSCs cultured in serum-free STP are able to generate neurospheres-like **dentosphere** 3D structures. Proliferation rate of hDPSCs cultured in DMEM + FBS 10% is significantly **higher** than the rate showed by these cells grown in STP, that showed **quiescence-like** stage, likely promoted by serum absence.

3. All tested **neurotransmitter receptors** and voltage dependent **calcium channels** have shown to be **functional** in both DMEM + FBS 10% and serum-free STP.

4. *NTRK2* and *NTRK3*, neurotrophin receptor are **overexpressed** in the cells cultured in serum-free STP.

5. Adding BDNF and NT3, respectively the natural ligands of *NTRK2* and *NTRK3*, in a serum-free STP, termed as STP + NTF2, **NC** and **pluripotential** markers are **overexpressed**. These results suggest the lineage step -back to NCSCs from **hDPSCs**, after passing out **quiescence** stage showed by cells cultured in genuine STP.

6. The hDPSCs cultured in **STP + NTF2**, show remarkable **higher neurogenic** ability than those cells cultured in both genuine STP and DMEM- FBS 10%.

7. hDPSCs cultured in Neurocult proliferation™, are able to generate **dentospheres** and express both **stem mesenchymal** and **endothelial** markers.

8. While hDPSCs cultured in neurocult differentiation™ are able to express **neuronal** and **glial** markers, they do **not** express **endothelial** markers.

9. The engraftment of hDPSCs pre-cultured in serum-free neurocult proliferation™ into mice brain, showed the ability to generate *de novo* **vasculature** in brain. Suggesting both *in vivo* **integration** and **endothelial** differentiation of hDPSCs *in vivo*.

Annex I

Patents

I Patent: *Organ and tissue revascularization by dental origin cells transplantantion (2018)*

Universidad   Euskal Herriko
del País Vasco   Unibertsitatea

Solicitud para el registro de Invenciones

SOLICITUD PARA EL REGISTRO DE INVENCIONES EN LA UPV/EHU

I. TÍTULO DE LA INVENCIÓN

REVASCULARIZACIÓN DE TEJIDOS Y ÓRGANOS POR TRASPLANTE DE CÉLULAS DE ORIGEN DENTAL

II. PALABRAS CLAVE PARA BÚSQUEDAS EN BASES DE DATOS:

Dental Stem Cells, Dental Pulp, Endotheliocytes, Serum-Free culture medium, Cell therapy, Cell transplant, Neovascularization, Blood-supply, Blood vessel, Regeneration, Angiogenic therapy, Angiogenesis, Vasculogenesis, Tissue Engineering, intracranial graft.