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Potential applications of DPSCs in regenerative medicine: differentiation ability and support in the angiogenesis process

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PhD Student

Fanny Pulcini

Coordinator

Prof.ssa Mariagrazia Perilli

Tutor

Prof.ssa Simona Delle Monache

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ABSTRACT

Dental pulp has been revealed as an accessible and rich source of mesenchymal stem cells (MSCs), and its biological potential is currently under intense investigation. MSCs from dental pulp stem/stromal cells (DPSCs) have been indicated as a heterogeneous population, originating from the neural crest, and physiologically involved in dentin homeostasis; moreover, they contribute to bone remodeling and differentiation into several tissues including cartilage, bone, adipose, hepatic, and nervous tissues. DPSCs have also been shown to influence the angiogenesis process, for example through the release of secretory factors or by differentiating into vascular and/or perivascular cells. This multifaceted nature underscores the potential therapeutic applications of DPSCs across various fields, ranging from tissue regeneration to vascular pathology.

Based on this information, the primary goal of this study was to investigate the capacity of DPSCs to differentiate into various cell types, including osteo-odontogenic, hepatic, neurogenic, and perivascular phenotypes. This investigation utilized both traditional two-dimensional (2D) and advanced three-dimensional (3D) culture methods. The ultimate aim of this research is to showcase the potential versatility of DPSCs for future applications in regenerative medicine research.

For the experiments, DPSCs were subjected to various treatments (osteo-odontogenic and hepatic differentiation media, hypoxia at 1%, and MACS separation). At the same time, a stationary technique was employed to generate organoids.

The results revealed a significant ability of DPSCs to differentiate into the osteo-odontogenic phenotype. Notably, these cells exhibited high expression levels of genes characteristic of osteo-odontoblasts and produced a matrix containing dentin and calcium phosphates, both in 2D and 3D environments.

DPSCs possess the capacity to differentiate in neuronal cells by specific media. Additionally, they can be influenced by the environment to differentiate into specific neuronal cells of the nervous system, such as under hypoxic conditions. In light of this, DPSCs were exposed to hypoxia (1% O_2) for 5 and 16 days, revealing that hypoxia-induced DPSCs differentiation was time-dependent. Moreover, conditioned media (CM) derived from DPSCs stimulated by hypoxia had the ability to induce neural differentiation in both SH-SY5Y neuroblastoma cells and undifferentiated DPSCs. This suggests that the differentiation of DPSCs mediated by hypoxia is likely to occur through an autocrine/paracrine mechanism.

To investigate the potential to differentiate into the peri-vascular cells were subjected to immunomagnetic separation (MACS) targeting the pericytic marker NG2, resulting in the isolation of three subpopulations of interest (Total, NG2-, and NG2+ DPSCs). The data showed that NG2+ DPSCs, displaying a pericyte-like phenotype, were able to stabilize tubules in vitro for 14 days by directly interacting with endothelial cells. This suggests that DPSCs have the capability to differentiate into different cell types, making them a valuable resource for regenerative medicine. Their potential offers hope for the treatment of various medical conditions.

1. INTRODUCTION

1.1 Mesenchymal Stem Cells (MSCs)

Mesenchymal stem cells (MSCs) represent a type of adult stem cells characterized by an undifferentiated or partially differentiated state and, they are localized in specific tissues of the adult organism. MSCs possess the inherent capacity for self-renewal, enabling them to undergo unlimited regeneration and replication, as well as the ability to differentiate into specialized cell types derived from the same germinal layer [Jackson, et al., 2007]. Specifically, MSCs are distributed in various tissues of the body, including bone marrow, adipose tissue, umbilical cord, and cartilage tissue [Uccelli et al., 2008].

The first evidence validating the existence of a mesenchymal stem cell population dates to 1970 when Alexander Friedenstein and his colleagues first cultured samples of human bone marrow. They observed the formation of a small colony of adherent cells composed of some cells very similar to fibroblasts. After several passages in culture, the colony of cells becomes increasingly homogeneous, assuming a spindle-shaped morphology [Friedenstein et al., 1970]. Subsequently, Caplan and colleagues demonstrated that these cells were able to generate chondrocytes, osteocytes, and adipocytes [Caplan et al., 2005; Bianco et al., 2008].

Mesenchymal stem cells are defined as "Multipotent Mesenchymal/Stromal Cells" because they adhere to plastic and expand massively like stromal cells, but they retain the term "mesenchymal" as they represent the subset that preserves the two cardinal properties of stem cells: self-renewal and the ability to differentiate into multiple cell lineages. In fact, they are capable of spontaneous differentiation, both *in vitro* and *in vivo*, into all specialized tissues of mesodermal embryonic origin: bone tissue, adipose tissue, and cartilage tissue. Nomenclature changed officially and was consolidated by the International Society for Cellular Therapy (ISCT) in 2019 [Dominici et al., 2006; Horwitz et al., 2005; Viswanathan et al., 2019].

Bone marrow represents the main origin site of MSCs, where they are located near the sinusoidal endothelium in association with resident hematopoietic stem cells, but they also are found in many other sites such as umbilical cord blood, placenta, peripheral blood, and adipose tissue [Fajardo-Orduña et al., 2015]. Moreover, several MSCs populations are localized near blood vessels and are a subset of pericytes residing on capillaries and venules.

From the literature, there are known similarities in surface antigens expression between pericytes and MSCs, with perivascular cells expressing markers characteristic of MSCs from both bone marrow and human dental pulp [Gronthos et al., 2001]. In particular, Crisan and colleagues discovered that cells positive for NG2, for melanoma cell adhesion molecule (CD146/MCAM), and for platelet-derived growth factors subunit β (PDGFR-β) are similar to pericytes found in multiple human tissues. Cells isolated with these markers showed a significant osteogenic potential *in vitro* and *in vivo*. Finally, despite the prevalent opinion that MSCs reside in perivascular niches, some populations of these cells may also inhabit vascular regions [Crisan et al., 2008].

Another typical characteristic of MSCs is their positivity (>95%) and negativity (<2%) for a wide panel of cell surface antigens. There are several stemness markers *(Table 1)* used for the identification of these stem cells [Bara et al., 2014].

Positive Stemness Markers	***Negative Stemness Markers***
CD73 (5'-nucleotidasi)	CD45 (lymphocyte common antigen)
CD90 (Thy-1)	CD14 o CD11b
CD105 (endoglin)	CD19 o CD79a
STRO-1	CD15 (Sialil-LewisX)
CD166 (activated leukocyte cell adhesion molecule protein)	HLA-DR (human leukocyte antigen subclass)
CD44	CD31 (platelet endothelial cell adhesion molecule 1)
CD29 (integrin β1)	CD40
CD146 (melanoma cell adhesion molecule)	
CD271 (nerve growth factor receptor)	
CD54 (Intercellular Adhesion Molecule 1)	
CD106 (vascular cell adhesion molecule 1)	
SSEA-4 (stage-specific embryonic antigen-4)	
Nestin	

Table 1*. Positive and negative stemness markers of mesenchymal stem cells [Dominici et al., 2006; Simmons et al., 1991; Sacchetti et al., 2007; Gang et al., 2007; Crisan et al., 2008].*

However, it is important to emphasize that there is no single universal marker for MSCs, and their identification relies on a combination of markers and functional characteristics. Additionally, the expression of some markers may vary depending on the tissue source and culture conditions of MSCs. The accurate use of stemness markers is crucial to ensure the purity and quality of isolated cell populations, as well as to establish standardized

criteria for MSCs identification in both clinical and research settings [Dominici et al., 2006; Horwitz et al., 2007; Bara et al., 2014].

MSCs exhibit a vast range of biological functions that render them invaluable tools in various therapeutic and research contexts. In addition to their intrinsic capacity to differentiate into mesenchymal tissue cells such as osteoblasts, chondrocytes, and adipocytes, MSCs demonstrate remarkable abilities in modulating components of the immune system and controlling neo-angiogenesis *(Figure 1)* [Le Blanc et al., 2007; Salem et al., 2010]. Indeed, MSCs play a role in the maturation of lymphocytes T and B, natural killer cells, and dendritic cells. Furthermore, they possess anti-inflammatory and immunomodulatory properties, being able to modulate both innate and adaptive immune responses through direct interactions with immune cells and secretion of soluble factors such as TGF-β1, HGF, NO, and PGE2 [Nauta et al., 2007; Salem et al., 2010; Caplan et al., 2009]. MSCs are also involved in tissue repair and wound healing processes, with the ability to migrate towards injury sites, release growth factors, and promote neo-vascularization and tissue regeneration. Mesenchymal stem cells release angiogenic factors that influence the survival, proliferation, and migration of endothelial cells. These factors are important for the initial vessel formation and subsequent stabilization (VEGF, FGF2, SDF1, ANG1, MCP1, HGF, and many others) [Bianco et al., 2008; Caplan et al., 2009].

In addition to these properties, another advantage of MSCs is their relatively easy to isolate and culture in the laboratory. Once harvested from bone marrow or adipose tissue, they can be expanded in large quantities without compromising their characteristics. This makes them an accessible resource for research and clinical applications, such as in the treatment of a wide range of conditions including musculoskeletal injuries, autoimmune diseases, cardiovascular disorders, bone regeneration, cartilage repair, and neurodegenerative diseases [Uccelli et al., 2008; Vasanthan et al., 2020]. However, despite their therapeutic potential, several challenges persist, such as the standardization of culture protocols, understanding of mechanisms of action, and management of potential risks, including the risk of tumor formation or adverse immune reactions. Research continues to focus on these issues to optimize the efficacy and safety of MSCs in clinical settings.

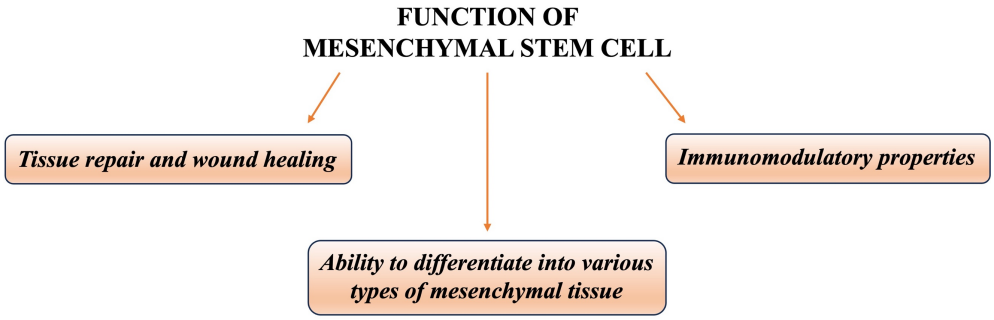


Figure 1. *Representative diagram of the main functions of MSCs*

1.2 Dental Pulp Stem/Stromal Cells (DPSCs)

In recent years, several studies have demonstrated the presence of cells with typical characteristics of mesenchymal stem cells in dental tissues [Stanko et al., 2018]. Gronthos and his collaborators were the first to isolate stem cells from human dental pulp, and these cells were called Dental Pulp Stem/Stromal Cells (DPSCs) [Gronthos et al., 2000]. Subsequently, were identified and characterized populations like dental mesenchymal stem cells from other tissues *(Figure 2)* [Bakkar et al., 2017; Nagata et al., 2021]:

- Stem Cells from Human Exfoliated Deciduous Teeth (SHEDs)
- Periodontal Ligament Stem Cells (PDLSCs)
- Stem Cells from Dental Follicles (DFSCs)
- Stem Cells from Apical Papilla (SCAPs)
- Gingival Fibroblastic Stem Cells (GFSCs)

Except for SHED, all the other cells are isolated from permanent teeth [Miura et al., 2003]. DPSCs and SHED are the most attractive cellular sources for regenerative medicine compared to similar populations of mesenchymal stem cells due to their easy accessibility [Stanko et al., 2018; Yoshida et al., 2020]. In particular, DPSCs are also characterized by trilineage differentiative potential, giving rise to endodermal, mesodermal, and ectodermal tissues *(Table 2)* [Mattei et al., 2021].

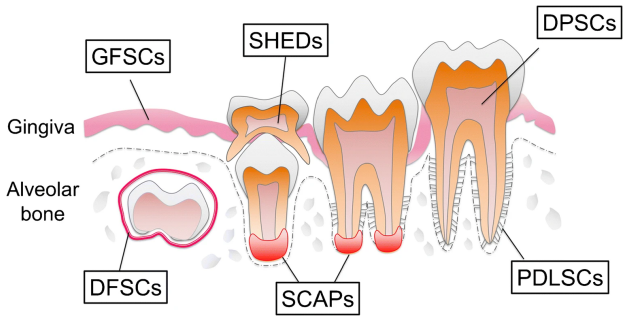


Figure 2. *Schematic diagram of isolated tooth-derived stem cells populations. From tooth pulp, dental pulp stem cells (DPSCs), stem cells from human exfoliated deciduous teeth (SHEDs) and cells from apical papilla (SCAPs) can be isolated. From supporting tissue, periodontal ligament stem cells (PDLSCs), gingival fibroblastic stem cells (GFSCs) and dental follicle stem cells (DFSCs) can be isolated [Nagata et al., 2021].*

Types	***Tissue sources***	***Markers***	***Differentiation Potency***
DPSCs	Adult dental pulp	STRO-1, CD146	Odontoblast-like cells, osteoblasts, adipocytes, neural cells, endothelial cells
SHEDs	Exfoliated deciduous teeth	STRO-1, CD146, CD90, CD29, CD44, CD166, CD105	Odontoblasts, osteoblasts, adipocytes, neural cells
PDLSCs	Periodontal ligament	STRO-1, CD146, CD73, CD90, CD105	Osteoblast-like cells, adipocytes, collagen-foming cells
SCAPs	Apical papilla	STRO-1, CD146, CD24	Odontoblasts
DFSCs	Dental follicle	STRO-1, CD105, CD90, nestin, notch-1	Periodontal ligament cells, osteoblasts, cementoblasts

Table 2. *Characteristics of different types of dental stem cells [Zhai et al., 2019].*

1.2.1 Structure of the dental pulp and localization of DPSCs

The dental pulp is a non-mineralized connective tissue located within the pulp chamber of the crown and root canals; the central mass of the pulp consists of stem cells, fibroblastic cells, lymphocytes, and macrophages immersed in an extracellular matrix, and odontoblasts, located at the periphery of the pulp tissue and able of producing dentin [Gronthos et al., 2000; d'Aquino et al., 2008]. The matrix, which surrounds and supports the structures, is composed of collagen type I and III and substances such as water and proteoglycans; this matrix represents the medium through which metabolites and waste products diffuse into the pulp [Yoshida et al., 2020].

DPSCs are stem cells derived from the ectoderm, originating from the migration of neural crest cells *(Figure 3)* [Lan et al., 2019].

These cells are typically extracted from adult human pulp tissue of included third molars (wisdom tooth) in patients with dysodontiasis, thereby making them available as waste material. DPSCs are considered cells with intense clonogenicity, marked proliferative activity, and the ability to form mineralized nodules [Govindasamy et al., 2010; Gronthos et al., 2002; Mattei et al., 2021].

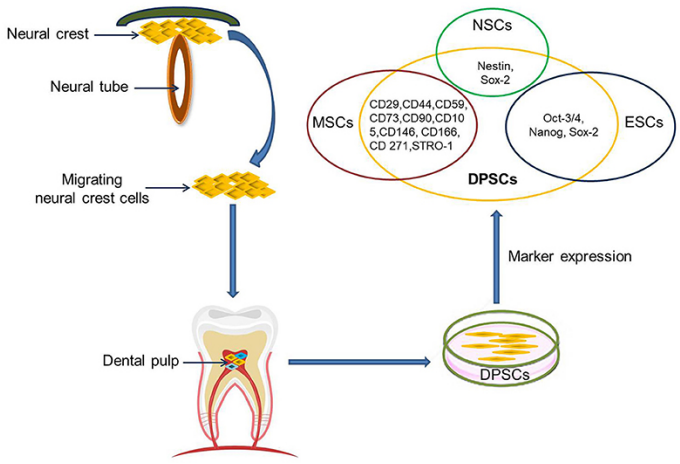


Figure 3. *DPSCs origin, isolation, and marker expression. Dental Pulp Stem/Stromal Cells originate from the migrating of neural crest cells, coming to rest in the dental pulp and expressing markers overlapping with MSCs, embryonic stem cells (ESCs), and neural stem cells (NCSs) [Lan et al., 2019].*

1.2.2 Isolation of Dental Pulp Stem/Stromal Cells

The isolation of DPSCs is a crucial process that enables obtaining a population of these valuable cells for research and clinical applications.

Several methods for the isolation and expansion of DPSCs have been published in recent years. Starting from the Gronthos protocol, different researchers have reviewed the DPSCs isolation, characterization, differentiation, and storage procedures, providing a revision of non-invasive techniques and ethical constraints [Ferro et al., 2012; Tirino et al., 2012; Rodas-Junco et al., 2017]. The main factors that can influence DPSCs' properties during the experimental procedures are the selection of the tooth type (e.g., molar, third molar), the stage of tooth development, the characteristics of the donors (e.g., sex, age, lifestyle), tooth transport and short-term storage [Gronthos et al., 2000]. In this context, Perry et al. demonstrated that DPSCs maintained viability up to five days following tooth storage at 4°C in phosphate-buffered saline (PBS). However, viability

significantly declined after 24 hours of storage, underscoring the importance of immediate treatment to ensure optimal cell recovery [Perry et al., 2008].

The literature describes three primary techniques for isolating DPSCs: tissue explants (TE), enzymatic digestion (ED), and mechanical methods, sometimes in combination [Naz et al., 2019; Ferrúa et al., 2017; Arora et al., 2022].

The TE method is a simpler and faster technique involving the direct placement of pulp fragments (1-2 mm^3 in size) into a culture dish, allowing the cells to grow from the pulp tissue explants *(Figure 4 A, B)* [Naz et al., 2019; Patil et al., 2018].

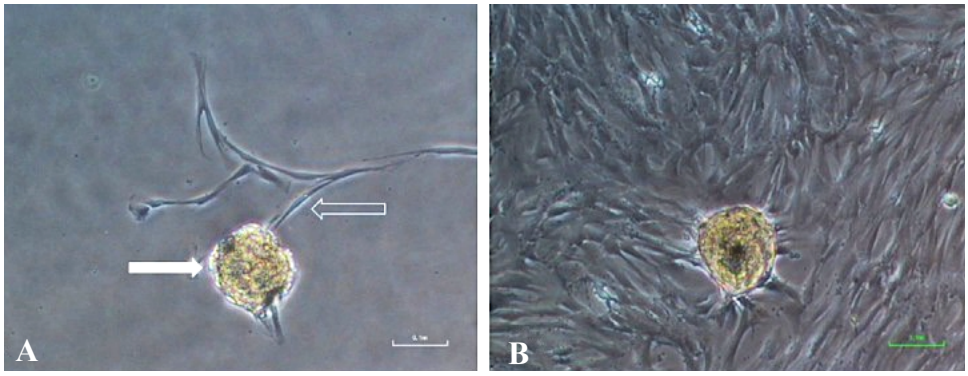


Figure 4. *Dental pulp stem cells emerging from tissue explant.* ***A*** *The image showed the dental pulp tissue explant (solid arrow) and newly emerging cells from the tissue explant (hollow arrow) on day 11 of culture. These cells exhibit typical morphology of fibroblast-like cells with long cytoplasmic extensions.* ***B*** *The image showed the same tissue explant after 19 days of culture with the confluent field of view. Magnification 100X [Naz et al., 2019].*

ED, on the other hand, allows the isolation of single-cell suspensions from primary tissues by digestion with various enzymes, including collagenase type I, II, IV, dispase, trypsin, and accutase [Gronthos et al., 2000; Takeda-Kawaguchi et al., 2014; Arora et al., 2022].

In particular, the isolation process begins with the recovery of pulp tissue from the pulp chamber of extracted teeth*;* subsequently, the tissue is minced into small fragments and transferred to a sterile saline solution supplemented with antibiotics and antifungals to prevent microbial and mycotic contamination (Hank's solution). The tissue is treated with proteolytic enzymes such as collagenase to facilitate cell dissociation from the extracellular matrix. Following enzymatic treatment, the tissue is centrifuged to separate the dissociated stem cells from the cell suspension. The obtained stem cells are cultured in a specific growth medium, such as DMEM Low Glucose, supplemented with fetal bovine serum (FBS) and antibiotics to promote cellular growth and proliferation *(Figure 5A)*. DPSCs adhere to the culture plate and expand, forming colonies. After several days,

the cell colonies are passed into larger culture plates to promote their growth *(Figure 5B)* [Delle Monache et al., 2019; Martellucci et al., 2019].

Several researchers have compared the two methods (TE and ED), observing that DPSCs isolated using the ED method showed higher rates of proliferation, differentiation, and expression of specific surface markers. Consequently, ED remains the most used method to obtain DPSCs [Huang et al., 2006; Karamzadeh et al., 2012; Raoof et al., 2014; Lindroos et al., 2008].

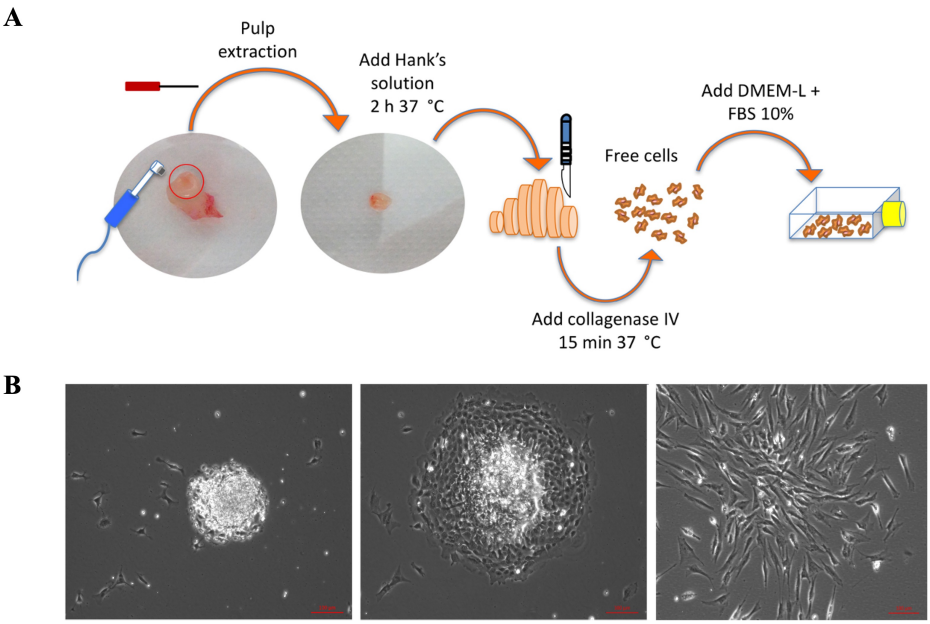


Figure 5. *Schematic representation of dental pulp stem cell isolation using enzymatic digestion (ED).* ***A*** *The tooth was opened with a cutter by coronal cutting pass parallel and tangent through the roof of the pulp chamber, and the pulp was gently removed under sterile conditions with a small excavator and placed in a test tube. The pulp, after Hank's solution treatment, was cut into slices treated with collagenase IV for 15 min and propagated in a T25 flask.* ***B*** *The image showed the morphology of DPSCs on different days (1,4,7) following dental pulp separation using a phase-contrast microscope. Scale bars = 100 µm [Martellucci et al., 2019].*

After isolating DPSCs, it is crucial to characterize them through flow cytometry. They must indeed be positive for specific markers of mesenchymal stem cells, such as CD90, CD73, CD105, CD44, and negative for CD14, CD19. These surface markers confirm the identity of dental pulp stem cells and eliminate any unwanted contaminants [Delle Monache et al., 2019; Kawashima et al., 2012]. Once DPSCs have been isolated, characterized, and expanded, they are ready to be used in a wide range of applications, including regenerative medicine, cellular development research, and gene therapy [Paino et al., 2017; d'Aquino et al., 2008].

1.2.3 Characterization of Dental Pulp Stem/Stromal Cells

Dental pulp stem cells are commonly characterized through the analysis of surface markers using techniques such as flow cytometry, which allows for quantitative and qualitative assessment of marker expression on individual cells. This approach provides detailed information about the cellular population composition and confirms the identity of DPSCs as multipotent mesenchymal stem cells *(Table 3).*

Surface markers are protein molecules present on the cell membrane that distinguish DPSCs from other cell types. Among the surface markers commonly used to characterize DPSCs, in addition to CD90, CD73, CD105, and CD44, are also CD146, STRO-1, and CD34, among many others [Santilli et al., 2024].

CD90, CD73, CD105, and CD44 are known to be expressed on mesenchymal stem cells and have been identified as crucial for the ability of DPSCs to differentiate into various cell types, as well as for their function in maintaining the stem cell phenotype and regulating proliferation [Bakopoulou et al., 2011; Lindroos et al., 2008; Ledesma-Martínez et al., 2016; Mattei et al., 2021].

CD146 and STRO-1 are also considered specific markers of mesenchymal stem cells and have been utilized to isolate and characterize DPSCs, contributing to their identification and separation from other cell types present in dental tissue [Gronthos et al., 2000]. Furthermore, DPSCs exhibit a 70% positivity for STRO-1, which is regarded as an early marker of mesenchymal stem cells [You et al., 2019].

In particular, DPSCs are also found to be CD34+, and since STRO-1+ cells are also CD34+, it can be concluded that DPSCs are present within the CD34+ population [Ledesma-Martínez et al., 2016]. The expression of these markers makes DPSCs a heterogeneous population of mesenchymal stem cells localized in a perivascular niche within the pulp. Due to the heterogeneity of DPSCs, specific markers for their identification have not been discovered. However, some researchers have investigated new markers that could be used for DPSCs identification; for instance, Feng and colleagues demonstrated how NG2, a proteoglycan used as a pericyte marker, can also be utilized as a marker for DPSCs [Feng et al., 2011]. DPSCs also express other markers typical of pericytes in addition to CD34 and NG2, such as desmin, PDGFR-β, CD146, and vascular endothelial VE–cadherin (CD144). Moreover, they express high levels of α-smooth muscle actin (α-SMA), indicating characteristics consistent with muscle cell activity [Bergers et al., 2005; Mattei et al., 2021].

DPSCs may express some neuronal markers even in undifferentiated conditions. This phenomenon could be attributed to their ectomesenchymal origin during embryonic development. In particular, DPSCs express markers such as nestin, a protein detectable in dividing cells during early development in the central nervous system (CNS), as well as in the peripheral nervous system (PNS). Additionally, they exhibit low basal levels of markers associated with mature CNS cell types, including the neuronal markers microtubule-associated protein 2 (MAP2), neurofilament (NF), β3-Tubulin, CNPase associated with oligodendrocytes, and the astrocytic marker glial fibrillary acidic protein (GFAP) [Yang et al., 2017; Martellucci et al., 2019; Darvishi et al., 2021].

The presence of many other positive markers has been observed in DPSCs, including CD10, CD13, CD106, stem cell factor receptor (CD117), CD29, CD166, and CD271 [Kawashima et al., 2012; Martens et al., 2012; Potdar et al., 2015]. Additionally, DPSCs express markers such as SH2, SH3/SH4, and SSEA-4 [Kawashima et al., 2017; Martinez et al., 2007; Petrenko et al., 2020; Santilli et al., 2022]. Moreover, DPSCs also express some pluripotent stem cells markers such as octamer-binding transcription factor 4 (Oct4), Nanog, Sox2, and c-Myc [Yasui et al., 2017].

Likely due to their location, DPSCs also express specific markers such as dentin sialophosphoprotein (DSPP), dentin matrix protein-1 (DMP-1), osteoblast-specific transcription factor osterix (OSX), osteocalcin (OCN), osteopontin (OPN), alkaline phosphatase (ALP), and type I collagen [Guo et al., 2013; Tziafas et al., 2010; Martellucci et al., 2019].

Recently, it has been observed that gangliosides act as molecular markers for the recognition of subclasses of multipotent stem cells. Gangliosides are glycosphingolipids ubiquitously expressed on the cell membrane, known to be involved in a range of cellular processes, including cell adhesion, differentiation, and proliferation [Kim et al., 2008; Santilli et al., 2022]. Studies have highlighted that undifferentiated DPSCs express certain gangliosides, including GM3, GM2, and GD1a, which have been associated with key roles in regulating cell differentiation and modulating cell-cell and cell-matrix interactions [Moussavou et al., 2013; Lee et al., 2010; Santilli et al., 2022].

On the other hand, DPSCs should test negative for markers associated with hematopoietic and monocytic cell lines, such as CD14, CD19, CD24, CD45, and HLA-DR markers [Agha-Hosseini et al., 2010; Raoof et al., 2014; Luisi et al., 2017; Mattei et al., 2021; Delle Monache et al., 2019; Ledesma-Martínez et al., 2016] as suggested by International Society for Cellular Therapy (ISCT) [Dominici et al., 2006]. The presence of these

markers on DPSCs may indicate sample contamination with other cell types, compromising the purity of the cell population.

In conclusion, the characterization of surface markers of DPSCs represents a fundamental step in the isolation and identification of these stem cells, significantly contributing to the understanding of the mechanisms regulating their function and therapeutic potential.

Cell Type	***Surface markers***	***References***
DPSCs	***Positive Markers:*** CD10, CD13, CD29, CD44, CD73, CD90, CD105, CD106, CD117, CD146, CD166, CD271, STRO-1, SH2, SH3/SH4, SSEA-4	Bakopoulou et al., 2011; Lindroos et al., 2008; Ledesma-Martínez et al., 2007/2016; Mattei et al., 2021; You et al., 2019; Kawashima et al., 2012/2017; Martens et al., 2012; Potdar et al., 2015; Petrenko et al., 2020; Santilli et al., 2022]
	Peri-Vascular Markers: CD34, NG2, Desmin, PDGFR-β, CD146, CD144, α-SMA	Feng et al., 2011; Bergers et al., 2005; Mattei et al., 2021
	Neuronal Markers: Nestin, MAP2, NF, β3-Tubulin, CNPase, GFAP	Yang et al., 2017; Martellucci et al., 2019; Darvishi et al., 2021
	Pluripotent Stem Cells Markers: OCT4, Nanog, Sox2, c-Myc	Yasui et al., 2017
	Osteoblastic Markers: DSPP, DMP-1, OSX, OCN, OPN, ALP, Type I Collagen	[Guo et al., 2013; Tziafas et al., 2010; Martellucci et al., 2019].
	Gangliosides markers: GM3, GM2, GD1a	Moussavou et al., 2013; Lee et al., 2010; Santilli et al., 2022
	Negative markers: CD14, CD19, CD24, CD45, HLA-DR	Agha-Hosseini et al., 2010; Raoof et al., 2014; Luisi et al., 2017; Mattei et al., 2021; Delle Monache et al., 2019; Ledesma-Martínez et al., 2016

Table 3. *Surface markers of DPSCs*

1.2.4 Differentiation ability of Dental Pulp Stem/Stromal Cells

Human dental pulp stem/stromal cells are a type of mesenchymal stem cells that display characteristics like plastic adherence and a fibroblast-like morphology. Studies by Mattei

et al. in 2015 have confirmed this [Mattei et al., 2015]. These cells, known as DPSCs have shown significant growth potential, as demonstrated by Gronthos and colleagues in several *in vitro* studies. They are also capable of renewing themselves and differentiating into various cell types under appropriate culture conditions. These include osteo-odontoblasts, cementoblast-like cells, collagen-forming cells, chondrocytes, adipocytes, neurons, myocytes, pancreatic cells, hepatic cells, endothelial cells, and pericytes [Shi et al., 2005; Mattei et al., 2021; Tsutsui et al., 2020; Gronthos et al., 2002; Zhai et al., 2019; Suchanek et al., 2009; Zou et al., 2019; Ishkitiev et al., 2012; Ahmed et al., 2016]. Additionally, other researchers are exploring novel differentiative capabilities of DPSCs; for instance, they can also differentiate into corneal epithelial cells and cardiomyocytes, thus demonstrating their multipotentiality [Mattei et al., 2021; Yoshida et al., 2020]. In summary, DPSCs possess the ability to differentiate into various cell types, highlighting their multilineage potential *(Table 4)*.

- ***Osteo-Odontoblastic differentiation of DPSCs***

DPSCs have been extensively studied for their ability to differentiate into various cell types, including osteo-odontoblasts, which are crucial for bone and tooth formation and maintenance. There are 6 main pathways involved in the osteo-odontoblastic differentiation of DPSCs [Zhou et al., 2023]:

1. Wnt signaling pathways.
2. Smad signaling pathways.
3. MAPK signaling pathways.
4. NF-kB signaling pathways.
5. PI3K/AKT/mTOR signaling pathways.
6. Notch signaling pathways.

1. Wnt Signaling Pathways

In these pathways *(Figure 6A)*, the absence of Wnts leads to the formation of a protein complex consisting of adenomatous polyposis coli (APC), glycogen synthase kinase-3β (GSK-3β), Axin, and casein kinase 1 (Ck1). This complex subsequently phosphorylates β-catenin, leading to its degradation and thereby inhibiting signal transduction in the Wnt pathway.

In contrast, the presence of Wnts triggers their interaction with the receptor and low-density lipoprotein receptor (LRP), leading to the disruption of the protein complex.
This disruption allows cytoplasmic β-catenin to accumulate and translocate into the nucleus, where it activates target genes and proteins crucial for osteo-odontoblastic differentiation, such as runt-related transcription factor 2 (RUNX2), alkaline phosphatase (ALP), dentin sialophosphoprotein (DSPP), and osteocalcin (OCN). As a result, β-catenin can activate RUNX2, thereby promoting the osteo-odontogenic differentiation of DPSCs [Kim et al., 2013; Han et al., 2014; Yoshida et al., 2016; Rahman et al., 2018].
On the contrary, Zhang et al. reported that increased levels of Wnt10A promote DPSCs proliferation but hinder their osteo-odontoblastic differentiation. Following the differentiation process, Wnt10A-overexpressing DPSCs downregulated the expression of DSPP, dentin matrix protein 1 (DMP-1), ALP, and collagen-type I-alpha1 (COL1A1) [Zhang et al., 2014]. This study highlights the significance of Wnt/β-catenin signaling pathways in controlling the differentiation of DPSCs, suggesting the need for further research using live animal models to better understand the molecular mechanisms involved.

2. Smad Signaling Pathways

The transforming growth factor-β (TGF-β) superfamily transduces intracellular signals through Smad proteins *(Figure 6B)*. TGF-β exhibits a dual role in vitro concerning osteo-odontogenic differentiation, where it hampers the early phases of progenitor development but promotes bone matrix production in cells undergoing advanced differentiation [Lee et al., 2003]. Bone morphogenetic protein (BMP), belonging to the TGF-β superfamily, is closely associated with Smad proteins *(Figure 6C)* [Wu et al., 2016]. BMP activates Smad proteins, which help transmit signals to the nucleus for the regulation of gene expression. Given its crucial involvement in tooth development, any impairment in BMP function can result in dental developmental disorders [Fraser et al., 2004; Salazar et al., 2016; Lowery et al., 2018]. Previous studies have demonstrated that BMP/Smad signaling pathways regulate DSPP in both lab settings and in living organisms, as well as dentin regeneration. [Chen et al., 2008; Cho et al., 2010; Woo et al., 2016; Hu et al., 2019]. Therefore, BMP2 induces the expression of OSX [Matsubara et al., 2008]. Thus, it is apparent that BMP/Smad signaling pathways play a crucial role in DPSCs differentiation towards the osteo-odontoblastic phenotype.

3. Mitogen-activated protein kinase Signaling Pathways

The MAPK signaling pathways present in the cytoplasm play an important role in cell proliferation, differentiation, and apoptosis [English et al., 2002]. A typical MAPK cascade consists of three main kinases: a MAPK (MPK), a MAPK kinase (MAPKK or MEK), and a MAPK kinase kinase (MAPKKK or MEKK). Upon detection of a stimulus, MAPKKKs phosphorylate and activate downstream MAPKKs, which subsequently phosphorylate and activate the MAPKs. In turn, the activated MAPKs can phosphorylate numerous downstream substrates and activate cellular responses [Chen et al., 2021; Zhang et al., 2022]. The MAPK family comprises three primary subfamilies: extracellular signal-regulated kinase (ERK), P38 MAPK, and c-Jun N-terminal kinase (JNKMAPK) [Pearson et al., 2001].

Ngo et al. demonstrated that leptin induces the differentiation of DPSCs into osteo-odontoblasts by activating MAPK signaling pathways. Treatment of DPSCs with leptin resulted in increased ALP expression and mineralization, along with elevated phosphorylation levels of ERK, P38 MAPK, and JNK [Ngo et al., 2018].

Several studies suggest that when certain factors promote the osteo-odontogenic differentiation of DPSCs, the related protein of MAPK signaling pathways could be enhanced. MAPK signaling pathways represent one of the crucial mechanisms regulating cell proliferation and osteo-odontogenic differentiation [Liu et al., 2014; He et al., 2015; Rodriguez-Carballo et al., 2016; Wu et al., 2019; Cui et al., 2019]. Nonetheless, further investigation is needed to elucidate the detailed mechanisms of ERK, P38, and JNK signaling pathways and their interrelationships.

4. Nuclear factor-kappa B Signaling Pathways

The NF-κB signaling pathways play an important role in various biological processes, including cell proliferation, apoptosis, inflammation, and immune response. Typically, NF-κB proteins remain inactive in cytoplasmic complexes bound to inhibitors of the κB (IκB) family. These pathways are classified into canonical and non-canonical signaling pathways *(Figure 6D).*

In canonical pathways, signals from numerous immune receptors can activate the TGFβ-activated kinase 1 (TAK1). TAK1 then phosphorylates IκB kinase-β (IKKβ), which in turn activates a complex called IκB kinase (IKK). The IKK complex is made up of two catalytic subunits (IKKα and IKKβ) and one regulatory subunit (IKKγ). Then, IκB family members, particularly IκBα, are phosphorylated by the IKK complex and eventually

undergo ubiquitylation and proteasomal degradation. Consequently, various NF-κB complexes, predominantly the p50/RelA dimers, translocate to the nucleus [Zhou et al., 2023].

The non-canonical pathway responds to tumor necrosis factor receptor (TNFR) members, which activate NF-κB-inducing kinase (NIK). IKKα is phosphorylated and activated by NIK, which then phosphorylates and degenerates p100 and generates p52. Subsequently, p52 and RELB translocate to the nucleus, activating target genes associated with osteo-odontogenic differentiation, such as RUNX2, ALP, DSPP, and OCN [Sun et al., 2011; Sun et al., 2017].

Wang et al. demonstrated that estrogen deficiency reduces the odonto-osteogenic potential of DPSCs through NF-κB signaling pathways [Wang et al., 2013]. Moreover, when factors promote the osteo-odontogenic differentiation of DPSCs, proteins related to NF-κB signaling pathways may be upregulated [Hozhabri et al., 2015; Pei et al., 2016; Wu et al., 2019]. So, NF-κB signaling pathways also play a crucial role in the osteo-odontogenic differentiation of DPSCs.

5. Phosphoinositide-3-kinase (PI3K) /protein kinase B (AKT) /mammalian target of rapamycin (mTOR) Signaling Pathways

The PI3K/AKT/mTOR signaling pathways *(Figure 6E)* are crucial for various cellular functions, including cell growth, survival, and metabolism. Upon stimulation by growth factors, receptor tyrosine kinases (RTK) activate PI3K, which converts phosphatidylinositol 4,5-bisphosphate (PIP2) into phosphatidylinositol 3,4,5-triphosphate (PIP3). This conversion can be reversed by the action of phosphatase and tensin homolog (PTEN). PIP3 subsequently activates PI3K-dependent kinase 1 (PDK1), leading to the phosphorylation and activation of AKT by PDK1 and mTORC2, respectively. AKT then phosphorylates target proteins, mediating various cellular responses [Keppler-Noreuil et al., 2016; Yu et al., 2016]. Several studies have suggested that when specific factors promote the osteo-odontogenic differentiation of DPSCs, proteins related to the PI3K/AKT/mTOR signaling pathways exhibit decreased expression. These findings suggest a negative regulatory role of the PI3K/AKT/mTOR signaling pathways in the odontogenic differentiation of DPSCs [Kajiura et al., 2021; Park et al., 2022]. However, further investigations are required to elucidate the underlying mechanisms in detail.

6. Notch Signaling Pathways

The Notch signaling pathways *(Figure 6F)* are evolutionarily conserved pathways facilitating cell-to-cell communication, influencing processes like proliferation, differentiation, and apoptosis during development [Artavanis-Tsakonas et al., 199]. These pathways include four transmembrane Notch receptors (Notch-1, Notch-2, Notch-3, and Notch-4) and two families of ligands (Delta and Jagged). Upon binding of a ligand from a neighboring cell, Notch receptors undergo proteolytic cleavages, releasing the Notch intracellular domain (NICD). Subsequently, NICD translocate into the nucleus and associates with members of the CSL (CBF-1, Suppressor of Hairless, and Lag-1) transcription factor family to activate Notch target genes [Lai et al., 2004; Espinoza et al., 2013; Nandagopal et al., 2018]. Notch signaling has been observed to promote osteo-odontogenic differentiation not only in DPSCs but also in human periodontal ligament stem cells, human adipose stem cells, human bone marrow stem cells, and stem cells derived from human exfoliated deciduous teeth [Lough et al., 2016; Liao et al., 2017; Tian et al., 2017]. However, the precise impact of Notch signaling on osteogenic differentiation remains to be fully elucidated.

Furthermore, the process of osteo-odontogenic differentiation of DPSCs is considerably complex, involving the activation of multiple signaling pathways simultaneously. However, most studies failed to explore the underlying mechanisms between activated signaling pathways.

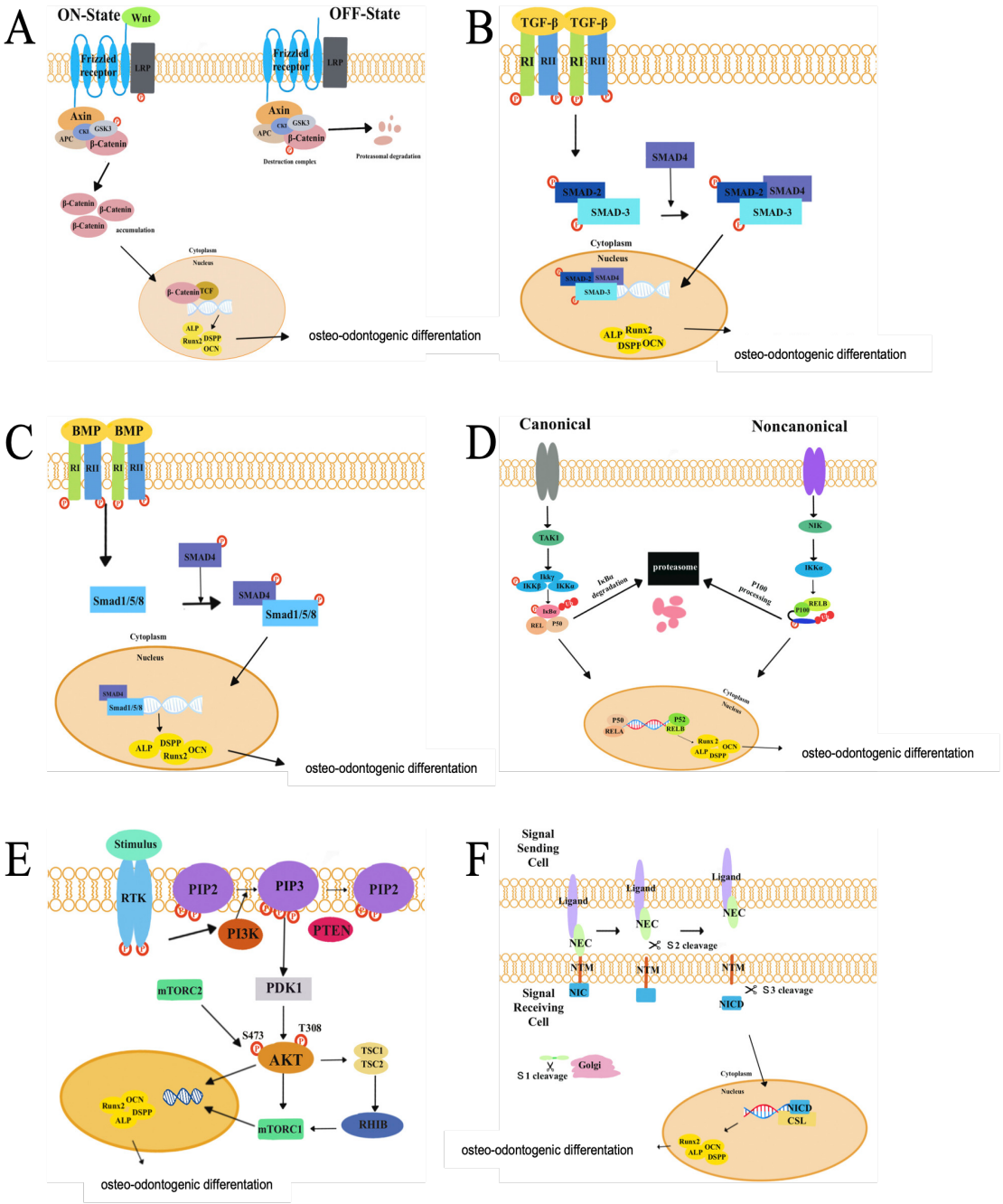


Figure 6. *Representative diagrams of the six main pathways involved in the osteo-odontoblastic differentiation of dental pulp stem/stromal cells.* ***A*** *Wnt signaling pathway.* ***B, C*** *Smad Signaling Pathways.* ***D*** *NF-kB signaling pathways.* ***E*** *PI3K/AKT/mTOR signaling pathways.* ***F*** *Notch Signaling Pathways [Zhou et al., 2023].*

To obtain osteo-odontogenic differentiation of DPSCs *in vitro*, these cells are cultured in a medium supplemented with osteo-inductive factors. The main ones include dexamethasone, which induces the expression of RUNX2, OSX, and bone matrix proteins; β-glycerophosphate and ascorbic acid, which promote increased secretion of

type I collagen and activity of ALP in the osteoprogenitors [Gupta et al., 2007; Igarashi et al., 2004; Zhang et al., 2012].

The evaluation of osteo-odontogenic differentiation can be conducted through various methods:

- Histological staining techniques such as Alizarin Red S and Von Kossa staining can highlight the deposition of mineralized matrix and the formation of mineralized nodules resembling bone or dental tissue [Awais et al., 2020; Ajlan et al., 2015; Wang et al., 2012; Kanafi et al., 2013; Lim et al., 2021].
- Scanning electron microscopy (SEM) and micro-computed tomography (micro-CT) analysis can assess the morphology and structure of calcified nodules, providing additional information on their composition and distribution [Wang et al., 2012; Kang et al., 2017; Luo et al., 2023; Hozhabri et al., 2015].
- Measurement of alkaline phosphatase (ALP) activity, an enzyme involved in bone and dental tissue mineralization [Ajlan et al., 2015; Wang et al., 2012; Cui et al., 2019].
- Evaluation of the expression of specific surface markers including OCN (secreted by osteoblasts in the mineralized matrix), OPN (phosphoglycoprotein secreted by osteoblasts before mineralization), type I collagen, RUNX2, DSPP, and ALP [Kang et al., 2017; Luo et al., 2023; Lim et al., 2021; Cui et al., 2019].

- ***Hepatic differentiation of DPSCs***

Human dental pulp stem/stromal cells have demonstrated the ability to differentiate towards a hepatic phenotype, thus expanding their therapeutic potential. Studies conducted by Hara et al. have highlighted that DPSCs when exposed to specific growth factors and culture conditions, can differentiate into hepatocyte-like cells [Hara et al., 2020]. In the intricate process of mesenchymal stem cells differentiating into hepatocytes, researchers delineated the procedure into three stages: initiation, differentiation, and maturation, mirroring the sequences of liver developmental processes involving specification and differentiation into mature hepatocytes [Ye et al., 2015].

1. Initiation stages: transition from mesoderm to endoderm

The hepatocyte is the primary liver cell type and originates from the embryonic endoderm [Sell et al., 2003]. It is known that DPSCs are cells derived from the ectoderm, originating from the migration of neural crest cells and have the potential to differentiate into both mesodermal and non-mesodermal lineages under specific culture conditions [Mattei et al., 2021; Lan et al., 2019]. Therefore, the transition phase towards the endoderm is essential, and it is considered the initiation stage in the process of hepatocyte differentiation *in vitro* [Zorn et al., 2007; Touboul et al., 2010; Ramasamy et al., 2013].

Activin/Nodal family members initiate mesodermal/endodermal transition when Activin A expression increases by 3-fold compared to baseline. Activin/Nodal family members release inhibitory signals generated by phosphoinositide 3-kinase (PI3K) through insulin/IGF *(Figure 7A)* [McLean et al., 2007].

Further studies have shown that the combination of Activin A and Wnt also induces differentiation towards hepatic lineages, as does the overexpression of β-catenin. This suggests that Wnt/β-catenin signaling autonomously induces non-hepatic endodermal cells to the liver fate [Toivonen et al., 2013; So et al., 2012].

Hence, in the initiation stage of hepatocyte differentiation, DPSCs can be induced to become a homogenous population of endodermal cells using a combination of Activin A and Wnt, together with PI3K inhibition [Touboul et al., 2010; McLean et al., 2007].

2. Differentiation stages: development of hepatoblasts

Hepatoblasts are considered somatic stem/progenitor cells in fetal livers due to their high proliferative potential and ability to differentiate into both hepatocytes and cholangiocytes during middle to late embryonic stages [Decaens et al., 2008; Touboul et al., 2010; Wei et al., 2008].

The proliferation and differentiation of hepatoblast cells are regulated by various soluble factors. By the addition of growth factors such as fibroblast growth factors (FGF4), hepatocyte growth factor (HGF), and epidermal growth factor (EGF), DPSCs can be directed towards cells co-expressing hepatocyte marker alpha-fetoprotein (AFP) and cholangiocyte marker cytokeratin 19, indicating some correspondence to the hepatoblast population [Schmelzer et al., 2007].

Forte et al. demonstrated the essential role of HGF in liver development and regeneration through comparative observations of short-term and long-term exposure to DPSCs *(Figure 7B).* Short-term exposure to HGF induces activation of its cognate Met receptor

and downstream effectors extracellular signal-regulated kinase 1/2 (ERK1/2), p38, MAPK, and PI3K/AKT. However, long-term exposure results in cytoskeletal rearrangement, cell migration, and inhibition of proliferation by arresting at the G1–S checkpoint [Forte et al., 2006].

Other studies have shown that HGF and c-Met are constitutively expressed by DPSCs and that TGF-β down-regulates the expression of HGF. Furthermore, HGF acts as a strong chemotactic stimulus to DPSCs, possibly enhanced by autocrine signaling through the HGF c-Met pathway [Neuss et al., 2004]. Zhou et al. have shown that HGF supports a mid/late hepatic phenotype such as albumin (ALB) and dipeptidyl peptidase IV expression, it fails to induce functional hepatocyte maturation [Zhou et al., 2007].

These investigations suggest that HGF stimulates rapid hepatoblast proliferation via Wnt/β-catenin signaling and the HGF c-Met pathway, playing a crucial role in hepatogenesis *in vivo* and considered a critical and preliminary growth factor in the process of hepatocyte differentiation *in vitro* [Ye et al., 2015].

Another family of FGFs has demonstrated efficacy in mediating early hepatic differentiation, as evidenced by studies conducted by Tsukada and Sekhon [Tsukada et al., 2006; Sekhon et al., 2004]. Additionally, numerous other growth factors have been implicated in hepatocyte differentiation. The process of differentiating DPSCs into hepatocytes appears to be catalyzed and induced by the synergistic action of HGF and FGF4 through the HGF/c-Met signaling pathway, coupled with interactions with Wnt signaling. This is supported by notable increases in the expression levels of ALB, CK18, and CK19, along with a significant reduction in AFP levels [Ye et al., 2015].

3. Maturation stages: development of hepatocyte

During the later phases of liver cell development in a lab setting, various research studies have identified oncostatin M (OSM), insulin–transferrin–selenium (ITS), dexamethasone, and nicotinamide as crucial elements. OSM, which belongs to the interleukin-6 (IL-6) subfamily, is secreted by certain blood cells during the early stages of embryo formation. Research literature indicates that OSM plays a key role in promoting the growth of liver cells through the activation of the transcription 3 (STAT3) signaling pathway, although it is not able to fully trigger the development of mature liver cells on its own *(Figure 7C)*. Furthermore, OSM by itself has limited effects on liver cell function; however, when combined with nicotinamide, it significantly boosts the production of ALB [Urbaenska-Ryes et al., 2000; Kamiya et al., 2001; Sakai et al., 2002;

Sate et al., 1999]. Moreover, Vollmer et al. demonstrated that treatment of hepatocytes with OSM resulted in increased protein levels of hypoxia-inducible factor-1alpha (HIF-1alpha) under normoxic and hypoxic conditions through the Janus kinase (JAK)/STAT3 and mitogen-activated protein kinase kinase (MAPKK)/ERK1/2 pathways. They further revealed that OSM-mediated up-regulation of HIF-1alpha was not attributed to an increase in HIF-1alpha protein stability but rather to enhanced transcription of the HIF-1alpha gene [Vollmer et al., 2009].

To summarize, various cytokines and growth factors implicated in the diverse stages of hepatocyte differentiation from dental pulp stem cells are closely associated with signaling pathways such as the Activin/Nodal pathway, PI3K/AKT pathway, HGF/c-Met pathway, ERK1/2, MAPK, JAK/STAT3, and hypoxia pathway, although further elucidation of precise mechanisms is warranted [Ye et al., 2015].

The assessment of hepatic differentiation of dental pulp mesenchymal stem cells can be carried out through various methods:

- Evaluation of gene expression [Bishi et al., 2013; Kumar et al., 2017]:

Endodermal Commitment: Sox17, HEX, GATA4, HNF4α, HNF3α/β/γ, HNF1, CCAAT enhancer binding protein (C/EBPα/β)

Early Differentiation: AFP, Alb, A1AT, TTR, TAT, G6P, PEPCK, OTC, GS, ASGPr

Hepatic Maturation: GS, ammonia metabolism (OTC), G6P, ASGPr, TN, nuclear hormone receptor genes (PXR, CAR, PPARα/β/γ), CYP450s (1A1, 1A2, 2C8/9/19, 2D6, 3A4/7, 7A1), bilirubin metabolism (UGT1A1), transporters (BSEP, MRP2, NTCP, OATP1), clotting factors (V, VII, IX)

- Functional analysis [Ji et al., 2012; Ishkitiev et al., 2010]:

Cytochrome P450 (CYP) activities: activities of enzymes such as 1A1, 1A2, 2B6, 2C9, 3A4, 3A7 are assessed via luminescent assays induced by prototypical inducers like rifampicin (Rif), Phenobarbital (PB), omeprazole (Omp), or beta-Naphthoflavone (BNF).

Phase II metabolism: the decrease of the Resorufin fluorescent compound signal is assessed after conjugation and measured by a fluorimetric spectrometer.

Albumin/A1AT ELISA: measurement of human ALB and A1AT through a competitive Enzyme-Linked Immunosorbent Assay.

Ammonia Metabolism: measurement of ammonia metabolism over 2-6 hours by adding NH_4Cl to the cultures, followed by measurements at 3-6 hours with ammonia test.

Urea Synthesis: determined by mass spec analysis of incorporated N15, a more specific assay compared to the commonly used colorimetric BUN assay.

- Ultrastructural analysis: electron microscopy techniques are utilized to analyze the ultrastructure of differentiated hepatic cells and evaluate their resemblance to mature hepatic cells [Ji et al., 2012; Bishi et al., 2013].

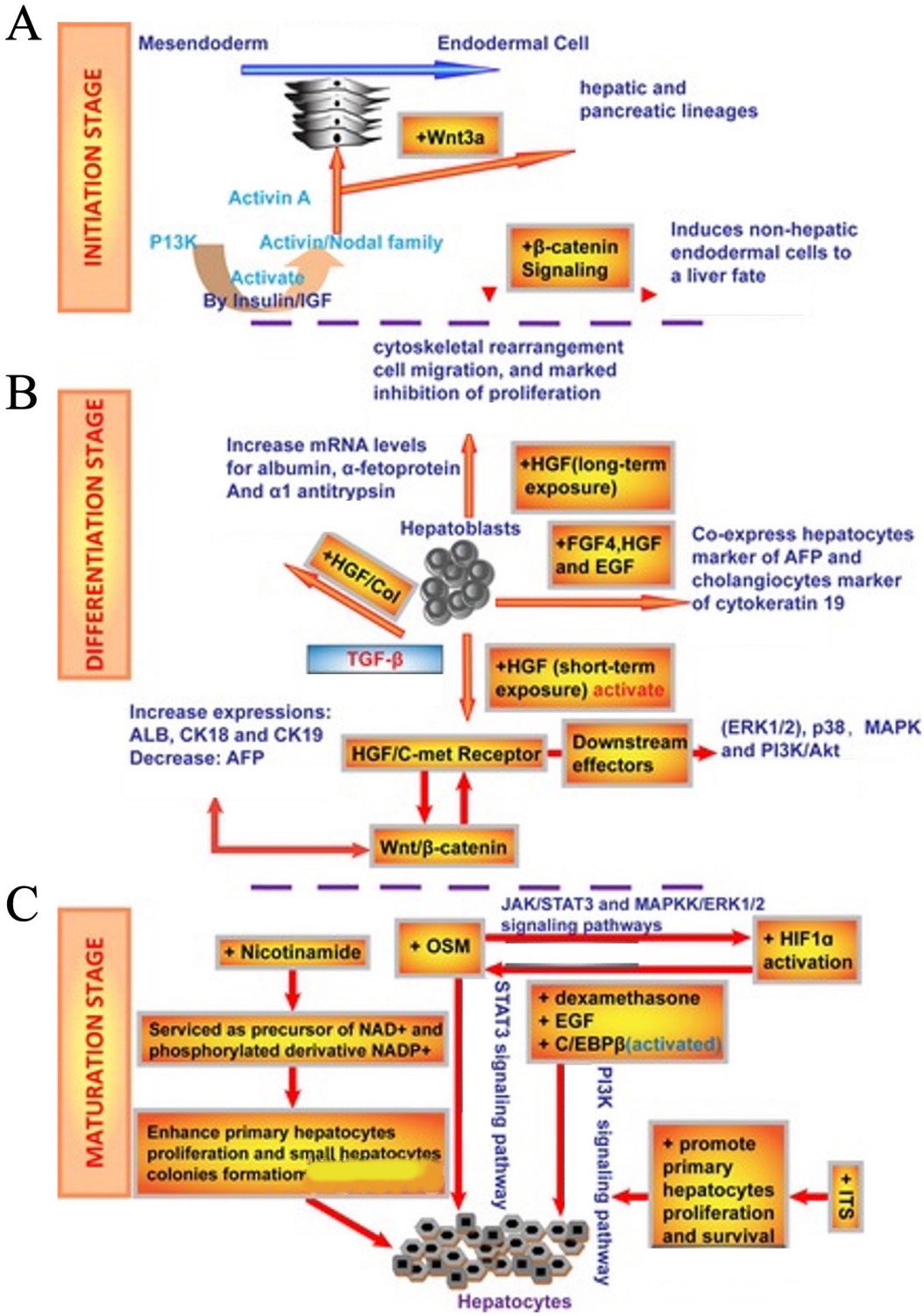


Figure 7. *Schematic signaling pathways of MSCs differentiating into hepatocytes in vitro.* ***A*** *Initiation stages, transition from mesoderm to endoderm.* ***B*** *Differentiation stages: development of hepatoblasts.* ***C*** *Maturation stages: development of hepatocyte [Ye et al., 2015].*

- ***Neuronal differentiation of DPSCs***

Neurodegenerative diseases lead to a progressive loss of mature and functionally active neurons. The replacement of damaged neurons as a therapeutic strategy has been extensively researched, and for regenerative medicine, it is crucial to identify autologous and transplantable sources of neurons and glial cells [Barker et al., 2018; Björklund et al., 2020; Volkman et al., 2017]. Accessible sources of stem cells can be found outside the central nervous system [Ming et al., 2011]. Multipotent MSCs, including DPSCs, have the ability to differentiate into specific lineage cell types [Dominici et al., 2006]. This high differentiation potential is associated with a significant capacity for self-renewal, easy accessibility, and high proliferation capacity.

In the differentiation of DPSCs towards a neuronal phenotype, three signaling pathways are critical *(Figure 8)* [Neirinckx et al., 2013; Sramkó et al., 2023]:

1. Cyclic-adenosine-monophosphate and PKA signaling pathway.
2. Retinoic acid signaling pathway.
3. Neurotrophic factors and downstream signaling pathways.

1. Cyclic-adenosine-monophosphate and PKA signaling pathway

Cyclic adenosine monophosphate (cAMP) is a well-known intracellular messenger synthesized physiologically from adenosine triphosphate by a membrane-anchored adenylyl cyclase when activated by a G protein-coupled receptor. In the cytoplasm, cAMP primarily activates protein kinase A (PKA), which then translocate to the nucleus where it facilitates the phosphorylation of a transcription factor, cAMP-responsive element-binding protein (CREB). Once phosphorylated, CREB binds to CREB-binding protein (CREBBP or CBP) and, with the assistance of various cofactors, binds to specific DNA sequences, regulating the expression of several genes such as brain-derived neurotrophic factor (BDNF) or tyrosine hydroxylase (TH) [Suzuki et al., 2011; Lim et al., 2000]. Intracellular cAMP degradation is mediated by phosphodiesterases (PDE), which convert cAMP to AMP, thereby regulating cytoplasmic cAMP concentrations. This cAMP-dependent pathway has been demonstrated to be critical in embryonic development,

neural cell survival, and processes like long-term memory and neuronal plasticity [Bourtchuladze et al., 1994; Dworkin et al., 2007; Mantamadiotis et al., 2002].

cAMP is commonly utilized in culture media to induce DPSCs into the neural lineage, along with other molecules that raise the intracellular cAMP levels. For example, forskolin activates adenylyl cyclase, dibutyryl-cAMP (db-cAMP) and 3-isobutyl-1-methylxanthine (IBMX) both act as phosphodiesterase inhibitors, and 8-bromo-cAMP activates PKA and exhibits long-acting effects due to its resistance to degradation by PDE [Jori et al., 2005; Kim et al., 2005].

While the cAMP pathway is supposed to play a role in the neural differentiation of mesenchymal stem cells, its precise contribution to this differentiation process requires further elucidation. Zhang et al. provided evidence for cAMP involvement in two differentially regulated processes: early transient changes in neuron-like morphology, such as cytoskeletal rearrangement, and subsequent expression of neural markers associated with neuronal function. However, they demonstrated that cAMP treated MSCs did not achieve complete differentiation [Zhang et al., 2011], so further studies are needed.

2. Retinoic acid signaling pathway

The retinoic acid (RA) signaling pathway involves the physiological conversion of retinol to RA through the sequential actions of cellular retinol-binding protein (CRBP), retinol dehydrogenase (RoDH), and retinaldehyde dehydrogenases (RALDHs). Once synthesized, RA binds to cellular RA-binding protein (CRABP) in the cytoplasm and then translocate into the nucleus, where it binds to its specific receptors, retinoic acid receptors (RARs), and retinoid X receptors (RXRs). These receptor complexes heterodimerize and bind to DNA sequences known as retinoic acid-response elements (RAREs), activating the transcription of target genes such as Hox genes and OCT4 [Gudas et al., 2011]. The RA signaling pathway plays a crucial role in brain development, particularly in defining the anteroposterior axis of the nervous system by regulating the expression of Hox genes in specific localized domains of the embryo [Maden et al., 2002].

Arthur et al. conducted RA treatment of human DPSCs, resulting in the acquisition of neural morphology and the expression of β3-Tubulin, Neurofilament M (NF-M), and Neurofilament H (NF-H). Importantly, these treated cells also exhibited electrophysiological activity characteristic of sodium voltage-gated channels, indicating their potential to differentiate into functional neurons [Arthur et al., 2008].

3. Neurotrophic factors and downstream signaling pathways

Neurotrophins (NT) are a family of secreted growth factors crucial for the development, survival, and functionality of neurons in the nervous system. This family includes prototypical members such as nerve growth factor (NGF), brain-derived growth factor (BDNF), and NT-3, 4/5, and 6. NTs promote neural cell survival, growth, differentiation, and function by binding to high-affinity tyrosine kinase receptors (TrkA, TrkB, and TrkC, respectively bound by NGF/NT-6, BDNF/NT-4, and NT-3) and a common low-affinity receptor, p75LNR. While p75LNR activation induces events like neurite formation, its role appears to facilitate NT binding to Trk receptors. After trans-phosphorylation, Trk receptors activate three main signaling pathways: mitogen-activated protein kinases (MAPKs), phospholipase C (PLC), and phosphatidylinositol-3-kinase (PI3K) [Reichardt et al., 2006; Huang et al., 2003; Mitsiadis et al., 2017; Luzuriaga et al., 2019].

The MAPK pathway involves a cascade of sequentially activated kinase proteins, including regulators such as alpha-foetoproteins (Raf), extracellular-regulated kinases (ERK), p38, or jun-kinase 1/2/3. This pathway results in the phosphorylation of transcription factors, regulating gene expression. MAPKs are highly expressed in the central nervous system (CNS), with ERKs playing key roles in neuronal maturation, survival, and synaptic functions [Chang et al., 2017].

The PLC signaling pathway primarily induces intracellular calcium mobilization and stimulates protein kinase C (PKC) via diacylglycerol (DAG) production. PKC can also be activated directly by 12-O-tetradecanoylphorbol-13-acetate (TPA), activating the MAPK pathway [Kiraly et al., 2009]. Finally, PI3K controls another downstream kinase called AKT (also known as protein kinase B or PKB), which is crucial for cell survival by regulating apoptosis and other cellular functions.

Kiraly et al. achieved neural induction of DPSCs by activating the cAMP and PKC signaling pathways. Following reprogramming with 5-azacytidine treatment, cells were treated with IBMX, db-cAMP, forskolin, TPA, NGF, and NT-3, resulting in increased expression of neurogenin-2, β3-Tubulin, neuron-specific enolase (NSE), NF-M, and GFAP. Electrophysiological recordings revealed voltage-dependent sodium channel activity [Kiraly et al., 2009].

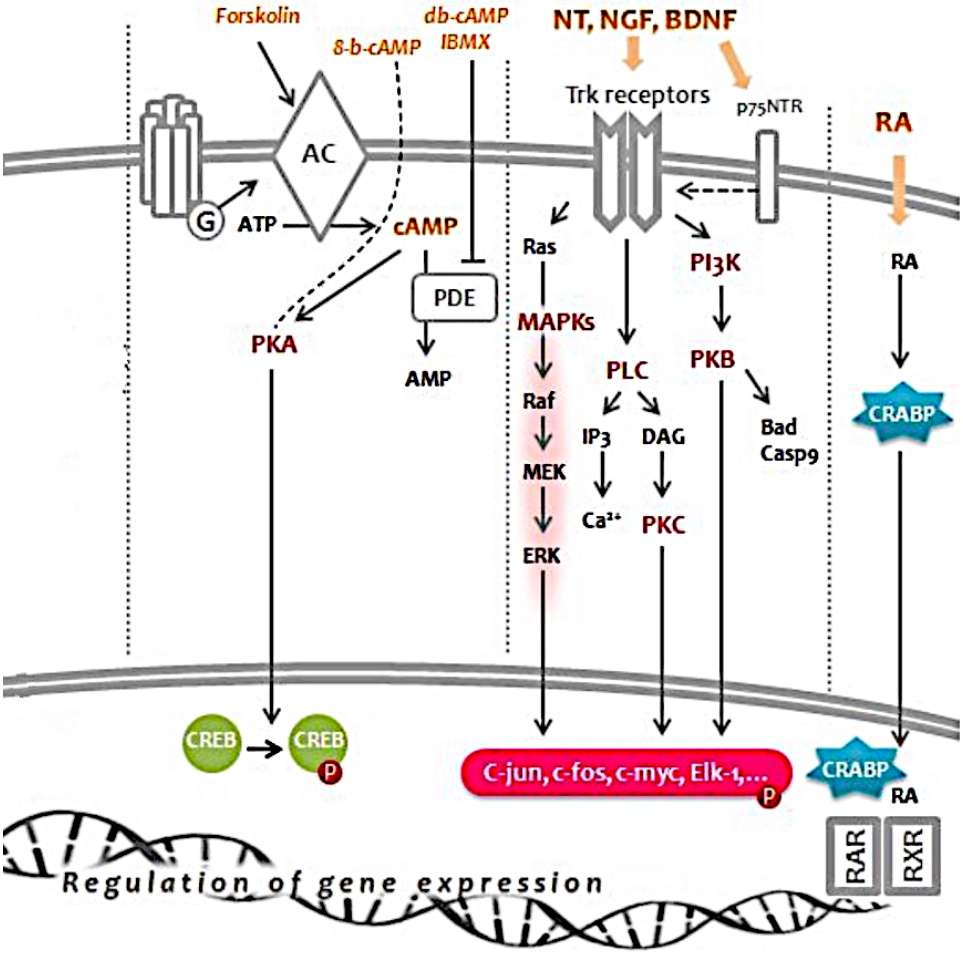


Figure 8. *Different signaling pathways are involved in the neural differentiation of DPSCs [Neirinckx et al., 2013].*

The main techniques used to verify the neuronal differentiation of DPSCs include:

- Morphological analysis: this technique involves observing cells under a microscope to assess any changes in shape and cellular structure indicative of neuronal differentiation [Al-Maswary et al., 2022; Delle Monache et al., 2022].
- Evaluation of gene expression (RT-PCR): this method is used to detect the gene expression of neuronal markers such as neurogenin-2, nestin, β3-Tubulin, GFAP, bFGF, NGF, EGF, GDNF, BDNF [Chang et al., 2014; Al-Maswary et al., 2022; Delle Monache et al., 2022; Rafiee et al., 2020; Osathanon et al., 2014].
- Immunofluorescence analysis: it allows visualization of neuronal proteins, such as β3-Tubulin, neurofilaments, GFAP, and nestin by labeling them with fluorescent antibodies [Al-Maswary et al., 2022; Chang et al., 2014; Delle Monache et al., 2022; Luo et al., 2020; Osathanon et al., 2014].

- Electrophysiological analysis: this technique involves recording electrical currents across the cell membranes of differentiated cells to assess the presence of electrical activity typical of mature neuronal cells [Al-Maswary et al., 2022; Osathanon et al., 2014; Urraca et al., 2015].

- *Peri-Vascular differentiation of DPSCs*

The development of a complex network of blood vessels is crucial for supplying cellular nutrients and supporting the proper growth and regeneration of damaged tissues and organs. Blood vessel formation can occur through angiogenesis, involving the sprouting and enlargement of existing vessels, or through vasculogenesis, which entails the differentiation of endothelial cells and the creation of new vasculature from precursor cells. Effective promotion and regulation of both processes are vital for tissue engineering endeavors [Luzuriaga et al., 2020].

Recent studies suggest that postnatal mesenchymal stem cells hold promise for generating functional and histointegrative blood vessels [Song et al., 2018]. In this context, selecting the appropriate stem cell source is critical. Factors such as minimizing host immune reactions, the capacity for *in vitro* expansion, ease of isolation, and successful integration within the transplanted tissue are all important considerations [Rabkin et al., 2002].

DPSCs emerge as promising candidates as a source of mesenchymal stem cells due to their ability to release pro-angiogenic factors and differentiate into peri-vascular cells [Liang et al., 2021]. Nevertheless, the mechanisms underlying the vasculogenic differentiation of mesenchymal stem cells remain unclear.

It is known that DPSCs can act as pericyte-like cells and secrete pro-angiogenic factors such as vascular endothelial growth factor (VEGF) for vascularization in dental pulp regeneration [Janebodin et al., 2013]. Indeed, DPSCs exposed to factors like TNF-α, hinokitiol, and lipopolysaccharide (LPS) activate the MAPK pathway (p38 and ERK), thereby upregulating the expression of pro-angiogenic factors like VEGF, HIF-1alpha, angiopoietin-1 (Ang-1), and von Willebrand factor (vWF) [Kim et al., 2014; Huang et al., 2015; Shin et al., 2015]. Additionally, it has been demonstrated that the signaling pathway EphrinB2/EphB4 enhances the secretion of VEGF by DPSCs via MEK/ERK1/2 and MAPK (p38) transduction, thereby stimulating endothelial cell angiogenesis [Gong et al., 2019].

Moreover, dental stem cells can directly differentiate into endothelial cells to promote angiogenesis [Bento et al., 2013]. The differentiation of DPSCs into peri-vascular cells involves various signaling pathways such as:

1. *Vascular endothelial growth factor (VEGF) pathway:* VEGF is a homodimeric protein that binds specifically to two receptor tyrosine kinases (RTKs), namely the fms-like tyrosine kinase Flt-1 (VEGFR1) and KDR (VEGFR2) [Leung et al., 1989; Breier et al., 1992]. While VEGFR1 expression is relatively ubiquitous, VEGFR2 expression is more limited to vascular endothelial cells [Shibuya et al., 2006]. Quiescent DPSCs strongly express VEGFR1 but not VEGFR2 and long-term VEGF treatment modifies this balance by inducing VEGFR2 expression through the activation of downstream ERK and AKT signaling pathways [Sakai et al., 2010; Zhang et al., 2016].
2. *Sema4D/PlexinB1 pathway:* semaphorins are a family of glycoproteins initially identified as axonal guidance molecules during nervous system development [Basile et al., 2004; Nkyimbeng-Takwi et al., 2011]. It has been reported that, in endothelial cells, when SEMA4D is binding to Plexin B1 it exhibits a potent pro-angiogenic activity *in vitro* and *in vivo* [Iragavarapu-Charyulu et al., 2020]. Plexin B1 regulates SEMA4D-induced vasculogenic differentiation of DPSCs by upregulating VEGF secretion through the phosphorylation of AKT and ERK1/2 [Zou et al., 2019].
3. *WNT/β-catenin pathway:* Wnt-β-catenin signaling plays critical roles in the regulation of cell proliferation and polarity, apoptosis, branching morphogenesis, inductive processes, and differentiation of stem cells [Wodarz et al., 1998; Willert et al., 2003]. In particular, in the central nervous system, canonical Wnt signaling regulates organ-specific assembly and differentiation of the vasculature [Wodarz et al., 1998; Willert et al., 2003; Stenman et al., 2008]. Treatment of DPSCs with VEGF or Wnt1 upregulates the expression of endothelial cell differentiation markers such as VEGFR2, VE-Cadherin, angiopoietin-1 receptor (Tie-2), and CD31 through the activation of the canonical WNT/β-catenin pathway, along with increased expressions of WNT receptors (LRP-6 and Fzd-6) and β-catenin [Zhang et al., 2016]. However, the exact role of this pathway in regulating the vasculogenic differentiation of postnatal mesenchymal stem cells remains poorly understood.

Overall, DPSCs not only act as pericytes secreting pro-angiogenic factors to induce endothelial cell vascularization but can also differentiate directly into peri-vascular cells through VEGF, HIF-1alpha, MAPK (p38 and ERK), canonical WNT/β-catenin, and PI3K/AKT signal transduction pathways *(Figure 9)* [Liang et al., 2021].

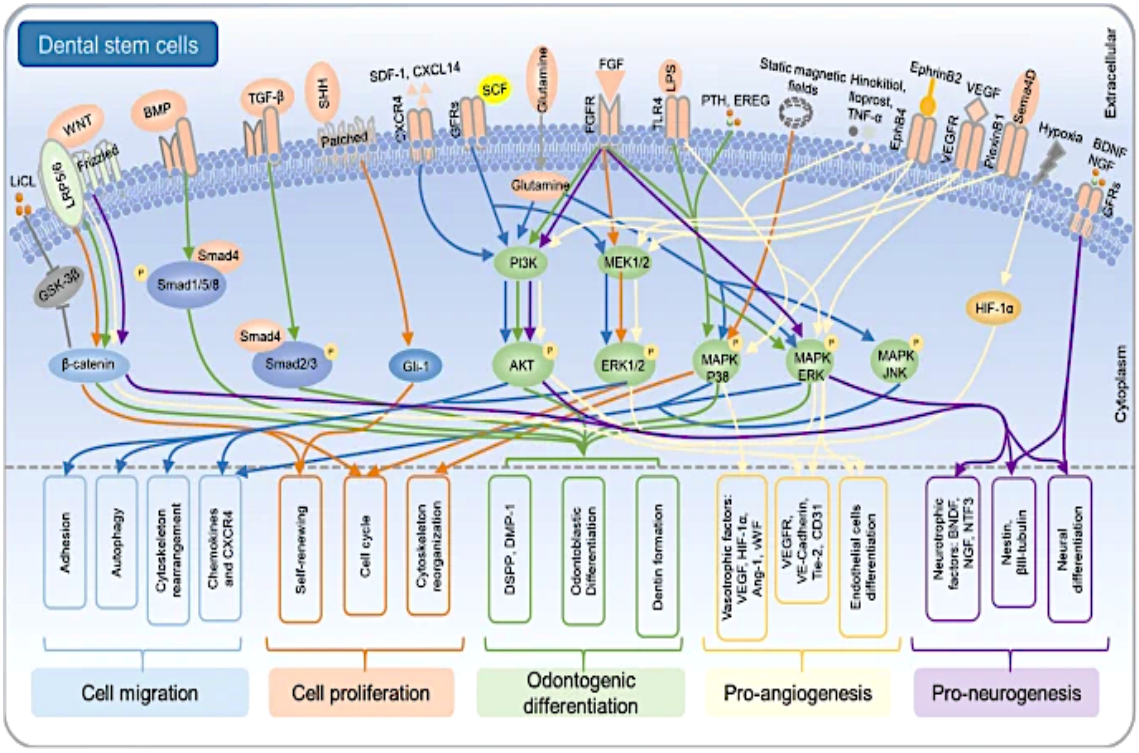


***Figure 9**. The key signaling pathways and downstream transductions contribute to the regenerative potential of DPSCs [Liang et al., 2021].*

The peri-vascular differentiation of DPSCs is essential for their use in tissue regeneration. To verify this process, various analysis techniques are employed, such as:

- Immunofluorescence: vWF, CD31, VEGFR1 [Aksel et al., 2017; Katata ET AL., 2021; Bergamo et al., 2021; Li et al., 2021; Zhang et al., 2016].
- Gene transcription analysis (RT-PCR): VE-Cadherin, VEGFR-1, VEGFR-2, Tie-2, CD31, Plexin-B1, VEGF, vWF, HIF-1alpha, ANG1 [Sasaki et al., 2020; Bergamo et al., 2021; Li et al., 2021; Zhang et al., 2016; Jin et al., 2018; Zou et al., 2019].
- In vitro angiogenesis test: for the evaluation of the differentiated DPSCs' ability to form tubules *in vitro*, simulating the process of angiogenesis. Various parameters such as total segment length and branching index are considered for quantitative analysis [Aksel et al., 2017; Mantesso et al., 202; Bergamo et al.,

20211; Silva et al., 2017; Li et al., 2021; Zhang et al., 2016; Jin et al., 2018; Zou et al., 2019].

DPSCs Types of Differentiation	***Signaling Pathways***	***Markers of Function***
Osteo-Odontoblastic Differentiation	Wnt Smad MAPK NF-kB PI3K/AKT/mTOR Notch	- Alizarin Red S and Von Kossa staining - SEM and micro-CT analysis - ALP activity - RT-PCR: OCN, OPN, Collagen I, RUNX2, DSPP, ALP
Hepatic Differentiation	Activin/Nodal Wnt/β-catenin MAPK PI3K/AKT JAK/STAT3 HGF/c-Met HIF-1alpha	- RT-PCR: Sox17, HEX, GATA4, HNF4α, HNF3α/β/γ, HNF1, CCAAT, AFP, ALB, A1AT, TTR, TAT, G6P, PEPCK, OTC, GS, ASGPr, TN, PXR, CAR, PPARα/β/γ, CYP450s, UGT1A1, BSEP, MRP2, NTCP, OATP1, clotting factors - Cytochrome P450 activities - Resorufin fluorescence analysis - Albumin/A1AT ELISA - Ammonia Metabolism - Urea Synthesis - Electron microscopy techniques
Neuronal Differentiation	cAMP and PKA RA NT	- Morphological analysis - RT-PCR: neurogenin-2, nestin, β3-Tubulin, GFAP, bFGF, NGF, EGF, GDNF, BDNF - Immunofluorescence analysis - Electrophysiological analysis
Peri-Vascular Differentiation	VEGF Sema4D/PlexinB1 WNT/β-catenin	- Immunofluorescence analysis - RT-PCR: VE-Cadherin, VEGFR-1, VEGFR-2, Tie-2, CD31, Plexin-B1, VEGF, vWF, HIF-1alpha, ANG1 - In vitro angiogenesis test

Table 4. *The differentiation ability of DPSCs (osteo-odontoblast, hepatic, neuronal, and peri-vascular differentiation). Signaling pathways are involved in the regulating of differentiation processes. Markers of function used to evaluate the differentiation of DPSCs.*

1.2.5 Applications of Dental Pulp Stem/Stromal Cells in Regenerative Medicine

Dental Pulp Stem/Stromal Cells have emerged as a promising tool in regenerative medicine due to their unique properties and accessibility. DPSCs, which are found within the dental pulp of teeth, possess multipotent differentiation capabilities, enabling them to give rise to various cell types including osteoblasts, chondrocytes, adipocytes, neuronal cells, and cardiac muscles [Delle Monache et al., 2012; Martellucci et al., 2018; Mattei et al., 2015]. This pluripotency makes them particularly valuable for tissue engineering and regenerative therapies. In regenerative medicine, DPSCs have been extensively studied and applied in the repair and regeneration of various tissues and organs including bone, cartilage, nerve, and dental tissues.

Furthermore, some researchers have highlighted that DPSCs, in addition to their differentiation ability can secrete various molecules into the medium that can be utilized in regenerative medicine. They demonstrated that conditioned media derived from DPSCs (DPSCs-CM) contain some molecules such as anti-inflammatory cytokines [Bousnaki et al., 2022; Matsumura-Kawashima et al., 2021], interleukin (IL)-10, IL-13, follistatin, TGF-β1, HGF, NCAM-1, adiponectin, etc. The production of these cytokines can reduce inflammation, increase progenitor cell proliferation, enhance tissue repair, and effectively reduce infection [Yamada et al., 2019].

Therefore, the potential of DPSCs to differentiate into specific cell types, along with their ability to influence the immune system makes them ideal candidates for repairing injured or diseased tissues *(Figure 10).*

In particular, several authors have reported that DPSCs represent a potential novel stem cell therapy for joint cartilage repair. Studies have demonstrated the efficacy of DPSCs in enhancing joint function and reducing pain [Fernandes et al., 2020]. The accessibility of dental pulp as a source of mesenchymal cells makes DPSCs a valuable alternative to primary chondrocytes due to their enhanced cartilage proliferation and regeneration capacity [Mata et al., 2017]. One potential application of DPSCs following chondrogenic

differentiation could be the treatment of symptomatic cartilage lesions and early osteoarthritis [Fernandes et al., 2020]. A study by Lo Monaco et al. has elucidated the role of DPSCs in osteoarthritis, a degenerative and inflammatory joint disease characterized by cartilage loss. The authors discuss the therapeutic potential of DPSCs in osteoarthritis through immunomodulation and cartilage regeneration [Lo Monaco et al., 2020; Popa et al., 2015].

Numerous authors have proven through both in vitro and in vivo studies that DPSCs exhibit the ability to stimulate osteogenesis and promote bone regeneration. These studies have consistently shown that DPSCs possess a high proliferation rate, excellent osteogenic differentiation potential, and advantageous paracrine and immunomodulatory properties [Liu et al., 2015; La Noce et al., 2014; Chamieh et al., 2016]. The combined findings from these investigations suggest that DPSCs hold significant promise for application in regenerative therapies targeting bone-related diseases and orthopedic surgeries [Ramamoorthi et al., 2015; Jensen et al., 2016].

Since DPSCs express specific neural markers such as nestin, S100-beta, GFAP, or molecules like the prion protein highly expressed in neuronal tissues, several authors have speculated that these cells could serve as an ideal tool for neural induction and regeneration [Martellucci et al., 2019; Mattei et al., 2021]. Indeed, DPSCs have demonstrated the ability to differentiate into neuronal-like cells or dopaminergic neuron-like cells [Ullah et al., 2016; Chun et al., 2016]. Moreover, other studies have shown that DPSCs can produce and release neurotrophic factors such as brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), and nerve growth factor (NGF) into the medium. This conditioned media from DPSCs has been found to enhance the growth rate of Schwann cells and induce neurite outgrowth in vitro [Kolar et al., 2017; Yamamoto et al., 2016]. These characteristics make DPSCs a promising cellular model for studying and treating neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's [Mediano et al., 2015; Martellucci et al., 2019].

Furthermore, it has been observed that the release of trophic factors such as BDNF and GDNF by DPSCs significantly improves motor functions when utilized in a murine model with spinal cord injury [Zhang et al., 2016; Asadi-Golshan et al., 2018]. Additionally, DPSCs release other factors (ED-Siglec-9 and monocyte chemoattractant protein-1), inducing notable functional recovery in a rodent spinal cord injury model by promoting an M2-dominant neuro-repairing microenvironment [Kano et al., 2017].

Indeed, recent studies have revealed that in addition to their already known differentiation capacities, DPSCs can differentiate into hepatocytes and insulin-secreting beta cells [Potdar et al., 2015].

Yamaguchi et al. suggest that administering conditioned media from DPSCs can protect the heart from ischemic damage by reducing cardiomyocyte death and suppressing inflammatory responses in myocardial cells. They found that treatment with conditioned media derived from DPSCs led to decreased myocardial infarction and improved cardiac function in mice following ischemia-reperfusion injury. Furthermore, they demonstrated that under specific conditions such as hypoxia and serum deprivation, conditioned media from DPSCs could induce cardiomyocyte survival and reduce pro-inflammatory mediators promoted by lipopolysaccharide [Yamaguchi et al., 2015; Sowa et al., 2018].

Over the last ten years, significant efforts have focused on improving techniques for vascularizing tissue grafts. It is well understood that the regenerative capacity of tissues is closely tied to the presence of progenitor stem cells capable of proliferating and differentiating into different cell types. Because of this, scientists have conducted thorough research on using cell-based treatments to improve the formation of new blood vessels after ischemic incidents like heart attacks or strokes, [Rohban et al., 2018]. Moreover, research has demonstrated that human MSCs can effectively repair ischemic tissue and restore tissue function by promoting the formation and stabilization of new blood vessels and modulating the immune response [Yan et al., 2015].

In particular, DPSCs exhibit phenotypes consistent with peri-vascular cell populations, supporting the processes of vasculogenesis and angiogenesis. DPSCs not only secrete pro-angiogenic factors like VEGF, but they also can differentiate into both endothelial cells and pericytes [Zou et al., 2019; Delle Monache et al., 2019; Sasaki et al., 2020]. As demonstrated by Dissanayaka et al., DPSCs are capable of triggering angiogenesis by secreting VEGF, which is utilized by human umbilical vein endothelial cells (HUVECs) to activate migration and vascular structure formation [Dissanayaka et al., 2015]. In addition to VEGF secretion, DPSCs secreted other factors involved in various phases of the angiogenesis process, such as proliferation or stabilization. For example, in a study conducted by Hilkens et al., the authors demonstrated that DPSCs express beta fibroblast growth factor (bFGF), matrix metalloproteinases, endostatin, thrombospondin-1, and insulin-like growth factor-binding protein-3 [Hilkens et al., 2014]. Moreover, some studies have demonstrated the proliferative and contraction/remodeling capabilities of DPSCs. They express matrix metalloproteinases, which can degrade biomaterial scaffolds

and regulate cellular functions in 3D environments, contributing to the process of vascular remodeling [Alraies et al., 2020].

Some researchers have developed a decellularized dental pulp (DDP) scaffold obtained through the decellularization process, enriched with extracellular vescicles (EVs) and 5-Aza-2′-deoxycytidine (5-Aza), for dental pulp regeneration. DPSCs were used as a cell source for DDP recellularization, given their recognized suitability for dental pulp regeneration. The study demonstrated that following recellularization DPSCs effectively attached, proliferated, and migrated on the DDP scaffold, indicating their compatibility for cell binding and growth [Mattei et al., 2023].

Specifically, EVs derived from MSCs have been investigated for their beneficial effects on tissue regeneration through paracrine action [Tsiapalis et al., 2020; Lai et al., 2015]. Several molecular factors released by EVs promote cell recruitment, with a significant potential role in endogenous tissue repair and regeneration [Silva et al., 2015; Yu et al., 2020]. Moreover, the study observed that EVs derived from DPSCs could enhance cellular functions, offering an alternative therapy for regenerative endodontic approaches [Zhang et al., 2020].

The authors employed 5-Aza treatment to induce odontogenic differentiation of DPSCs without an odontogenic medium. They noted a reduction in DNA methylation levels of some odontogenic differentiation-associated genes (such as ALP and DLX5). The combined treatment of EVs and 5-Aza on DPSCs seeded on DDP upregulated the expression of odontogenic and osteogenic markers (ALP, RUNX2, COL1A1, Vinculin, DMP1, and DSPP) compared to DPSCs without the decellularized scaffold.

These findings suggest that DDP enriched with DPSCs and EVs holds significant potential as a promising scaffold for dental pulp regeneration, promoting DPSCs odontogenic differentiation [Mattei et al., 2023].

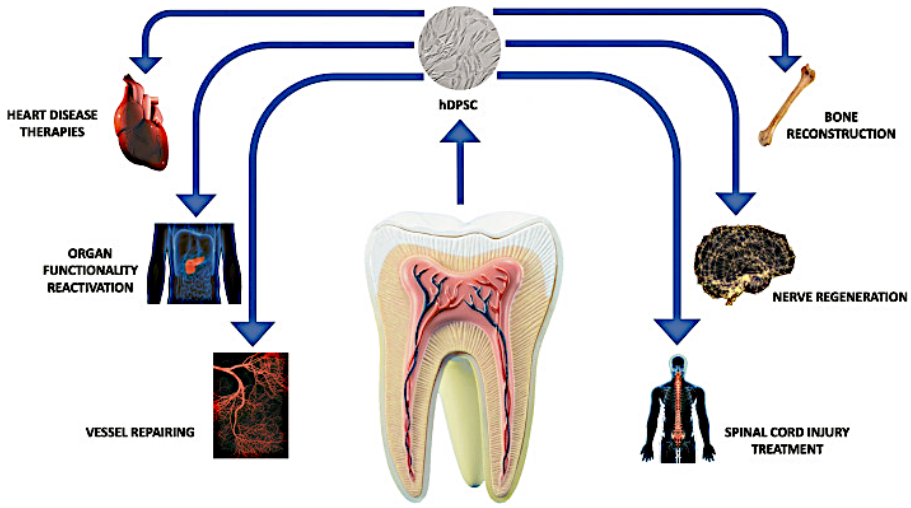


Figure 10. *Differentiative ability and regenerative potential of Dental Pulp Stem/Stromal Cells (DPSCs) [Mattei et al., 2021].*

1.3 Exploring Organoid Development and Applications

Although bidimensional (2D) cell culture models are still widely used, they do not fully reproduce the complexity of biological processes as they occur in vivo. For these reasons, recent advances in organoid technology have revolutionized in vitro culture tools for biomedical research, creating powerful three-dimensional models (3D) capable of initiating and expanding organ-like structures [Clevers et al., 2016; Dutta et al., 2017; Lancaster et al., 2014]. Today, organoids can be cultured in an attempt to replicate different organs, displaying a striking resemblance to their in vivo counterparts. Furthermore, human organoids are used to study models of infectious diseases, genetic diseases, and tumors and are obtained starting from different types of stem cells [Broutier et al., 2017; Dart et al., 2018; Lancaster et al., 2014; Mann et al., 2014].

Organoids and spheroids are both commonly used 3D cell culture models in the field of biomedical research to study cell-cell interactions and cellular processes. Despite their similarities, they also exhibit distinct characteristics that set them apart. Organoids originate from stem cells and are cultivated in a specialized medium or substrate containing growth factors and molecules that facilitate the development of organ-like structures. Typically, organoids form within days and serve as models for organ or tissue development. Spheroids result from an aggregation of cells in a medium, resulting in a more spherical structure compared to organoids. The spheroids typically develop within hours. These can also be used to study cell-cell interactions, drug testing, and a wide range

of cellular processes, including cell migration, differentiation, and apoptosis [Baker et al., 2012; Lee et al., 2018; Sang et al., 2019; Tebroke et al., 2019]. Among the numerous types of adult stem cells (ASCs), MSCs are certainly the most interesting and studied. Specifically, DPSCs exhibit notable regenerative potential and are readily accessible as they can be easily isolated from discarded or extracted teeth, particularly from the third molars of young donors [Arthur et al., 2008; Govindasamy et al., 2010; Gronthos et al., 2000; Mehlhorn et al., 2006; Seo et al., 2004]. As previously mentioned, DPSCs demonstrate the ability to differentiate into various tissues. Consequently, DPSCs represent excellent candidates for generating organoids, capitalizing on their remarkable plasticity in differentiating into diverse cell lineages, thereby enabling the creation of experimental organ models [Gronthos et al., 2000; Delle Monache et al., 2021].

Since 1907 when Henry Van Peters Wilson described the first attempt to regenerate organisms *in vitro*, demonstrating that dissociated spongy bone tissue cells can self-organize to restore an entire organism, numerous experiments aiming to generate different organ types have been conducted *(Figure 11)* [Evans et al., 1981; Li et al., 2008; Martin et al., 1981; Shannon et al., 1987; Steinberg et al., 2004; Takahashi et al., 2006; Thomson et al., 1998; Yu et al., 2007].

Research moved from 2D to 3D when researchers successfully generated cerebral cortex tissue from embryonic stem cells (ESCs) using the 3D aggregation culture method [Eiraku et al., 2008]. In 2009, a landmark study by Sato et al. showed that adult intestinal stem cells expressing G protein-coupled receptor 5 (Lgr5) could form matrigel 3D intestinal organoids capable of self-organize and differentiate into villous and crypt structures in the absence of mesenchymal niche. This was the first report of creating a 3D organoid culture derived from a single ASC, which set the way for further research on organoids in various biological systems, including the mesendoderm (stomach, liver, pancreas, lung, and kidney) and neuroectoderm (brain and retina) using ASCs or ESCs *(Figure 11)* [Corrò et al., 2020].

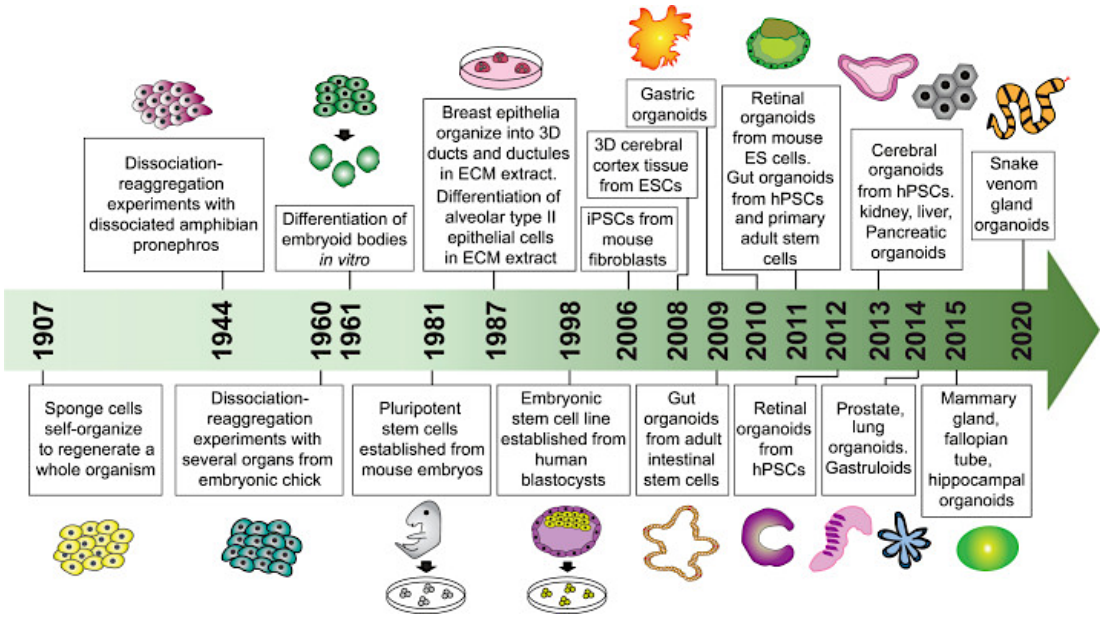


Figure 11. *Timeline for the development of organoid cultures. A summary of key landmark studies and breakthroughs leading to the establishment of various organoid technologies. Extracellular Matrix (ECM); Embryonic Stem Cells (ESCs); Human Pluripotent Stem Cells (hPSCs); Induced Pluripotent Stem Cells (iPSCs) [Corrò et al., 2020].*

1.3.1 Methods for cultivating Organoid

Although several techniques for generating and culturing organoids have been described in recent years, currently there isn't a standard reference procedure.

Organoids are typically created through different methods aimed at aggregating cells while promoting cell adhesion and preventing cell-surface interactions [Hardelauf et al., 2011].One commonly used strategy involves continuously agitating the cell suspension within spinner flasks, which spontaneously forms spherical aggregates and prevents adhesion to other substrates.

An alternative to spinner flask cultures involves the use of circular stirrers or rotating tubes, which keep the cells suspended between rotating cylindrical walls due to microgravity. Both methods enable the production of a high number of organoids of heterogeneous sizes, making them suitable for large-scale production. However, these procedures require significant amounts of culture medium and the availability of specific equipment [Hardelauf et al., 2011].

Stationary culture techniques are simpler and involve the application of the cell suspension onto a non-adherent surface, often utilizing plates coated with organic matrices such as agar, poly-HEMA, matrigel, or collagen [Friedrich et al., 2009]. The use of U-bottom 96-well plates coated with agar allows for cell aggregation through

sedimentation on the concave well surface, enabling subsequent monitoring and manipulation of the spheroids individually. While this method facilitates the formation of uniformly sized organoids and enables analysis of individual structures, they require careful manipulation and are hindered by limited production capacity.

An alternative technique known as the "hanging drops" method provides a promising approach for organoid production. This approach is simple and adaptable, making it suitable for different cell lines to generate uniformly sized and compact organoids [Friedrich et al., 2009]. The rounded bottom of the suspended drop facilitates cell aggregation, and organoid size can be adjusted simply by varying the cell count in the suspension. The method involves depositing a small volume of cells (20-40µl) on the lid of a Petri dish or a 96-well plate. By inverting the plate, a "hanging" drop is formed, and the cells, due to gravity, accumulate at the bottom of the drop, tending to aggregate into organoids [Kurosawa et al., 2007]. This technique has the advantage of forming individual organoids of uniform size, but there are disadvantages. Involves intensive manual labor for collecting individual organoids, changing the culture medium is practically impossible, and therefore, it is useful only for short-term cultures [Kurosawa et al., 2007].

Recently, automated methods have emerged, primarily aimed at the larger-scale production of tumor spheroids for preclinical screening [Burdett et al., 2010].

1.3.2 Applications of Organoid Technology

Organoid technology can satisfy a crucial need in medical research and holds great promise for a wide range of translational applications *(Figure 12)* [Tang et al., 2022].

1. *Models for Genetic Diseases*

Some researchers created intestinal crypt organoids from adult stem cells, which were applied to study cystic fibrosis (CF), a genetic disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. The study developed a rapid test for quantifying CFTR function, which could expedite diagnosis, functional research, drug screening, and personalized medicine strategies [Muñoz et al., 2012].

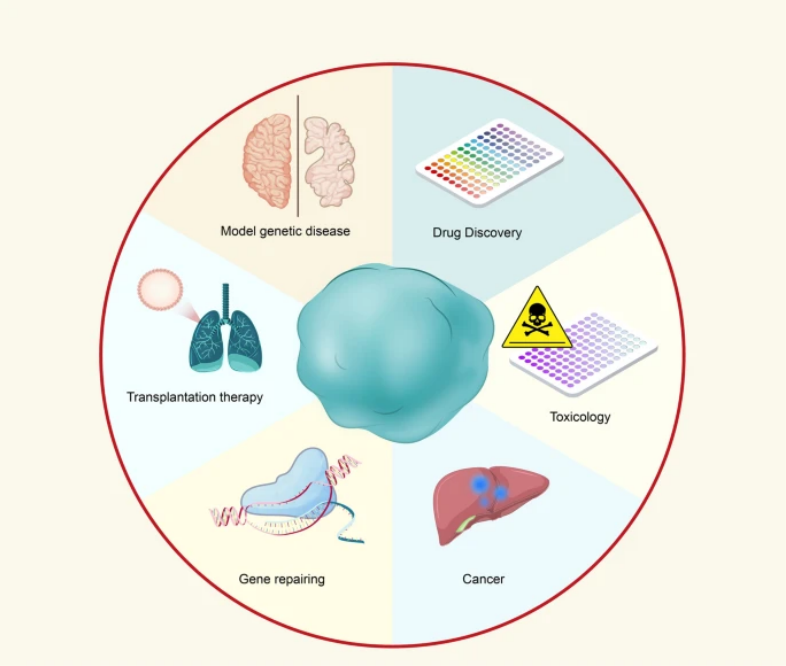


Figure 12. *Schematic depiction of various applications of organoid technology [Tang et al., 2022].*

2. *Drug Discovery and Toxicity Assessment*

In 2017, Crespo and colleagues developed colon organoids from patients with familial adenomatous polyposis (APC), a disease caused by the mutation of the APC gene. These organoids have been used to test compounds to limit excessive cell proliferation [Crespo et al., 2017].

During pharmacological experimentation, the toxicity of a drug may only gradually become evident in clinical studies; therefore, organoids could be utilized for pharmacological testing in pre-clinical stages. Given the similarity of their reactions to physiological tissue responses, organoids can be used for assessing drug toxicity and evaluating side effects on the liver, heart, and kidneys [Takasato et al., 2015; Vlachogiannis et al., 2018].

3. *Cancer Research*

What makes the use of organoids significant is their construction using patient-derived cells, enabling the imitation of tumor characteristics, and thus facilitating personalized therapy. Currently, various types of organoids generated with tumor cells exhibit excellent phenotypical mimicry of human tumors, allowing for pharmacological screening, as well as basic research [Van de Wetering et al., 2015; Sachs et al., 2018].

4. Personalized Medicine

In recent years, significant efforts have been made to successfully establish biobanks of organoids derived from various tumors, including prostate [Beshiri et al., 2018], lung [Li et al., 2020], colorectal [Fujii et al., 2016], liver [Broutier et al., 2017], pancreatic [Driehuis et al., 2019], and gastric tumors [Yan et al., 2018]. Through pharmacological sensitivity testing on organoids, the most suitable drug for patients can be identified. Consequently, the most effective pharmacological treatment plan tailored to each patient can be formulated, potentially minimizing drug side effects and reducing tumor recurrence [Tang et al., 2022].

5. Gene Therapy

CRISPR/Cas9 technologies have been applied to gene therapy, owing to their high efficiency in genetic modification [Schwank et al., 2013; Geurts et al., 2020]. Studies conducted on cystic fibrosis and mutations in the DF508 gene have demonstrated mutation correction using a homology-dependent repair system mediated by CRISPR/Cas9, showcasing the functionality of gene repair, and providing a potential gene therapy for cystic fibrosis patients [Schwank et al., 2013]. Considering this progress, genetic editing can correct genetic mutations in patient-derived organoids, rapidly advancing gene repair into the clinical phase.

6. Organ Transplantation

Organoids have emerged as potential donors, offering a promising platform for autologous transplant therapy. For instance, Watson et al. transplanted an intestinal organoid into a murine model and found that the transplanted organoid displayed marked expansion and maturation [Watson et al., 2014]. Although transplanted organoids are relatively immature compared to the host's natural organs due to their incomplete functional maturation and potential heterotypic cellular interactions, therapeutic use of organoids will nevertheless represent a significant alternative to organ transplantation.

2. AIM OF THE WORK

Mesenchymal stem cells (MSCs) are adult stem cells (ASCs) and are among the most extensively studied. They are multipotent stem cells characterized by high plasticity; following specific stimuli and interaction with the surrounding microenvironment, they can differentiate into various cell lines such as adipocytes, chondrocytes, osteocytes, perivascular cells, and neural cells [Uccelli et al., 2008]. MSCs, due to their characteristics such as extensive proliferative capacity, high differentiative potential, and easy accessibility, represent an ideal resource for stem cells for regenerative medicine without encountering bioethical issues. Although bone marrow represents the most common source of MSCs, these cells have also been isolated from other tissues such as adipose tissue, umbilical cord, amniotic fluid, blood, and dental pulp [Fajardo-Orduña et al., 2015]. In recent years, dental pulp derived MSCs have been attracting more attention and recognition for their potential applications. Dental Pulp Stem/Stromal Cells (DPSCs) are characterized by high regenerative potential and even easier accessibility, as they can be isolated from discarded or removed teeth, especially from the third molars of young donors; this makes them highly utilized in various fields of regenerative medicine [Gronthos et al., 2000; Mattei et al., 2021]. Due to their high capacity to differentiate into different cell lineages and their immunomodulatory properties, DPSCs are excellent candidates for tissue regeneration and repair. Particularly, they are seen as potentially applicable to bone, cartilage, dental tissue, and cardiac tissue regeneration, and they also promote neo-vascularization.

DPSCs can organize and aggregate to form organoids; therefore, their versatility could be exploited to obtain experimental organ models [Li et al., 2023].

In vitro monolayer (2D) models are excellent tools for studying the mechanisms underlying diseases, and pharmacological processes, and are thus widely used in preclinical studies, but they do not fully reflect the tissue architecture and its complexity. For this reason, it is essential to recreate tissue-specific conditions to obtain results that are increasingly similar to the *in vivo* condition. Three-dimensional (3D) *in vitro* models such as organoids overcome the limitations inherent in two-dimensional cultures and open up frontiers for optimal understanding of organ physiology in response to damage or pharmacological treatment.

Based on this information, this thesis project aimed to study the differentiating ability of DPSCs. Specifically, the goal was to examine the ability of dental pulp stem cells to

acquire osteo-odontoblastic, hepatic, neuronal, and peri-vascular phenotypes when exposed to specific conditioning factors, intending to use them for translational studies in the field of regenerative medicine. The work was carried out using both two-dimensional and three-dimensional approaches.

In the 2D cultures, DPSCs were subjected to osteo-odontogenic differentiation by utilizing a specialized culture medium, and the culture was sustained for 21 days. Throughout this timeframe, alterations in shape and morphology were identified and recorded. At the endpoint, were performed Alizarin Red staining to visualize mineralization, immunofluorescence assays to target specific markers (CD44, CD105, OSX, RUNX2), and RT-PCR analysis to evaluate the expression levels of genes associated with osteo-odontogenic differentiation (OCT-4, OSX, ALP, RUNX2).

For hepatic differentiation, DPSCs were cultured for 35 days in a specialized medium supplemented with the human hepatic matrix. The resulting cells were then examined for liver-related functions, such as cytochrome P450 activity, 1A1, and 3A7 expression, as well as resorufin conjugation activity, to verify their transformation into liver-like cells under controlled culture conditions.

Neuronal differentiation of DPSCs was induced through hypoxic conditions (1% O_2). The cells were maintained in hypoxic environment for 16 days, with evaluations conducted at two time points (5 days and 16 days). Morphological changes were analyzed through phalloidin staining, and proliferation tests were performed. Additionally, the expression levels of various mesenchymal and neuronal markers (CD44, CD90, CD73, CD105, STRO1, nestin, β3-Tubulin, NFH, and GAP43) were evaluated using flow cytometry. RT-PCR was employed to analyze the expression of neuronal-related genes (nestin, GFAP, bFGF, EFG, NGF, BDNF, GDNF) to assess the role of hypoxia in driving neuronal differentiation in DPSCs. The levels of EGF and bFGF in DPSCs conditioned media (DPSCs-CM) were quantified using ELISA, comparing hypoxic conditions with normoxic conditions (21% O_2). Finally, to investigate the potential of DPSCs to induce neuronal differentiation through autocrine/paracrine mechanisms mediated by growth factor secretion, the effects of DPSC-CM on SH-SY5Y cells and DPSCs themselves were examined. Immunofluorescence and flow cytometry analyses were used to detect the presence of neuronal markers β3-Tubulin and NFH.

Lastly, DPSCs underwent magnetic-activated cell sorting (MACS) targeting the NG2 marker to select cells with pericyte-like characteristics. Subsequently, three subpopulations of interest were obtained: Total DPSCs, DPSCs NG2-, and DPSCs NG2+,

and flow cytometry was used to confirm the expression or absence of stem cell markers and NG2 in these cells. To assess whether the expression of the pericyte marker NG2 could influence the DPSCs' ability to stabilize tubes in vitro, a co-culture test on matrigel (with HUVECs) was conducted. Additionally, to evaluate the paracrine function of DPSCs, conditioned media (CM) from these three subpopulations were collected, and their effects on both the proliferation and wound healing capacity of HUVECs were assessed. An ELISA test was also performed to evaluate the concentration of VEGF secreted by all DPSCs. Finally, to verify the potential direct role of DPSCs in vessel stabilization, Wound Healing assays were conducted using the three DPSCs subpopulations, along with a co-culture on matrigel with HUVECs, to determine the effective role of DPSCs on newly formed tubes.

In relation to the 3D cultures, organoids were constructed using a stationary technique and were maintained in parallel culture for differentiated and control conditions, like the methods discussed previously for 2D cultures. Morphological changes were monitored using the Incucyte® system.

3. MATERIALS AND METHODS

3.1 Cell Culture

- *Dental Pulp Stem/Stromal Cells (DPSCs)*

The DPSCs used for this project were obtained from dental pulp extracted from impacted third molars of selected young patients, aged between 13 and 19 years, affected by dysodontiasis (difficulty in the eruption of teeth, typically due to lack of space or incorrect orientation of the tooth), for whom the third molar represented discarded material and thus easily accessible for research purposes. All subjects were provided with an informed consent form to be signed after explaining the nature and procedures of the study, in accordance with the Declaration of Helsinki and the experimental protocol approved on January 26, 2017, by the Ethics Committee of the Umberto I Policlinic of Rome (Project identification code: 4336).

DPSCs were cultured in DMEM Low Glucose medium, with 10% FBS (Euroclone, Milan, Italy), 100 IU/mL penicillin, and 100 µg/mL streptomycin (Euroclone, Milan, Italy) in a humidified incubator under an atmosphere of 5% CO_2 at 37°C. The culture medium was replaced every 3 days, and when 90% confluence was achieved, cells were harvested using 0.05% Trypsin-EDTA (Euroclone, Milan, Italy). Cells were cultured between 2 and 5 passages for the subsequent experiments, and each was repeated at least three times.

- *Human Neuroblastoma Cell (SH-SY5Y)*

SH-SY5Y was cultured as a monolayer in polystyrene dishes and maintained in DMEM with high glucose formula (Gibco BRL) containing 10% of FBS (Euroclone, Milan, Italy), 100 IU/mL penicillin, and 100 µg/mL streptomycin (Euroclone, Milan, Italy) in a humidified incubator under the atmosphere of 5% CO_2 at 37°C. When 85% confluence was achieved, cells were harvested using 0.05% Trypsin-EDTA (Euroclone, Milan, Italy) and were passaged. The culture medium was replaced twice a week.

- *Human Umbilical Vein Endothelial Cells (HUVECs)*

HUVECs were purchased from Lonza (Walkersville, MD, USA) and were maintained in culture in endothelial growth medium 2 (EGM-2), obtained by adding specific endothelial supplements and 2% FBS provided by the company (Hydrocortisone, bFGF, VEGF, R3-

IGF-1, Ascorbic Acid, hEGF, GA-1000, Heparin) to the endothelial basal medium 2 (EBM-2) (Lonza, Basel, Switzerland). The cells were kept under controlled temperature and atmosphere conditions, respectively at 37°C and 5% CO_2. Cells were cultured with between 2 and 5 passages for the subsequent experiments, and the medium was replaced twice a week.

3.2 Flow Cytometry Analysis

For each experiment, at least 1×10^5 cells were fixed with 4% paraformaldehyde for 10 min at 4°C and permeabilized with permeabilizing solution (0.1% Triton X-100 in PBS) for 10 min at 4°C. After washing, cells were subsequently incubated overnight at 4°C with various primary antibodies, including: mouse Anti-CD44 mAb; mouse Anti-CD90 mAb (included in a Human MSC Analysis kit; Merck, Millipore, Milan, Italy); mouse Anti-CD105 mAb (BD Biosciences, Milan, Italy); mouse Anti-STRO1 mAb and mouse Anti-CD73 mAb (Merck, Millipore, Milan, Italy); mouse Anti-CD166 mAb (BD Biosciences, Milan, Italy); mouse Anti-CD271 mAb (BD Biosciences, Milan, Italy); mouse Anti-CD146 mAb (BD Biosciences, Milan, Italy); mouse Anti-CD19 mAb (BD Biosciences, Milan, Italy); mouse Anti-CD45 mAb (BD Biosciences, Milan, Italy); mouse Anti-nestin mAb; mouse Anti-β3-Tubulin mAb; mouse Anti-NFH mAb; mouse Anti-NG2 mAb (Merck, Millipore, Milan, Italy), and rabbit Anti-GAP43 mAb (Cell Signaling Technology, Danvers, MA, USA). Next, cells were washed, centrifuged, suspended in 50 µL of PBS (Euroclone, Milan, Italy), and stained by PE-conjugated Anti-mouse IgG H&L and Anti-Rabbit IgG H&L (Cy5) (Abcam, Cambridge, MA, USA) in the dark for 30 min. All samples (at least 40,000 events were acquired) were analyzed with a FACScan cytometer (BD Accuri C6 Flow cytometer) equipped with a blue laser (488 nm) and a red laser (640 nm) (BD Biosciences, Milan, Italy).

3.3 Cell Title-Glo (CT)

Cell Title-Glo is a commercial kit (Promega) designed to test cellular viability by quantification of intracellular ATP. The commercial test was used according to the manufacturer's instructions. Briefly, 40 µl of cell suspension was transferred in three wells of a 96-well white plate and immediately supplemented with 40 µl Cell Titer-Glo®

reagent. The plate was incubated for 20 minutes at room temperature. Afterward, luminescence was read with spectrophotometer/luminometer CLARIOstar® (BMG Labtech) with an integration time of 1 second per well. Luminescence produced by the assay is proportional to the amount of ATP present in the cells. Results are expressed as LCU/min and normalized to a million viable cells.

3.4 Proliferation Assay

DPSCs were seeded in a 96-well plate (Euroclone, Milan, Italy) at a density of 5×10^3 cells/well and kept in a hypoxia incubator at continuous low oxygen tension (1%) for 5 and 16 days.

The same assay was performed on HUVECs in contact with the conditioned media from the three subpopulations of DPSCs (Total, NG2-, NG2+). Endothelial cells were plated, and the following day, the medium was replaced with the respective CM (diluted 1:4) and left for 24 hours. DMEM Low Glucose was used as a negative control.

After the specific treatments, cell proliferation was evaluated by staining the cells with crystal violet (1%) (Merk Life Science, Sigma Aldrich, Milan, Italy) and solubilized using a solubilization solution (1% SDS and 50% methanol). The plate was read at 595 nm.

3.5 Differentiation Medium

- *Osteo-Odontogenic medium*

Osteo-odontogenic differentiation was induced by incubating DPSCs in a specific medium for 21 days, with medium replacement every 2-3 days.

DMEM Low Glucose	
PES (Penicillin and Streptomycin)	1%
Ascorbic Acid	50 mg/ml
β-Glycerophosphate	10 nM
Dexamethasone	100 nM

- *Hepatic medium*

Hepatic differentiation was induced by incubating DPSCs in a specific medium for 35 days, with medium replacement every 2-3 days.

DMEM Low Glucose	
PES (Penicillin and Streptomycin)	1%
HGF	20 ng/ml
EGF	2 ng/ml
Dexamethasone	100 nM
ITS Premix (Insulin, Transferrin, Selenous Acid)	1X

- *Neuronal medium (2D)*

Neuronal differentiation was induced by incubating DPSCs in a specific medium for 14 days, with medium replacement every 2-3 days.

Neurobasal A medium	
B27	2%
bFGF	40 ng/ml
EGF	20 ng/ml

- *Neuronal medium (3D)*

Neuronal differentiation was induced by incubating organoids of DPSCs in a specific medium for 26 days, with medium replacement every 2-3 days.

Neurobasal A medium	
PES (Penicillin and Streptomycin)	1%
Insulin	10 μg/ml
Indomethacin	200 μM
3-Isobutyl-1-methylxanthine (IBMX)	0,5 mM
B27	2%
bFGF	40 ng/ml
EGF	20 ng/ml
β-NGF	20 ng/ml

3.6 Hypoxic Culture Condition

To evaluate the effect of hypoxia on the DPSCs neuronal differentiation process, DPSCs incubated under the O_2 1% condition were compared to DPSCs maintained in normoxia at the same time interval as indicated in *Table 5.* In brief, DPSCs were cultured in continuous low oxygen tension (1%) in a hypoxia incubator for 5 and 16 days. For normoxic conditions, the DPSCs were grown in a standard incubator under O_2 atmosphere conditions and 5% CO_2 at 37°C for the same time.

Abbreviations	*Type of Treatment*
5N	DPSCs for 5 days in normoxia
5H	DPSCs for 5 days in hypoxia
16N	DPSCs for 16 days in normoxia
16H	DPSCs for 16 days in hypoxia

Table 5. *Summary of culture conditions for neuronal differentiation.*

3.7 Hepatic Culture Condition

To evaluate the effect of hepatic differentiation medium on DPSCs, the cells were maintained in contact with the medium for 20 days. The DPSCs were then divided into two groups; in one group, a human liver matrix was added along with the differentiation medium for an additional 15 days, while the other group was maintained in the differentiation medium alone for 15 days. At the endpoint (35 days), all cells were subjected to the resorufin assay. Subsequently, they were treated with specific inducers, such as Rifampicin (RIF), Phenobarbital (PB), DMSO, and β-Naphthoflavone (BNF), respectively, for 3 and 6 days *(Table 6).*

Abbreviations	*Type of Treatment*
DPSCs	*For 35 days in hepatic medium*
DPSCs+M	*For 20 days hepatic medium+15 days hepatic matrix*
DPSCs+RIF(3d)	*For 35 days hepatic medium+3 days Rifampicin*
DPSCs+RIF(3d)+M	*For 20 days hepatic medium+15 days hepatic matrix +3 days Rifampicin*
DPSCs+RIF(6d)	*For 35 days hepatic medium+6 days Rifampicin*
DPSCs+RIF(6d)+M	*For 20 days hepatic medium+15 days hepatic matrix +6 days Rifampicin*
DPSCs+PB(3d)	*For 35 days hepatic medium+3 days Phenobarbital*

DPSCs+PB(3d)+M	*For 20 days hepatic medium+15 days hepatic matrix +3 days Phenobarbital*
DPSCs+PB(6d)	*For 35 days hepatic medium+6 days Phenobarbital*
DPSCs+PB(6d)+M	*For 20 days hepatic medium+15 days hepatic matrix +6 days Phenobarbital*
DPSCs+BNF(6d)	*For 35 days hepatic medium+6 days β-Naphthoflavone*
DPSCs+BNF(6d)+M	*For 20 days hepatic medium+15 days hepatic matrix +6 days β-Naphthoflavone*
DPSCs+DMSO(6d)	*For 35 days hepatic medium+6 days DMSO*
DPSCs+DMSO(6d)+M	*For 20 days hepatic medium+15 days hepatic matrix +6 days DMSO*

Table 6. *Summary of culture conditions for hepatic differentiation.*

3.8 Magnetic-Activated Cell Sorting (MACS)

DPSCs were immunomagnetically separated using the MACS Cell Separation Kit (Miltenyi Biotec, Bologna, Italy). DPSCs ($5x10^7$ cells/ml) were collected, passed through a 40 μm nylon filter to remove cell clumps, counted, and washed with 300 μl of separation buffer prepared with PBS pH 7.2 supplemented with 0.5% BSA and 2 mM EDTA for 10 minutes at 300xg. Subsequently, were incubated with MicroBeads conjugated with a monoclonal antibody against human NG2 for 30 minutes at 4°C with slow and continuous rotation. Afterward, the cells were washed for 10 minutes at 300xg, the supernatant was removed, and the cells were resuspended in 500 μl of buffer. For separation, an LS MACS Column, previously activated, was used in the MidiMACS Separator (Miltenyi Biotec, Bologna, Italy), and the cell suspension was passed through the column to separate the two subpopulations: DPSCs NG2- and DPSCs NG2+.

3.9 Preparation of the Conditioned Media

To assess the potential influence of DPSCs' secretome on neuronal differentiation and their paracrine effect on tubule stabilization, the culture medium of DPSCs was replaced with serum-free media for 48 hours. The cells were then incubated in a humidified atmosphere with 5% CO_2 at 37°C before starting the experimentation. The conditioned medium (CM) from each sample was then centrifuged at 2600× g at 4°C for 10 minutes, and the collected CM, normalized with respect to cell number, was either used immediately or frozen at −80 °C until needed for the experiments. For CMs of DPSCs treated with hypoxia see *Table 7.*

Abbreviations	*Type of Conditioned Media*
5N CM	Conditioned media derived from normoxic DPSCs for 5 days
5H CM	Conditioned media derived from hypoxic DPSCs for 5 days
16N CM	Conditioned media derived from normoxic DPSCs for 16 days
16H CM	Conditioned media derived from hypoxic DPSCs for 16 days

Table 7. *Summary of conditioned media treatment.*

3.10 Alizarin Red Staining

Following induction of osteo-odontogenic differentiation with the appropriate complete medium, DPSCs underwent Alizarin Red staining at 7, 14, and 21 days of culture. DPSCs were initially fixed with 4% paraformaldehyde for 15 minutes at room temperature, followed by PBS washing. Subsequently, a 2% Alizarin Red solution (Sigma-Aldrich, Milan, Italy) in distilled water was added, and the cells were incubated at 37°C for 30 minutes. The procedure was finalized with a gentle rinse using distilled water. Images of staining were captured using the Nikon Eclipse TS100 microscope.

3.11 Immunofluorescence Analysis

After 21 days of incubation with osteo-odontogenic differentiation medium, the DPSCs underwent immunofluorescence analysis.

Also, SH-SY5Y and DPSCs were subjected to immunofluorescence. These cells were seeded at a density of 2×10^4 cells/mL in 6-well plates and standard culture medium for 24 h. After seeding, both cell lines were stimulated for 48 h and 10 days with DPSCs-CM. At the end of the treatment, the obtained samples were used for immunofluorescence analysis.

All cells treated as above were fixed with 4% paraformaldehyde for 10 min at 4°C and permeabilized with permeabilizing solution (0.1% Triton X-100 in PBS) for 10 min at 4°C. After washing in PBS, cells were incubated with mouse Anti-CD44 mAb (Cell Signaling Technology, Danvers, MA, USA), mouse Anti-CD105 mAb (Cell Signaling Technology, Danvers, MA, USA), mouse Anti-OSX mAb (Cell Signaling Technology, Danvers, MA, USA), mouse Anti-β3-Tubulin mAb and mouse Anti-NFH mAb (Cell Signaling Technology, Danvers, MA, USA), rabbit Anti-RUNX2 mAb (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C, followed by Anti-mouse IgG Alexa

fluor 488 and Anti-rabbit IgG Alexa Fluor 594 (ThermoFisher Scientific, Rockford, IL, USA) in the dark for an additional 60 minutes. Finally, cells were observed with a Zeiss Axio Vert.A1 fluorescence microscope (Zeiss, Milan, Italy). Quantitative analysis of the fluorescence intensity for each protein of interest was measured by Image J.

To visualize the cellular cytoskeleton, DPSCs were stained with Phalloidin. Briefly, cells were seeded in 6-well plates at 2×10^5/well density, and grown for 24 h. Afterward, the cells were subjected to specific treatment. At the end of the experiment, the cells were fixed with 4% paraformaldehyde (Euroclone, Milan, Italy) for 10 minutes. Non-specific binding sites were blocked with 3% bovine serum albumin (BSA) (Euroclone, Milan, Italy) and 0.1% Triton X-100 (Merk Life Science, Sigma Aldrich, Milan, Italy) for 30 minutes, then DPSCs were labeled with FITC-Phalloidin (Fluorescein Isothiocyanate) (Merk Life Science, Sigma Aldrich, Milan, Italy) 1:1000 in PBS for 1 h at room temperature, and then washed three times with PBS. Finally, nuclei were counterstained with DAPI solution (Euroclone, Milan, Italy), and the images were acquired with a Zeiss Axio Vert.A1 fluorescence microscope (Zeiss, Milan, Italy).

The organoids were washed in PBS and fixed in 1% paraformaldehyde for 30 minutes at 4°C. After a brief washing in PBS, the organoids were embedded in Optimal Cutting Temperature (OCT) Embedding Medium for Frozen Tissues (Scigen Scientific Gardena, CA 90248, USA) by molds and rapid freezing in nitrogen was performed by adding a cryoprotectant (FBS-DMSO 9:1). Next, were cryosectioned, using a cryostat (Leica CM1859), to obtain 10µm thick sections. The sections obtained were mounted on Superfrost Plus slides (Menzel-Glaeser, Braunschweig, DE) and stored at -80°C until they were used. The sections were then left at room temperature for a few minutes to allow for the gradual increase in temperature and, subsequently, were used for immunofluorescence detection of active proliferating cells by means of Ki-67. Slides were dipped in PBS for 15 minutes (3 x 5-minute washes) to remove OCT residues. Triton X-100 permeabilizing agent at 0.05% in PBS was used for 10 minutes at 4 °C, followed by three washes of 5 minutes each in PBS. Non-specific site blockade was made using a 3% BSA solution for 1 hour at room temperature, and 150µl of primary antibody per slide was applied to cover the organoids. Ki-67 was a rabbit mAB (Cell Signaling Technology Inc, Danvers, MA, 32 USA, #9129), dilution 1:50, followed by an incubation with an anti-mouse secondary antibody labeled with Alexa fluor 594 (goat anti-mouse IgG, Alexa fluor 594, Invitrogen, A11012). The secondary antibody was used at a 1:1000 dilution in PBS and incubated for 1 hour at room temperature, protected from light. The slides were then washed in PBS

for 15 minutes (3 washes/5 minutes per wash) and lightly dried again, for nuclei staining 10µl of DAPI (D1306, Invitrogen, ThermoFisher Scientific, USA) diluted 1:100 in PBS was added to each section on to the slide. Next, the samples were washed in PBS for 15 minutes (3 washes/5 minutes per wash), dried, and mounted with a coverslip using glycerol as a mounting medium. Finally, the samples were analyzed, and images were acquired with the A1 Zeiss Axiovert fluorescence microscope (Zeiss, Milan, Italy).

3.12 Reverse Transcription-Quantitative PCR (RT-PCR) Analysis

Osteo-odontogenic DPSCs and DPSCs in hypoxic conditions were analyzed by RT-PCR analysis. Total cellular RNA was extracted from 100,000 DPSCs using TRIzol® Reagent (ThermoFisher Scientific, Rockford, IL, USA), and its quality and quantity were evaluated on a NanoDrop spectrophotometer (ThermoFisher Scientific, Rockford, IL, USA). For the PCR assay, cDNA was synthesized from 500 ng of total RNA with SuperScript (Euroclone, Milan, Italy). Next, the PCR was carried out using the SYBR green master mix (Luna), according to the manufacturer's protocols. The amplification reaction was performed on a MiniOpticon Real-Time PCR System (Bio-Rad, Milan, Italy) using the following program: the RT reaction was set at an initial denaturation step at 95°C for 1 minute, followed by 95°C for 15 seconds. The reaction mixture was heated for 30 seconds, followed by amplification that consisted of 40 cycles of denaturation at 95°C for 15 seconds and annealing and extension at 60°C for 30 seconds, according to the manufacturer's protocol. The relative expressions of the genes investigated were calculated using the relative quantification $2^{(-\Delta\Delta C(T))}$ $2^{-\Delta\Delta Cq}$ method [Livak et al., 2001], with GAPDH as a reference gene commonly used for this purpose. The primer sequences used in this RT-PCR analysis are shown in *Table 8.*

Gene	*Forward Primer*	*Reverse Primer*
RUNX2	CCGCTTCAGTGATTTAGGGC	GGGTCTGTAATCTGACTCTGTCC
ALP	GGAACTCCTGACCCTTGACC	TCCTGTTCAGCTCGTACTGC
OSX	ACGGGTCAGGTAGAGTGAGC	GGGATCCCCCTAATCAAGAG
OCT-4	GTATTCAGCCAAACGACCATC	CTGGTTCGCTTTCTCTTTCG
GFAP	TAGAGGGCGAGGAGAACCG	GTGGCCTTCTGACACAGACTTG

bFGF	CTGTACTGCAAAAACGGG	AAAGTATAGCTTTCTGCC
NGF	CATGCTGGACCCAAGCTCA	GACATTACGCTATGCACCTCAGTG
EGF	GGTCAATGCAACCAACTTCA	GGCATTGAGTAGGTGATTAG
GDNF	TCAAATATGCCAGAGGATTATCCTG	GCCATTTGTTTATCTGGTGACCTT
BDNF	AGCTCCGGGTTGGTATACT	CCTGGTGGAACTTCTTTGCG
NES	TGGCAAAGGAGCCTACTCCAAGAA	ATCGGGATTCAGCTGACTTAGCCT
GAPDH	CTGCACCACCAACTGCTTAG	ACCTGGTGCTCAGTGTAGCC

Table 8*. Primer sequences used in RT-PCR analysis.*

3.13 Resorufin conjugation

Phase 2 was measured by resorufin conjugation [Gramignoli et al., 2012). After transferring 40 µl of hepatocyte suspension to a 96-well black plate (in triplicate), 40 µl of the fluorescent compound Resorufin (Sigma-Aldrich, Milan, Italy) was added, and the plate was incubated for 30 minutes at 37°C. Afterward, the amount of metabolized resorufin was quantified with a spectrophotometer/luminometer CLARIOstar® and expressed as a pmol/min/millions of viable cells.

3.14 CYPs activities

Phase I metabolic activity was tested on hepatic DPSCs for the enzymes CYP1A1, and CYP3A7. The procedure was performed using different CYP450-GloTM probes (Promega), a luminescent method to selectively measure CYP activities by using pro-luminescent substrates that are converted by CYP subfamilies into a luciferin product. These products can be quantified by adding luciferase: Luciferin Detection Reagent (LDR) and the activity of CYPs will be proportional to the light emitted. Procedure: 10 µl of each single assay reagent was transferred (in triplicate) to a 96-well white plate then 40 µl of cell suspension was added and incubated for 30 minutes at 37°C. Then, 50 µl of LDR (with cysteine) was added. Luminescent signals were read directly in a luminometer (CLARIOstar®) with an integration time of 1 second per well. Luminescence produced by the assay is proportional to the amount of enzyme activity present in the cell sample. Results are expressed as LCU/min and normalized to a million viable cells.

3.15 ELISA

EGF, bFGF, and VEGF concentration in the CM of DPSCs, by ELISA, were evaluated. The quantity of EGF, bFGF, and VEGF, in each sample, was evaluated using the Human EGF ELISA Kit, the Human FGF basic ELISA Kit (Abcam, Cambridge, MA, USA), and the Human VEGF ELISA Kit (Enzo, Farmingdale, New York) following the manufacturer's instructions. All experimental points were analyzed in triplicate and normalized vs the total cell number.

3.16 In vitro Tubule Formation Assay

For the assay, a 96-well plate (40µl of Matrigel per well) (Euroclone, Milan, Italy) was utilized. Once the wells were coated, the plate was incubated at 37°C for 1 hour to allow matrigel (Merck, Millipore, Milan, Italy) polymerization. The cells of interest (Total DPSCs, DPSCs NG2-, DPSCs NG2+, and HUVECs) were detached, and after centrifugation, the cells were resuspended and counted. For the experiment, HUVECs were seeded simultaneously at 2×10^4cells/well density and Total DPSCs, DPSCs NG2-, and DPSCs NG2+ at 1×10^4cells/well density. The three subpopulations were previously stained with calcein (2µM) to distinguish them and observe their distribution in the nodes and on the newly formed tubules. For staining, the cells were incubated in the dark for 10 minutes at 37°C. Furthermore, HUVECs plated alone were used as controls. Once the cells were seeded, the plate was incubated at 37°C and monitored using the Incucyte® system for a period of 14 days. The degree of angiogenic response was assessed by considering the branching index, which was obtained by measuring the number of junctions formed per field. The acquired images were quantified using the ImageJ analysis system, and average values and standard deviation (SD) were determined for each analysis.

The same co-culture conditions on matrigel were maintained to verify the positioning of DPSCs on newly formed tubules. In this case, DPSCs were labeled with the vital dye red PKH26 (Sigma-Aldrich, Milan, Italy). The staining was performed following the manufacturer's instructions. In this case, the nuclei of all cells were stained with DAPI.

3.17 Wound Healing Assay

In order to assess cell migration, HUVECs were seeded onto a 24-well plate at 6×10^4cells/well density (Euroclone, Milan, Italy). Once the cells reached 80% confluence, the sample was synchronized for 6 hours by serum starvation, and a scratch was created in the cell monolayer using a 200 µl pipette tip. The cells were washed with PBS, and endothelial medium along with the respective conditioned media from the three subpopulations of DPSCs were added (3:1). The scratch was monitored and photographed every 2 hours for 24 hours using the Incucyte® system (4X magnification). As for the DPSCs, the assay was performed in the same way by seeding Total DPSCs, DPSCs NG2-, and DPSCs NG2+ and monitoring the scratch for 24 hours.

3.18 Development of Organoids

DPSCs were grown in DMEM Low Glucose until passage 4 at 37°C with 5% CO_2. The culture medium was changed twice a week, and the cell line morphology was monitored under phase contrast microscopy. Once the fifth passage and 80% confluency were reached, the cells were washed with PBS, trypsinized, and centrifuged for 5 minutes at 259 x g. DPSCs were counted with Burker's chamber; 75,000 cells were used for each organoid. DPSCs were resuspended in ECMatrixTM (Chemicon, Millipore, USA), a substrate consisting of a mixture of basement membrane components such as laminin, type IV collagen, growth factors like bFGF, TGF-β, and proteins. The organoids were made by placing drops of a mixture consisting of 10µl ECMatrixTM and 75,000 cells on sterilized parafilm and then left in an incubator for 30 minutes at 37°C to allow polymerization. Finally, the generated droplets were transferred to a 96-well U-bottom plate (Carli Biotech, Rome, Italy) with 100µl of DMEM Low Glucose, and the medium was replaced every two days. The organoids were maintained in culture under controlled conditions, by monitoring cell aggregation through the Incucyte® system *(Figure 13)*.

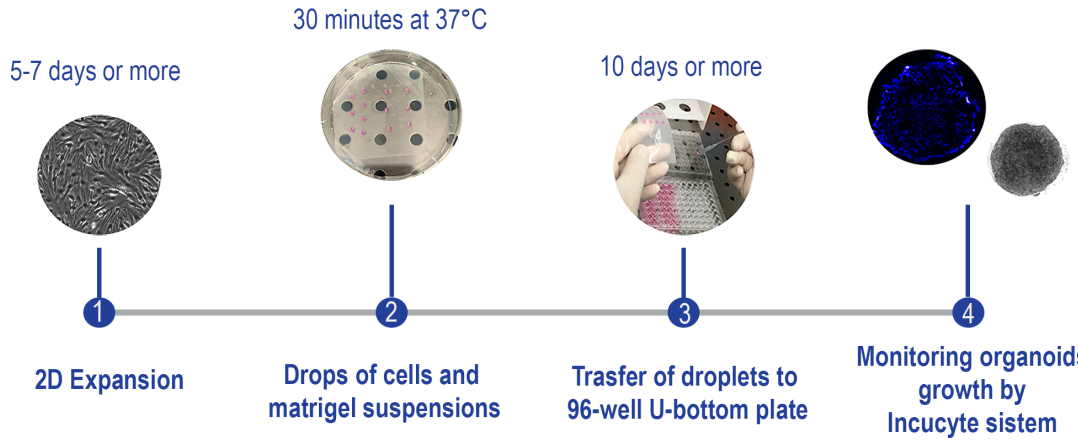


Figure 13. *Organoids setup*

3.19 Statistical Analysis

The data are expressed as mean ± standard error (SE) obtained from 3 experimental replicates (experimental replicates = 3 for each treatment/time point). Statistical analysis was performed using Prism 6 (GraphPad). Student's t-test or one-way ANOVA were used for multiple comparisons and performed on data sets. Values were considered statistically significant at $p \leq 0.05$.

4. RESULTS

4.1 Cytofluorimetric Characterization of DPSCs

Following the isolation of DPSCs, they were subjected to phenotypic characterization using flow cytometry. The results of this analysis confirmed a broad expression of the classical stem cell markers CD90 (99.9%), CD105 (94.6%), and CD73 (99.8%), which are commonly found in MSCs [Li et al., 2023]. Additionally, the cells showed partial positivity for CD146 and CD271, while they were negative for CD19 and CD45, as highlighted in the *Tabel 9:*

CD90	CD73	CD105	CD166	CD271	CD146	CD19	CD45
99,9%	99,8%	94,6%	100%	9%	9,5%	0,3%	0,2%

Table 9. *Cytofluorimetry analysis of positive and negative stemness markers of DPSCs.*

4.2 Determination of DPSCs Viability by ATP concentration

ATP, known as adenosine triphosphate, is the main energy source for all living cells and plays a role in numerous essential biochemical reactions. When cells die, they no longer produce ATP, causing the existing ATP supply to quickly break down. Therefore, ATP is widely accepted as a marker of viable cells; a higher concentration of ATP indicates a greater number of living cells [Deskins et al., 2013; Li et al., 2023]. To assess the viability of DPSCs in culture, the Cell Title-Glo (CT) assay was performed. This assay measures the amount of ATP present, and the luminescence reading that results is directly proportional to the number of living cells present. The assay was performed 3 times, and as shown in *Figure 14*, DPSCs on the second day of culture already exhibited a high ATP concentration, which increased by 200% e by day 4, indicating high metabolic activity. On the 8th day, a similar ATP concentration was measured as in the initial culture (100 LCU/min/ngDNA), followed by a new increase already from the 10th day with a peak on the 12th day (a 77% increase). Therefore, DPSCs exhibited high vitality and metabolic activity with a variable trend and a significant peak of ATP production was observed after a few days of culture.

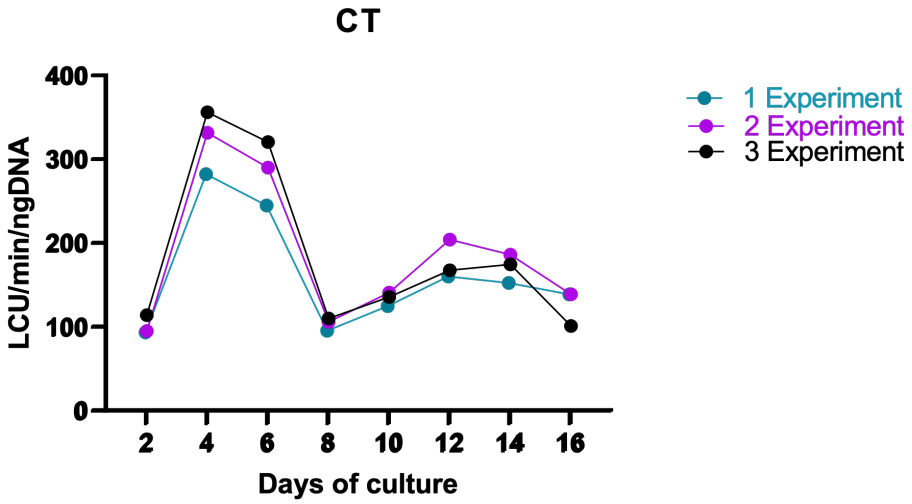


***Figure 14**. Intracellular ATP in DPSCs (expressed as LCU/min/ngDNA).*

4.3 Osteo-Odontoblastic differentiation of DPSCs

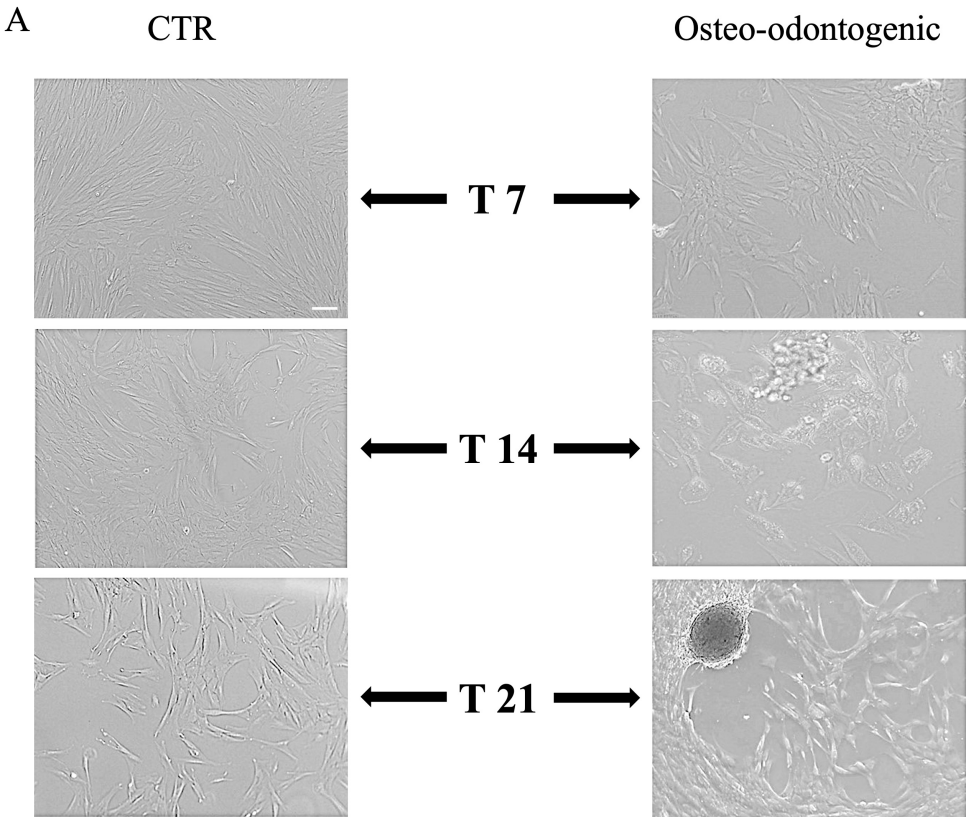
- ***Effect of Osteo-Odontogenic medium on the Morphological Features of DPSCs***

DPSCs at passage 3 were incubated in an osteo-odontoblastic differentiation medium for a total of 21 days (T21), with the medium being changed every 2-3 days. Throughout the differentiation process, the growth, and morphological transformations of DPSCs were observed using an Optika XDS-3LT optical microscope (Ponteranica, Bergamo BG) to compare differentiating cultures to those cultured in a standard medium (CTR). DPSCs cultured in the differentiation medium displayed time-dependent changes in growth and morphology. A notable development of spheroid-like structures was observable after 21 days of culture, which tended to detach *(Figure 15A)*.

- ***Alizarin Red Staining on DPSCs***

When DPSCs are cultured under suitable conditions for osteo-odontogenic differentiation, they can accumulate calcium and minerals in their extracellular matrices. Alizarin Red is a chemical dye that binds to mineralized structures, such as calcium, making it particularly suitable for highlighting the formation of minerals like hydroxyapatite in osteo-odontogenic cells [Liao et al., 2020]. Therefore, Alizarin Red staining was performed on DPSCs kept in contact with osteo-odontogenic differentiation

medium (T7, T14, T21) and on those of the control group (CTR) *(Figure 15B)*. Specifically, there is an increase in calcium phosphate deposition in osteo-odontogenic DPSCs at T7 and T14 compared to CTR. In osteo-odontogenic DPSCs at T21, calcium phosphate deposits appeared to decrease, but the cells continued to exhibit a different morphology compared to CTR; in fact, the cells appeared larger and resembled those of osteo-odontogenic at T7 and T14. These findings can be attributed to the characteristic ability of osteo-odontoblasts to release matrix containing dentin and calcium phosphates [Huo et al., 2021].



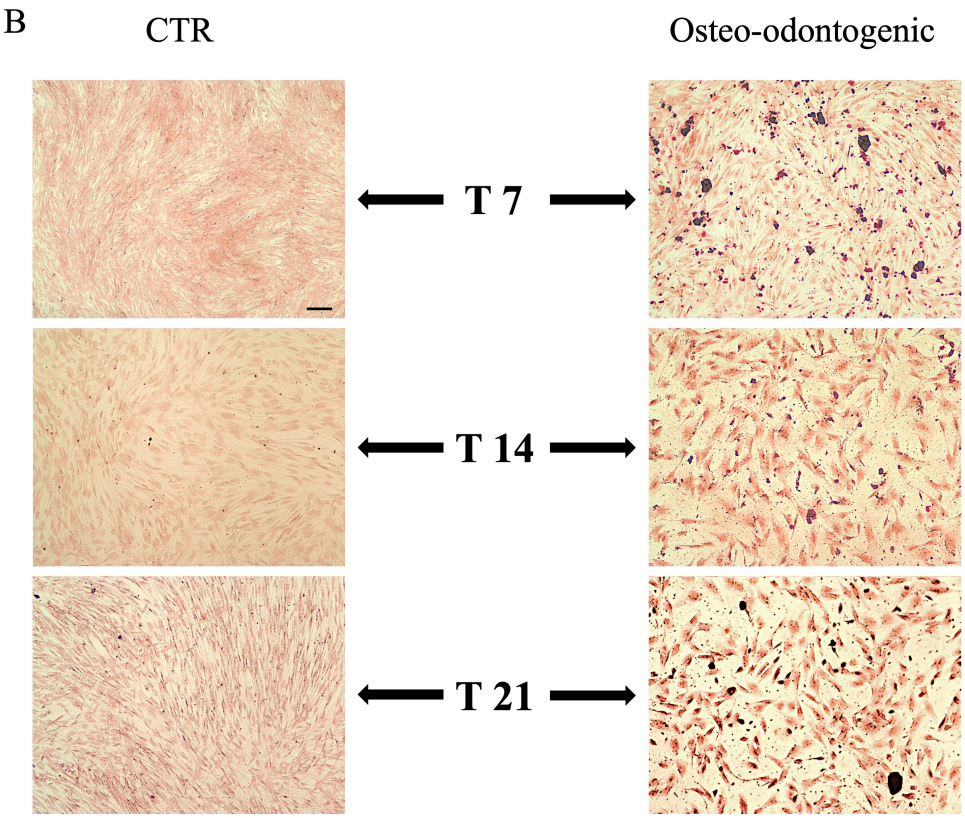


Figure 15. *Representative images of DPSCs cultured in osteo-odontoblastic differentiation medium compared with DPSCs maintained in standard medium (CTR) at various times: 7 days (T7), 14 days (T14), and 21 days (T21); 4X magnification.* ***A*** *Morphological assessment by optical microscope.* ***B*** *Alizarin Red staining; 200µm.*

- ***Evaluation of Osteo-Odontogenic Differentiation of DPSCs by Immunofluorescence***

To confirm the differentiation of DPSCs into the osteo-odontogenic phenotype, immunocytochemistry was performed for specific surface markers including CD44, CD105 (stem cell markers), OSX, and RUNX2 (osteo-odontogenic markers). As shown in *Figure 16A,B*, DPSCs held in the differentiation medium exhibited a time-dependent reduction in the stem cell marker CD44 (T21) compared to T0, accompanied by a concomitant increase in the osteo-odontogenic marker OSX. Similarly, CD105 displayed a gradual decrease over time in culture under specific differentiation conditions, following the same pattern as CD44. The increase in RUNX2 at T21 compared to T0 confirms the differentiation of DPSCs towards osteo-odontoblasts-like cells.

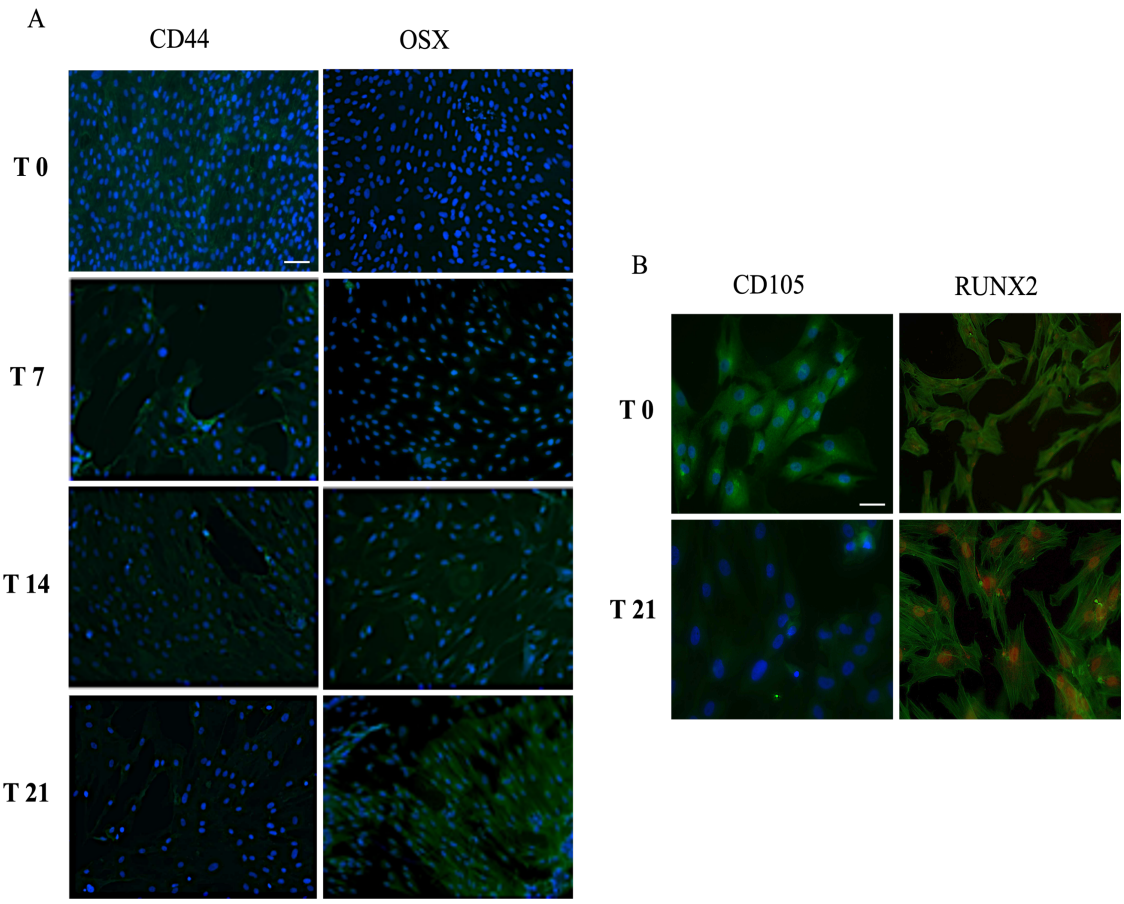


Figure 16. *Evaluation of osteo-odontogenic differentiation of DPSCs by Immunocytochemistry.* ***A, B*** *Immunofluorescence analysis of DPSCs, for the stem cell markers CD44, and CD105, and for osteo-odontogenic markers OSX and RUNX2; scale 100µm. In Panel* ***A*** *CD44 and OSX are shown in green, while DAPI was used for nuclear staining in blue. Images were acquired at a 20X magnification. In Panel* ***B*** *CD105 is shown in green, while DAPI was used for nuclear staining in blue. For RUNX2, dual staining was performed, with RUNX2 identified in red and phalloidin in green. Images were acquired at a 40X magnification.*

- ***Profiling of mRNA Expressed in DPSCs under Osteo-Odontoblastic or Standard Medium***

The expression of specific stem cell markers (OCT-4) and osteo-odontogenic differentiation markers (OSX, ALP, RUNX2) was evaluated by RT-PCR analysis *(Figure 17)*. DPSCs cultured in differentiation medium (T7, T14, T21) were compared to those cultured in standard medium (CTR). Histograms show an increase in the expression levels of genes associated with the osteo-odontoblastic lineage, in particular, OSX showed a progressive and significant increase compared to CTR. More specifically, its expression increased from 1.4 (T7) to 1.7 (T14), ultimately reaching 3.5 after 21 days of culture, in accordance with observations from immunofluorescence analysis. Notably, there was an

increase in ALP expression at T7 followed by a significant decrease at T14 compared to CTR. ALP serves as an early indicator of osteogenesis involved in the initial stages of mineralization [Darjanki et al., 2023; Wrobel et al., 2016].

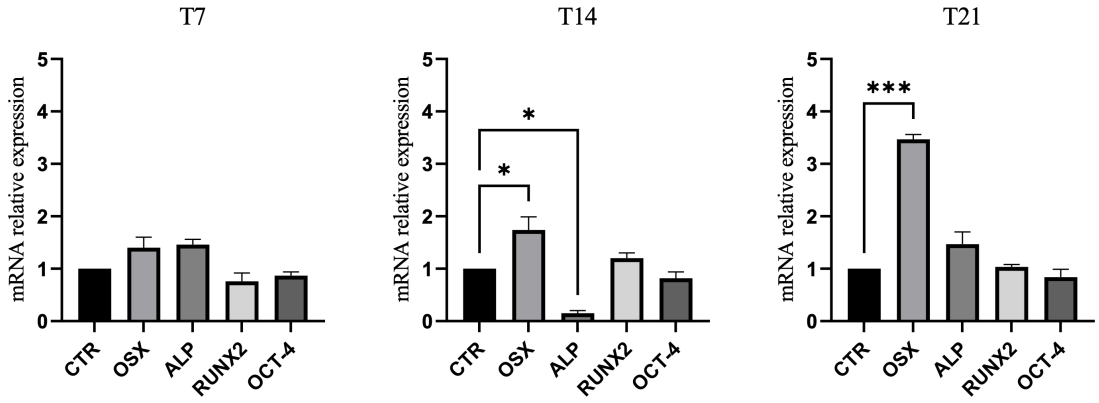


Figure 17. *Evaluation of osteo-odontogenic differentiation of DPSCs by RT-PCR. Analysis to assess the expression level of stem cell and osteo-odontogenic markers in DPSCs after 7, 14, and 21 days (T7, T14, and T21) in specific differentiation medium. * p< 0.05; *** p < 0,005*

4.4 Hepatic differentiation of DPSCs

- *Evaluation of Hepato-Specific Functional Parameters*

To differentiate DPSCs into hepatic-like cells, they were cultured in a hepatic differentiation medium for 20 days before being divided into two groups. In one group, the culture medium was supplemented with a hepatic matrix, and the cells were allowed to continue culturing for an additional 15 days. Meanwhile, the second group received only the differentiation medium for the same duration (20+15 days) *(Figure 18A).* Subsequently, hepato-specific functional parameters were measured in all cells, and the two cultures were compared side by side. Various Phase I enzymes (cytochrome P450, particularly 1A1 and 3A7) as well as resorufin conjugation activity (Phase II) were measured.

The activity of Phase II was assessed by quantifying the consumption of resorufin by hepatocytes. Hepatocytes metabolize resorufin, producing a metabolic product that can be detected through its fluorescence. Therefore, the more active the hepatic cells are, the faster the rate of resorufin metabolism [Gramignoli et al., 2012].

Resorufin conjugation in DPSCs cultured for 35 days in differentiation medium (20 days in medium alone and an additional 15 days with hepatic matter) was 0.5 pmol/min/millions of viable cells, which was significantly lower compared to the activity of freshly isolated hepatocyte. Even after treating DPSCs with specific inducers such as RIF and PB (for 3 days and 6 days), there were no changes in resorufin conjugation, indicating low Phase II activity of DPSCs *(Figure 18B).*

In the other group, DPSCs cultured for 35 days in differentiation medium alone, without hepatic matrix, a value of 0.3 pmol/min/millions of viable cells was measured. In this case, treatment with RIF and PB for 6 days increased significantly resorufin conjugation by the cells, compared with DPSCs without inductors (*Figure 18C).*

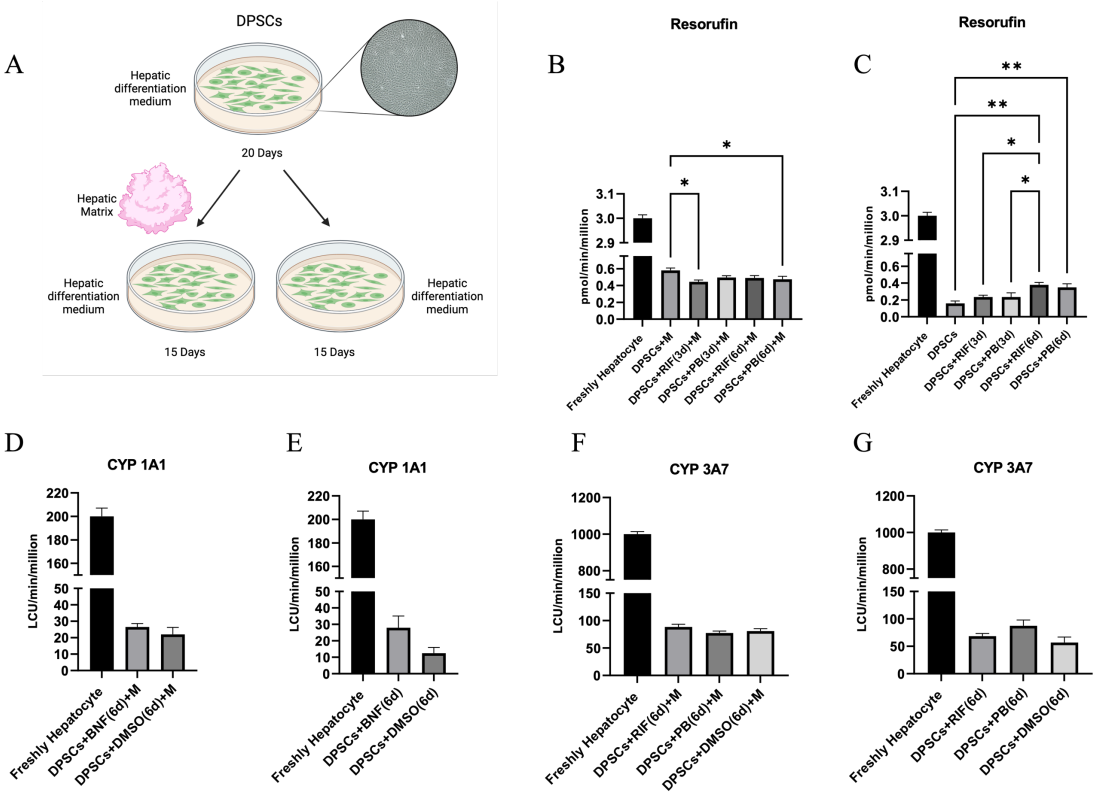


Figure 18. *Evaluation of hepatic differentiation of DPSCs by hepato-specific functional parameters* ***A*** *Representative diagram of culture conditions.* ***B, D, F*** *Conjugation of Resorufin and Cytochrome 1A1 and 3A7 activity on DPSCs cultured in hepatic medium with the supplement of hepatic matrix.* ***C, E, G*** *Conjugation of Resorufin and Cytochromes 1A1 and 3A7 activity on DPSCs cultured only in hepatic medium.*

As hepatocytes are expected to perform metabolic functions, particularly in Phase I, CYP 1A1 and 3A7 activities were measured [Gramignoli et al., 2012].

In both experimental groups, diminished activity of CYP 1A1 was observed concerning fresh hepatocytes. The 1A1 cytochrome activity was quantified at approximately 25

LCU/min/million cells following treatment with β-naphthoflavone (BNF), which is lower than the 200 LCU/min/million cells of freshly isolated hepatocyte. Moreover, treatment with dimethyl sulfoxide (DMSO) also resulted in a reduction of CYP 1A1 activity, indicating a low metabolic capability of the DPSCs cultured under these conditions *(Figure 18D,E).* CYP 3A7 is widely considered another important CYP form. Often referred to as the fetal isoform, CYP 3A7 is retained in adulthood by nearly 10% of the general population. A similar scenario was observed with CYP 3A7; indeed, the cytochrome's activity was low, less than 100 LCU/min/million cells in both culture groups, not comparable to the activity of freshly isolated hepatocytes, even after treatment with inducers such as RIF, PB, and DMSO *(Figure 18F,G).*

4.5 Neuronal differentiation of DPSCs

- *Effect of Hypoxia on the Proliferation and Morphological Features of DPSCs*

DPSCs were exposed to low oxygen levels (hypoxic conditions) for 5 and 16 days, as per the established procedure. Representative images in *Figure 19A* illustrate the changes in DPSCs' appearance after 5 days (5H) of hypoxic exposure compared to normoxic DPSCs (5N). Although 5H cells exhibited a minor alteration in cell shape relative to 5N cells, they appeared largely similar. Notably, hypoxic conditions appeared to enhance actin expression compared to normoxic cells. However, there was no significant impact on cell proliferation at this stage *(Figure 19B).*

In contrast, DPSCs exposed to hypoxia for 16 days (16H) displayed a distinct phenotype characterized by elongated and slender morphology *(Figure 19A).* Furthermore, the proliferation rate of cells subjected to 16 days of hypoxia was markedly reduced compared to other culture conditions *(Figure 19B).*

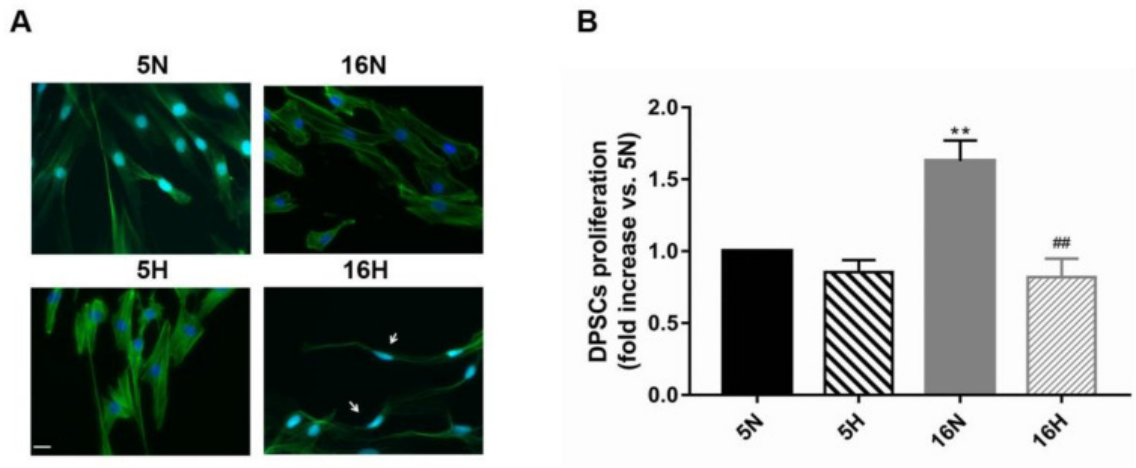


Figure 19. *Effects of hypoxia on DPSCs morphology and proliferation.* ***A*** *Representative image of phalloidin-stained DPSCs exposed or not to hypoxia for 5 and 16 days. White arrows indicate cell morphology changes. Scale bar 20 µm.* ***B*** *Proliferation assay of DPSCs subjected to hypoxia or not. Histograms indicate the means ± SE of three different cultures, each of which was tested in triplicate. ** $p < 0.01$ vs. 5N, and ## $p < 0.01$ vs. 16N.*

- ***Impact of Hypoxia on the ability of DPSCs to regulate the expression of Stem and Neuronal Markers***

In order to investigate the potential impact of hypoxia on the induction of neuronal differentiation, DPSCs were exposed for either 5 or 16 days at 1% O_2 (5H, 16H) or kept in normoxia at 21% O_2 (5N, 16N).

Table 10A illustrates a notable decrease in the expression levels of various mesenchymal stem cell markers (CD44, CD90, CD105, STRO1, and CD73), typically expressed by DPSCs, following exposure to hypoxia for 5 days [de Carvalho et al., 2020]. Particularly noteworthy was the significant drop in CD105 expression, reaching a reduction of 47%. The evaluation of the same stem cell markers at 16 days (16H and 16N) showed an even decrease. Interestingly stem cell markers in 16H decreased greatly, almost reaching zero. On the contrary, there was a slight yet significant increase in neuronal markers found that were exposed to hypoxia for 5 days (5H), with a more pronounced rise after 16 days (16H). Only the nestin marker decreased from 16N to 16H, suggesting the specific role of this marker in neuronal differentiation. Moreover, the phenotypic expression of DPSCs exposed to 16 days of hypoxia closely mirrored those of DPSCs treated with EGF and bFGF factors for 14 days, a culture condition known to induce neuronal differentiation, as depicted in *Table 10B* [Martellucci et al., 2019].

A

	CD44	CD90	CD105	STRO1	CD73	Nestin	β3Tub	NFH	GAP43
5 N	76,4±6,2	75,1±4,9	63,9±5,8	73,4±6,9	84,2±7,7	44,7±3,4	1,8±0,9	2,2±0,4	3,7±1,4
5 H	69,5±5,8	70,8±5,8	34,2±4,1	68,1±5,4	74,4±4,4	56,6±6,3	9,1±1,4	5,00±1,4	17,1±2,4
16 N	73,6±6,9	72,1±7,4	63,7±5,5	61,3±4,9	75,8±6,1	50,8±3,4	2,6±1,2	2,9±1,1	9,3±1,4
16 H	2,4±1,1	2,0±0,4	0±0,5	0,1±0,4	15,5±2,0	30,4±4,0	53,3±5,1	47,1±3,4	43,3±2,4

B

	CD44	CD90	CD105	STRO1	CD73	Nestin	β3Tub	NFH	GAP43
Control	79,3	62,9	62,1	68,3	71,0	45,1	2,4	3,1	4,0
EGF/bFGF	8,3	5,1	2,1	3,8	3,0	19,3	49,1	54,3	51,1

Table 10. *Effects of hypoxia on the phenotypic expression profile of DPSCs.* ***A*** *Mesenchymal stem cell and neuronal markers of DPSCs subjected to hypoxia and normoxia for 5 and 16 days.* ***B*** *Mesenchymal stem cell and neuronal markers of DPSCs treated with EGF and bFGF factors for 14 days.*

- ***Impact of Hypoxia on the mRNA Expression Profile***

To validate the impact of hypoxia on inducing phenotypic alterations in DPSCs a comparative analysis of mRNA expression profiles between DPSCs exposed (5H, 16H) and unexposed (5N, 16N) to hypoxic conditions was conducted.

The expression levels of both early markers (nestin, GFAP, bFGF, and EFG) and late markers nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial cell line-derived neurotrophic factor (GDNF), associated with neural differentiation, were evaluated.

The results from RT-PCR analysis showed a significant increase in the expression of most marker genes after 5 days *(Figure 20A)*. However, it is worth noting that there was no change in the expression of bFGF. Interestingly, the marked increase in nestin and GFAP, as well as the GDNF and BDNF mRNA expression observed in 5H suggests that these cells may be derived from neural crest origin, making them potentially more responsive to treatments targeting a neurogenic phenotype. Hypoxia at 16 days determined a new profound change in mRNA marker expression levels. The levels of expression of nestin, EGF, BDNF, GFAP, and GDNF were significantly reduced in 16H cells compared with 5H cells *(Figure 20A)*. Conversely, NGF and bFGF mRNA levels were strongly increased

compared with 5H cells, reaching values of 4-fold and 16-fold greater than the controls, respectively.

- ***Influence of Hypoxia on the Secretion of Factors by DPSCs***

Based on previous findings, hypoxia determined a switch in the expression profiles of DPSCs, addressing them toward a neurogenic phenotype. In particular, the levels of mRNA expression of EGF and bFGF at 5 days and 16 days of hypoxia, respectively, were particularly increased.

To explore whether these changes corresponded to an increase in the secretion of these growth factors were performed ELISA assays for human EGF and bFGF on CM collected from DPSCs exposed or not exposed to hypoxia for 5 and 16 days. The analysis of the CM from the DPSCs stimulated with hypoxia for 5 days showed a significant increase in EGF expression, consistent with the mRNA expression data. EGF increased even more in CM from DPSCs stimulated with hypoxia for 16 days, probably because of the observed increase in mRNA at 5 days of hypoxia *(Figure 20B)*. Moreover, bFGF secretion was highly detectable in the CM of DPSCs treated with hypoxia for 16 days, mirroring its mRNA expression pattern.

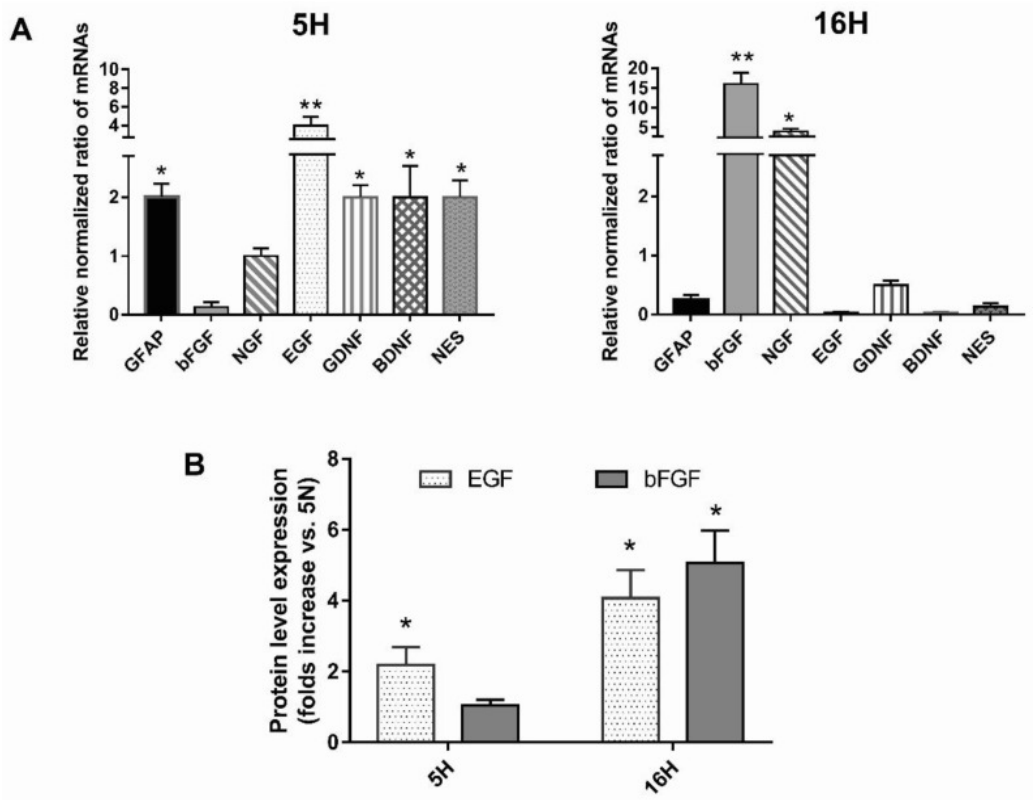


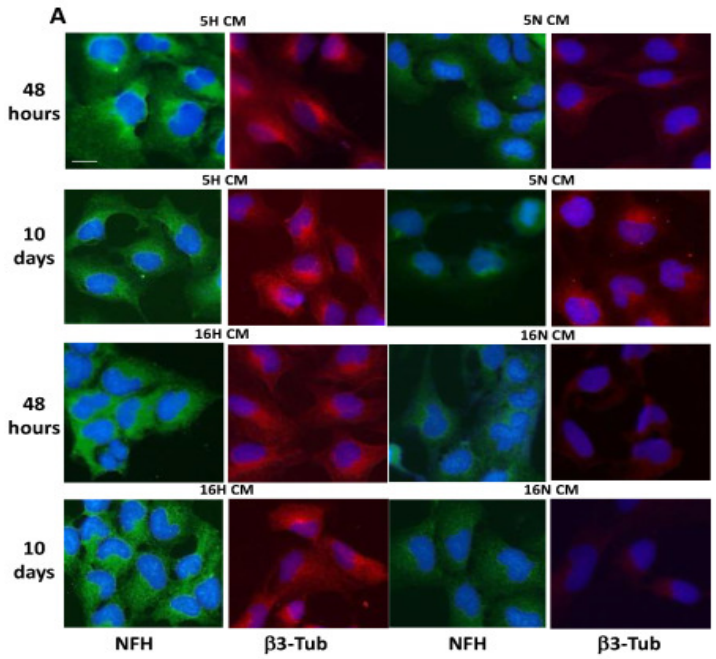
Figure 20. *Effects of hypoxia on the mRNA profile expression of DPSCs.* ***A*** *Real-time RT-PCR analysis of the levels of expression of early and late markers of neural differentiation (nestin, GFAP, EGF, NGF, bFGF BDNF, GDNF) after 5 days and after 16 days of hypoxic exposure.* ***B*** *Detection of growth factors bFGF and EGF in the conditioned media of DPSCs exposed or not exposed to hypoxia for 5 days and 16 days by human EGF and FGF basic ELISA Kits. Histograms indicate the means ± SE of three different cultures, each of which was tested in triplicate. * $p < 0.05$; ** $p < 0.01$ vs. 5N.*

- ***Impact of CM derived from DPSCs (Paracrine Signaling) on promotion of Neuronal Characteristics***

To investigate the potential of DPSCs to induce neuronal differentiation through an autocrine/paracrine mechanism mediated by growth factor secretion were examined the effects of DPSCs-CM (5H, 16H) to differentiate SH-SY5Y neuroblastoma cells into a more mature, neuron-like phenotype. Previous studies have demonstrated that undifferentiated SH-SY5Y cells express immature neuronal markers and lack mature neuronal markers. However, under the influence of differentiation-inducing agents, SH-SY5Y cells become morphologically more like primary neurons [Kalinovskii et al., 2022].

When SH-SY5Y cells were exposed to CM from DPSCs subjected to hypoxic conditions for 5 days (5H) and 16 days (16H), different phenotypic changes were observed compared to cells exposed to 5N and 16N CM. Immunofluorescence analysis revealed a notable increase in the expression of neuronal markers NFH and β3-Tubulin in all groups of SH-SY5Y cells treated with DPSCs' hypoxic CM (5H, 16H) compared to those treated with normoxic CM (5N, 16N) *(Figure 21A).*

Furthermore, flow cytometry analysis of NFH and β3-Tubulin expression in SH-SY5Y cells, either treated or untreated, confirmed, quantitatively, these results. Specifically, after 10 days of exposure to 16H CM, SH-SY5Y cells exhibited NFH and β3-Tubulin expression levels of 96.1% and 92.8%, respectively *(Figure 21B).*



B

CM from DPSCs	Time	NFH %	β3-Tub %
5H CM	48 hours	68.0 ± 4.4	51.4 ± 3.8
5N CM	48 hours	32.8 ± 4.1	5.4 ± 1.0
5H CM	10 days	66.8 ± 5.1	66.7 ± 5.7
5N CM	10 days	9.9 ± 1.2	1.2 ± 0.8
16H CM	48 hours	87.6 ± 6.8	71.8 ± 7.2
16N CM	48 hours	0.9 ± 0.3	2.3 ± 1.1
16H CM	10 days	96.1 ± 3.2	92.8 ± 6.8
16N CM	10 days	1.90 ± 0.8	1.1 ± 0.7

***Figure 21**. Immunofluorescence and flow cytometry analysis of SH-SY5Y. **A** Immunofluorescence staining of SH-SY5Y cells exposed to DPSC-CM (5H, 16H, 5N, and 16N). **B** Flow cytometry analysis of NFH and β3-Tubulin expression in SH-SY5Y-treated groups or untreated cells. Scale bar 20 µm.*

Similarly, the potential of 5H and 16H CM to induce differentiation of undifferentiated DPSCs towards a neuronal phenotype was tested.

Immunofluorescence analysis revealed a significant increase in the expression of neuronal markers NFH and β3-Tubulin in DPSCs treated with hypoxic DPSCs-CM (5H CM and 16H CM) compared to normoxic DPSCs-CM (5N CM and 16N CM). Specifically, the increase in neuronal marker expression was visible in the DPSCs' long processes, such as those of primary neurons *(Figure 22)*.

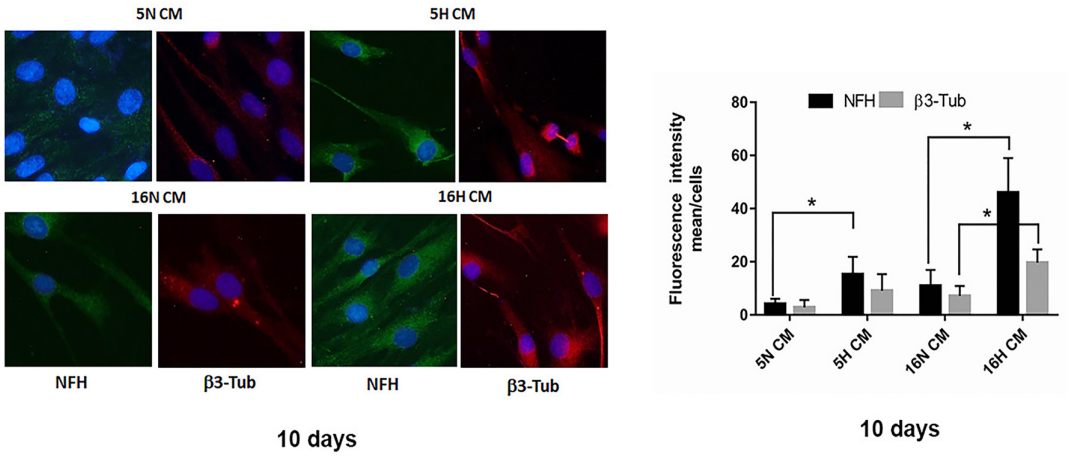


Figure 22. *Immunofluorescence analysis of DPSCs.* ***A*** *Immunofluorescence analysis of NFH and β3-Tubulin in DPSCs treated with DPSCs-CM (5H, 16H, 5N, and 16N). Scale bar 20 µm.* ***B*** *Analysis of fluorescence intensity for NFH and β3-Tubulin in DPSCs treated with DPSCs-CM (5H, 16H, 5N, and 16N). Data are represented as mean ± SE. * $p < 0.05$; 5H and 16H vs. 5N and 16N.*

- ***The Influence of Hypoxia on Triggering Phenotypic Characteristics in DPSCs***

The DPSCs' treated groups, 5H CM and 16H CM, were compared with the 5H and 16H groups by flow cytometry to determine the profile of expression of several neuronal and stem markers. As shown in *Figure 23*, the stem cell markers CD44 and CD73 were still quite high in the 5H group. Only CD90 was lowered in 5H CM. Moreover, the neuronal markers investigated (nestin, β3-tubulin, NFH, and GAP43) were still low and similarly expressed in the 5H and 5H CM groups.

Intriguingly, the 16H CM groups showed a dramatic decrease in stem cell markers, reaching values below 10% positivity. Conversely, neuronal markers strongly increased in 16H CM, reaching values higher than 16H.

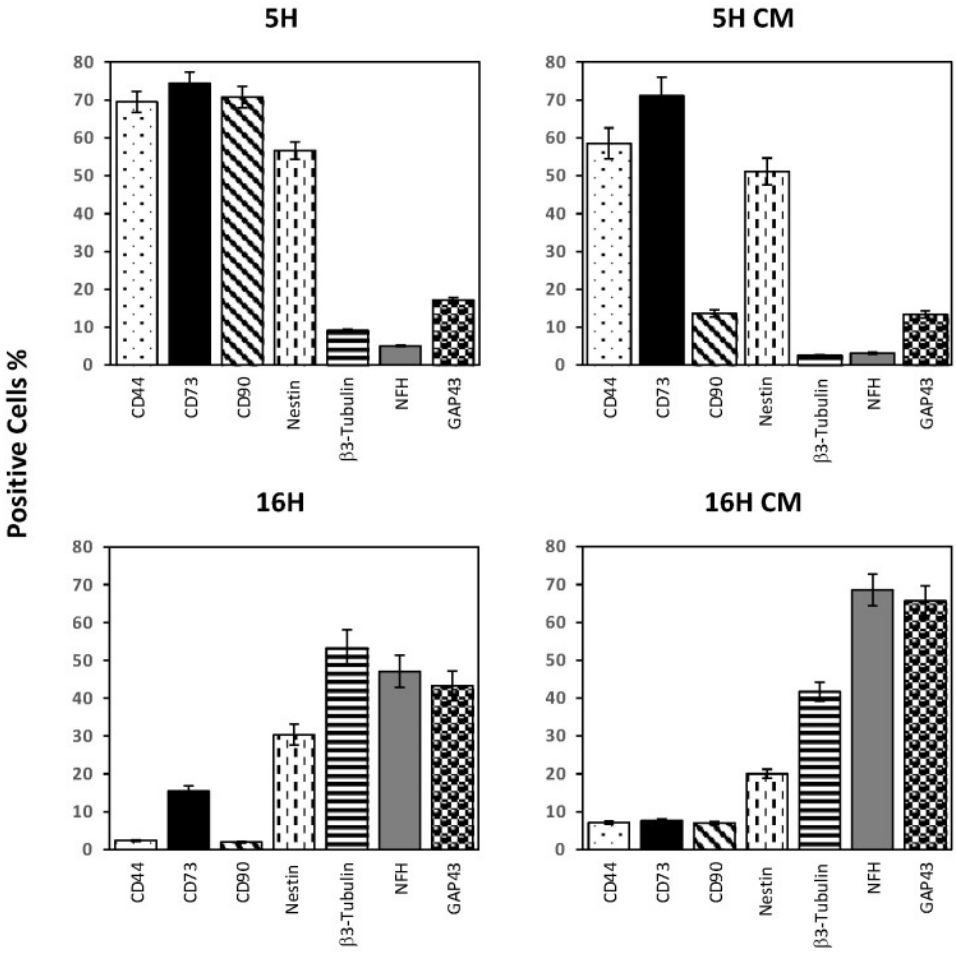


Figure 23. *Flow cytometry analysis of DPSCs-treated groups: 5H CM and 16H CM were compared with the 5H and 16H groups to determine the profile of expression of several neuronal and stem markers. Histograms indicate the means ± SE of three different experiments.*

4.6 Peri-Vascular differentiation of DPSCs

- *Analysis of DPSCs following Immunomagnetic Separation (MACS) based on NG2 marker*

Following the immunomagnetic separation procedure outlined in the Materials and Methods section, the three subpopulations obtained were phenotypically characterized based on the pericytic marker NG2 and stem cell markers (CD44, CD73, CD90, STRO1). As shown in *Table 11*, DPSCs NG2+ exhibit a high expression of the pericytic marker, reaching 96%, in contrast to the Total DPSCs expressing 48.7% and DPSCs NG2- which only reached 5%. However, DPSCs NG2+ only partially maintain stemness characteristics, especially compared to the other two subpopulations.

Cell Population (DPSCs)	*Number of cells*	*CD44*	*CD73*	*CD90*	*STRO1*	*NG2*
Total	$1*10^7$	72.4%	73.1%	58.4%	60.5%	48.7%
NG2-	$4.8*10^6$	80.2%	80.2%	73.1%	73.2%	5.3%
NG2+	$4.4*10^6$	55.2%	57.1%	57.4%	60.4%	96.0%

Table 11. *Phenotypic characterization of the three subpopulations (Total DPSCs, DPSCs NG2-, and DPSCs NG2+) based on the pericytic marker NG2 and the stemness markers (CD44, CD73, CD90, and STRO1).*

- ***Investigation into the Function of Total DPSCs, DPSCs NG2-, and DPSCs NG2+ in Maintaining Tubule Stability***

To test the hypothesis that the expression of the pericytic marker NG2 could influence DPSCs' ability to stabilize tubes *in vitro*, a co-culture assay was conducted on matrigel using HUVECs and different populations of DPSCs (Total DPSCs, DPSCs NG2-, and DPSCs NG2+). The assay was monitored over time for up to 14 days using the Incucyte® system. To distinguish between HUVECs and DPSCs, each subset of DPSCs, (Total DPSCs, DPSCs NG2-, and DPSCs NG2+) was marked with an intracellular fluorescent dye, calcein.

In *Figure 24A*, it can be observed that HUVECs were able to form tube-like structures within about 10 hours, but these structures completely broke down by the 7th and 14th day. On the other hand, in all co-cultures of HUVECs and DPSCs (Total DPSCs, DPSCs NG2-, DPSCs NG2+), were observed a stabilization of these tubular vascular structures over time, up to 14 days post-seeding.

For quantitative analysis, the percentage of tubule retention was considered, and the histogram indicates that the DPSCs NG2+ subpopulation in co-culture with HUVECs is the most capable of stabilizing tubes over time *(Figure 24B).* Specifically, HUVECs+DPSCs NG2+ exhibited a tubule retention percentage of 100% after 10 hours, decreasing to 80% after 7 days and 70% after 14 days. In comparison, HUVECs+DPSC NG2- showed values exceeding 100% after 10 hours of culture, decreasing to 40% and 15% after 7 and 14 days, respectively. Therefore, DPSCs NG2+ stabilize tubes over time compared to DPSCs NG2-. Comparing DPSCs NG2+ to Total DPSCs, there was still a greater stabilization of tubes over time, although not significantly so.

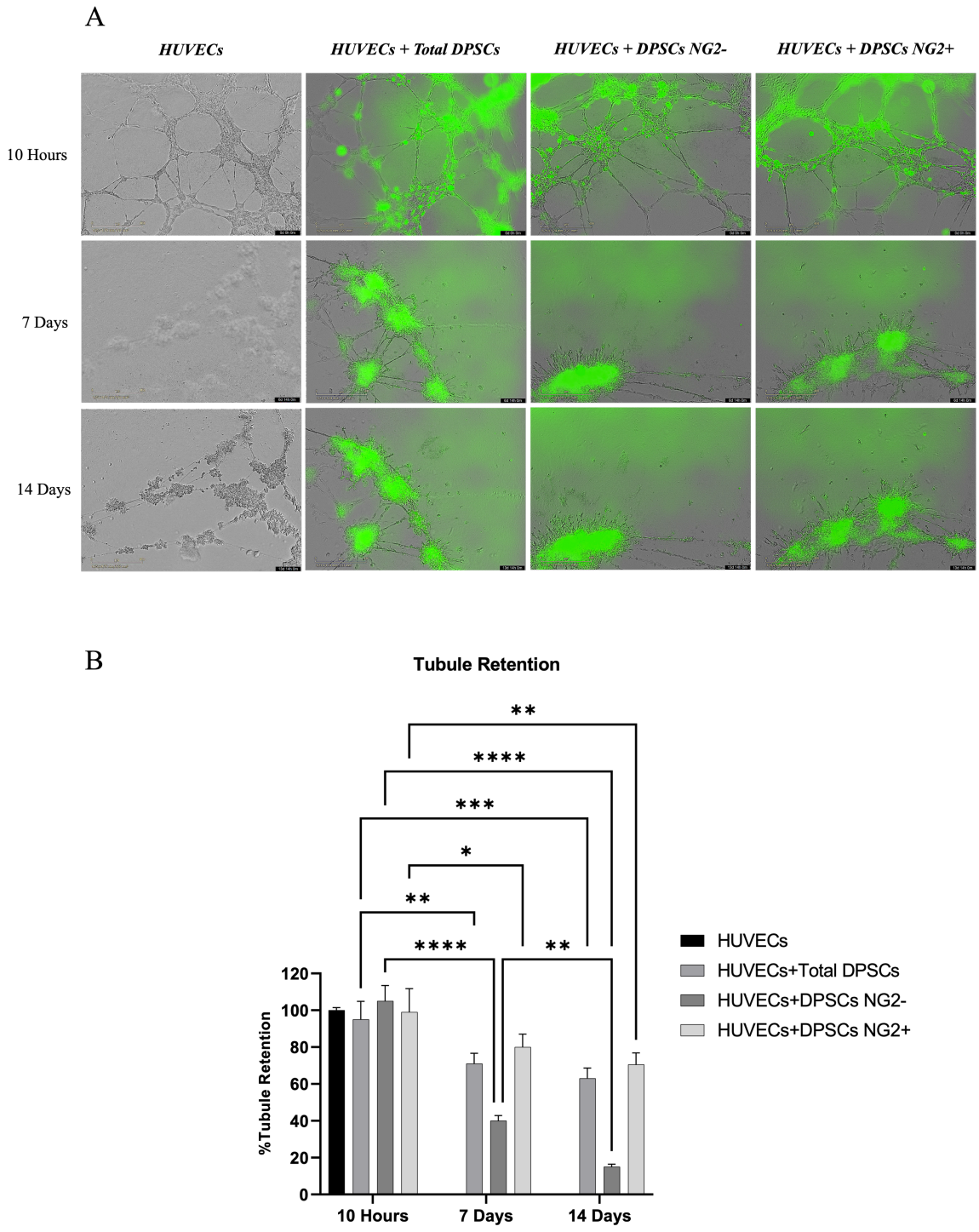


Figure 24. *DPSCs' ability to stabilize tubes in vitro.* ***A*** *Representative image in time-laps of HUVECs+DPSCs co-cultures at 10 hours, 7 days, and 14 days post-seeding. Green fluorescence was attributed to calcein, used to label DPSCs subpopulations. The images were captured using the Incucyte® system. magnification 4X.* ***B*** *Quantitative analysis of HUVECs+DPSCs co-culture (Total DPSCs, DPSCs NG2-, DPSCs NG2+). * p < 0.05; ** p<0,005; *** p < 0,0005; ****p<0,0001.*

- ***Potential Paracrine Role of DPSCs on the HUVECs Proliferation***

It is known that DPSCs can act as pericyte-like cells and secrete pro-angiogenic factors important for vascularization in regeneration tissue [Janebodin et al., 2013].

So, to clarify whether DPSCs play a role in vessel stabilization through paracrine signaling, the effects of CM, derived from three subpopulations of DPSCs were evaluated and compared on the growth of HUVECs.

As shown in *Figure 25A*, the CM from DPSCs did not affect the morphological feature of HUVECs. Furthermore, both CM of DPSCs NG2- and NG2+ mildly stimulated HUVECs proliferation compared to Total DPSCs CM, albeit without statistical significance *(Figure 25B).*

- ***Exploring the Potential Paracrine Role of DPSCs on the Ability of HUVECs to Promote Migration***

To evaluate the paracrine effect of DPSCs on HUVECs migration, conditioned media, from the three subpopulations, were utilized to conduct a Wound Repair assay on endothelial cells. Data from the histogram and images *(Figure 25C, D),* clearly show that after 24 hours, the wound closes completely in all experimental conditions. Particularly noteworthy is the finding that in the presence of CM from Total DPSCs, HUVECs migrated more rapidly compared to those stimulated by conditioned media from DPSCs NG2+ and DPSCs NG2-. Indeed, at 12 hours, the wound coverage percentage was nearly 70% *(Figure 25D)*. Conversely, the same situation was not observed for HUVECs cultured in the presence of conditioned media derived from DPSCs NG2+, specifically, at 12 hours, the wound coverage percentage was 60%, suggesting that CM from DPSCs NG2+ did not significantly stimulate endothelial cell migration *(Figure 25D).* The Wound Repair assay was monitored through time-lapse analysis using the Incucyte® system.

- ***Exploring the VEGF Release by DPSCs***

Considering the potential paracrine effects of DPSCs (Total DPSCs, DPSCs NG2-, DPSCs NG2+) in promoting angiogenesis, the concentration of the VEGF factor in their conditioned media was assessed. Using a specific ELISA assay were measured the levels of VEGF, and the results, depicted in the graph *(Figure 25E),* show that VEGF levels (pg/ml) in the conditioned media of DPSCs NG2+ (0.87 pg/ml) are significantly lower

compared to those of Total DPSCs (1.86 pg/ml). This suggests that DPSCs NG2+, exhibiting pericytic characteristics, support this process but not preferentially through the release of the pro-angiogenic factor VEGF.

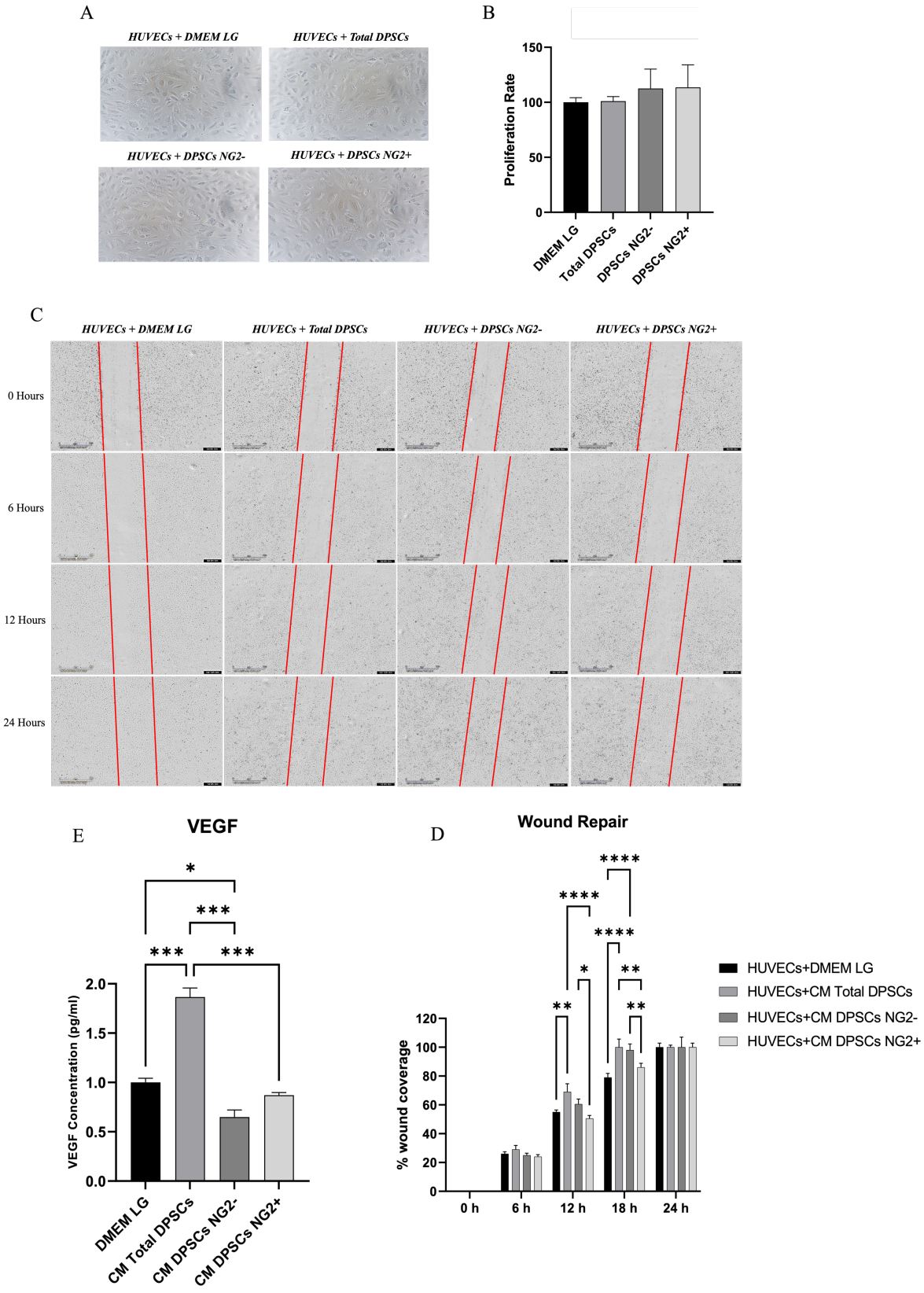


***Figure 25**. Potential paracrine role of DPSCs in the stabilization of tubular-like structure. **A** Representative image of the proliferation assay of HUVECs in the presence of conditioned media (CM) obtained from three subpopulations of DPSCs (Total DPSCs, DPSCs NG2-, DPSCs NG2+). magnification 10X. **B** Corresponding histogram for quantitative analysis. DMEM LG was used as a control. **C** Images of HUVECs monitored during the wound healing assay using CM derived from the three subpopulations of DPSCs (Total DPSCs, DPSCs NG2-, DPSCs NG2+). The scratch was monitored and photographed at various time points (0h, 6h, 12h, 24h) using the Incucyte® system; magnification 4x. **D** Quantitative analysis of the wound healing assay on HUVECs. HUVECs cultured in DMEM LG served as control. * p<0,05; **p<0,005; ****p<0,0001. **E** Evaluation of the concentration of the pro-angiogenic factor VEGF secreted by DPSCs (Total DPSCs, DPSCs NG2-, DPSCs NG2+) expressed in pg/ml. *p<0,05; ***p<0,0005.*

- ***Analysis of Wound Repair Ability of Total DPSCs, DPSCs NG2- and DPSCs NG2+***

Based on prior observations, it appears that the supportive role of DPSCs NG2+ in angiogenesis may not predominantly involve paracrine mechanisms.

To verify the direct pro-angiogenic potential of DPSCs and their similarity with physiological pericytes, a Wound Repair assay was conducted. Analysis of the images *(Figure 26A)* and corresponding results *(Figure 26B)* reveals a marked migratory capability of DPSCs NG2+ compared to other subpopulations.

Indeed, at 24 hours, DPSCs NG2+ demonstrated a wound coverage percentage of 31% in contrast to 13% observed for Total DPSCs. Additionally, DPSCs NG2- also demonstrate good migratory ability, as they exhibit a wound coverage percentage of 27% at 24 hours.

Furthermore, to further verify whether DPSCs behave similarly to pericytes and possess the ability to migrate on HUVECs, DPSCs were labeled with a red fluorescent dye PKH26. Subsequently, a co-culture assay with HUVECs in the matrix was conducted *(Figure 26C)*. DAPI was utilized to stain the nuclei of all cells.

Analysis of the acquired images indicated that fluorescently labeled DPSCs demonstrated a propensity to adhere to HUVECs. Notably, within 24 hours, they adhered to interconnected points and subsequently migrated along HUVECs tubes, thereby corroborating their resemblance to pericytes and their capacity to stabilize the tubules.

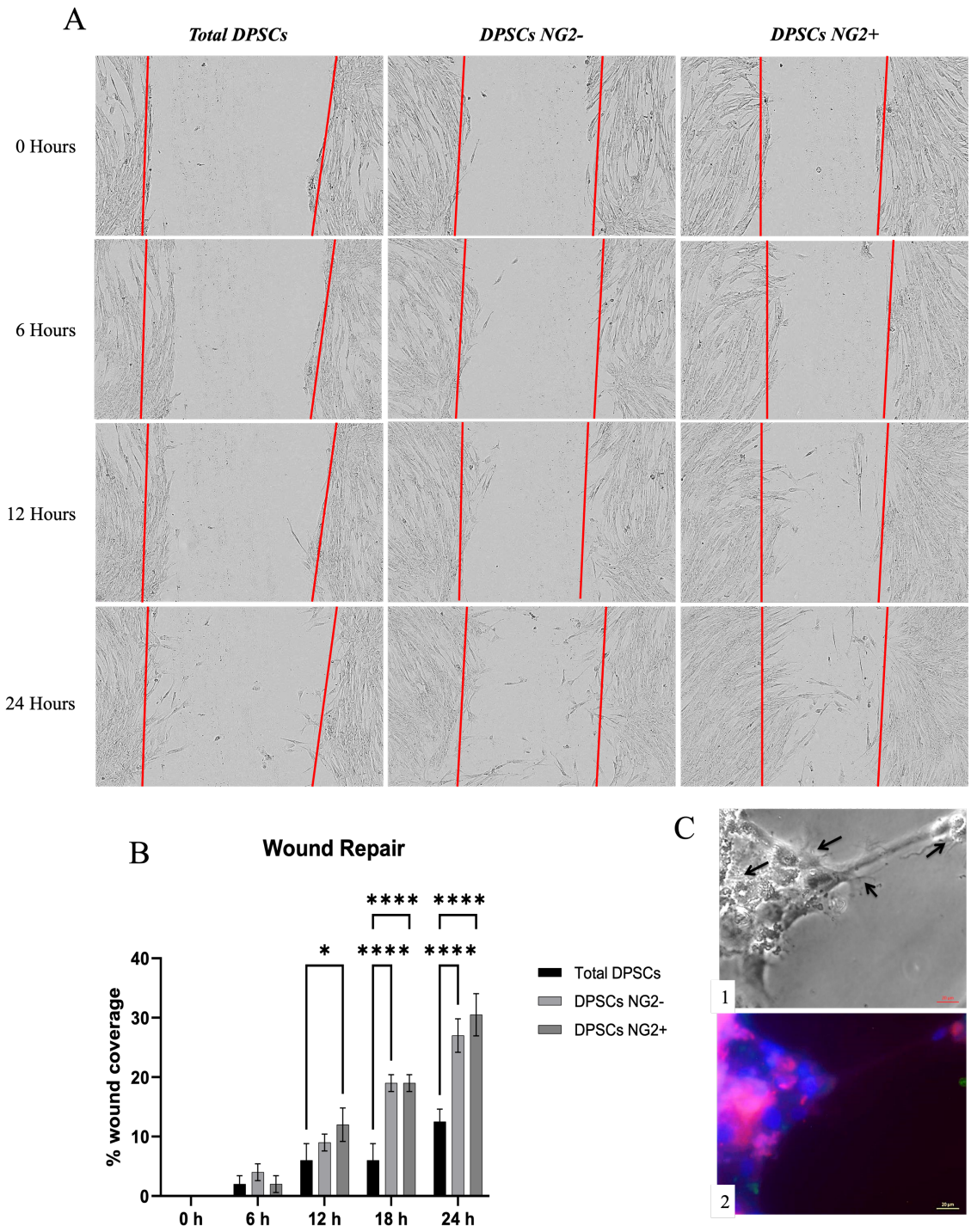


Figure 26. *Direct pro-angiogenic potential of DPSCs.* ***A*** *Representative image of DPSCs (Total DPSCs, DPSCs NG2-, DPSCs NG2+) monitored during the wound healing assay. The scratch was observed and photographed for 24 hours using the Incucyte® system; magnification 4X.* ***B*** *Histogram related to the wound healing assay of DPSCs (Total DPSCs, DPSCs NG2-, DPSCs NG2+) *p<0,05; ****p<0,0001.* ***C*** *Co-culture images of HUVECs and DPSCs on matrigel.* ***1.*** *Phase-contrast images, with arrows indicating the position of DPSCs;* ***2.*** *Fluorescence images, with DPSCs shown in red and DAPI in blue. magnification 40X.*

4.7 Three-Dimensional Analysis (3D)

- *Generation of Organoids from Dental Pulp Stem/Stromal Cells*

A stationary technique was employed to establish a standardized protocol for organoid generation from DPSCs. This assay involves culturing cells in 96-well U-bottom plates using ECMatrix™, as previously described by several researchers [Hisha et al., 2013; Howes et al., 2014; Jeong et al., 2020].

The optimal number of starting cells was determined by titolation, starting from 25,000 to 100,000 cells per well, picking 75,000 cells per well as the optimal concentration *(Figure 27A)*. Additionally, the optimal concentration of ECMatrix™ was determined through preliminary experiments, testing various concentration ratios with DMEM- Low Glucose. Absolute ECMatrix™ was found to be the most conducive condition for forming cell aggregates *(Figure 27B),* thus it was used for subsequent experiments.

After three days of culture in DMEM-Low Glucose, a significant aggregation of stem cells was observed, which was reproducible in each well, designated as Time Zero (T0). Following the methodology of Jeong et al., the growth of organoid-like structures was monitored using the Incucyte® system, generating a time-lapse video of their formation [Jeong et al., 2020].

Moreover, to confirm the viability of the DPSCs organized in the 3D structure, after 6 days of culture, the organoid was subjected to immunofluorescence for the Ki-67 marker *(Figure 27C)*. Ki-67 is a nuclear protein involved in cell cycle regulation; its expression is closely associated with cell proliferation, as it is present only in actively dividing cells [Cordero-Espinoza et al., 2021]. As observed in the image, it is evident that the cells are perfectly viable both externally and in the inner zone.

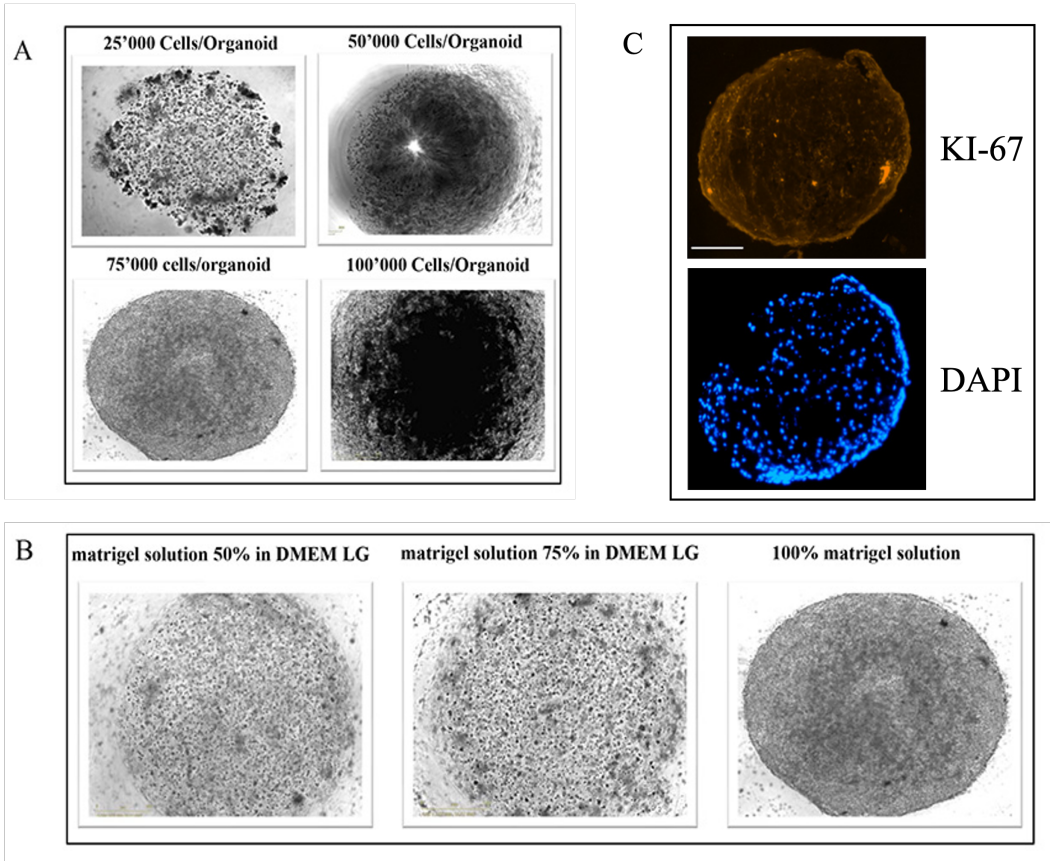


Figure 27. *Generation of organoids from Dental Pulp Stem/Stromal Cells.* ***A*** *Representative image of titolation to determine the optimal cell quantity.* ***B*** *Representative images of the experiments conducted to determine the appropriate concentration of ECMatrix™ (4X magnification).* ***C*** *Immunofluorescence analysis showing DAPI and Ki-67 staining after 6 days of culture (10X magnification). The scale bars correspond to 200 µm. Samples were analyzed, and images were captured using the A1 Zeiss Axiovert fluorescence microscope (Zeiss, Milan, Italy).*

4.7.1 Osteo-Odontoblastic differentiation of DPSCs organoids

- ***Morphological Analysis of Osteo-Odontoblastic Organoids***

Once the organoids were generated, the aggregation of DPSCs was analyzed from T0 onwards. This analysis involved monitoring the increase in cell density over time using a time-lapse imaging system, Incucyte®. Generally, a steady rise in cell density was observed until it reached approximately 1.5 mm in size after 26 days of culture.

To assess the organoids' capacity for differentiation, at T10 the standard growth medium was replaced with specific differentiation media. The samples were divided into two groups: one group remained in DMEM Low Glucose (CTR), while the other underwent osteo-odontogenic differentiation.

As shown in *Figure 28A*, there was a noticeable increase in cell density (indicated by the darker color of the organoid itself) in the control group compared to the group subjected to osteo-odontogenic differentiation. Specifically, organoids cultured in DMEM Low Glucose showed no significant growth differences between T0 and T6. However, there was a substantial increase in cell density starting from T12, with even greater density observed at T26. In contrast, the organoids subjected to differentiation exhibited a slower growth rate and a lesser increase in cell density compared to the controls.

Between T12 and T18, the differentiating organoids appeared less dense and compact than the control group. This could be attributed to the slower growth rate of the latter. These observations support the hypothesis of a cell choice-dependent reduction in proliferation toward cell differentiation.

Using the Axio Zoom V6 stereomicroscope, a device that allows image reconstruction through the acquisition of multiple focal planes, it was possible to obtain images of the organoids incubated in both differentiation medium and control medium (CTR).

As highlighted in *Figure 28B*, the organoids maintained in the osteo-odontogenic differentiation medium for 26 days observed mineralization, likely due to an increased release of mineralized matrix by the differentiated cells compared to the control organoids. Images acquired with the Incucyte® had not revealed this characteristic; on the contrary, in the organoids subjected to osteo-odontogenic differentiation, a lower cell density was observed.

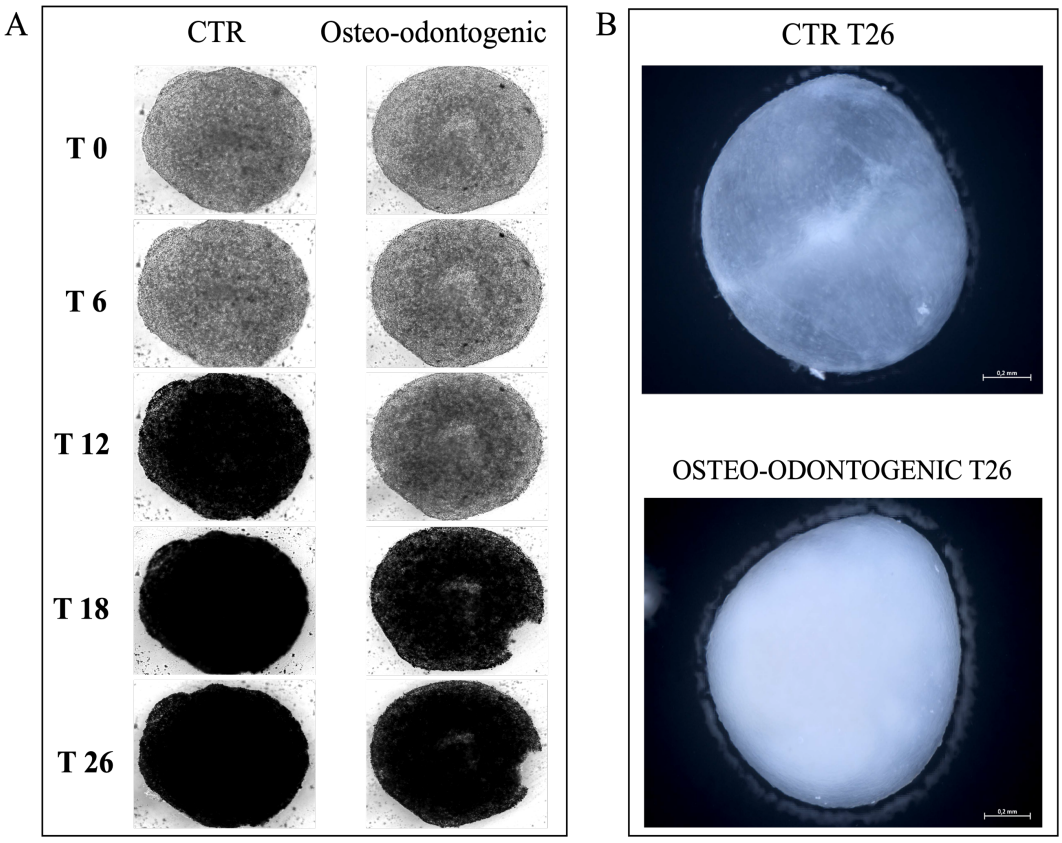


Figure 28. *Morphological analysis of osteo-odontoblastic organoids* ***A*** *Time-lapse image acquired with the Incucyte® system from T0 to T26. Development of organoids maintained in DMEM LG (CTR) and organoids undergoing osteo-odontogenic differentiation (4X magnification). The scale corresponds respectively to 300µm.* ***B*** *Representative images acquired with Axio Zoom V6 stereomicroscope of organoids at T26 (63X magnification).*

4.7.2 Hepatic differentiation of DPSCs organoids

- ***Morphological Analysis and Functional Parameters of Hepatic Spheroids***

The DPSCs were cultured in a specific medium aimed at inducing hepatic differentiation. At time point 0 (T0), a monolayer of cells was observed, while after 6 days of culture (T6), cells began detaching from the culture plate edges and curling upon themselves, forming a thick layer of cells *(Figure 29A)*. Cell growth and self-curling continued (T12), culminating in the spontaneous formation of a spheroid after 20 days of culture (T20). A hepatic matrix was added to the spheroid and left for 15 days, and then hepato-specific functional analyses were conducted *(Figure 29B)*.

Phase II activity was evaluated by resorufin conjugation. As shown in the histogram, the spheroid cultured in differentiation medium for 20 days followed by 15 days with hepatic

matrix exhibited a resorufin conjugation rate of 0.5 pmol/min/million cells. However, after a 3-day treatment with the inducer RIF, this activity increased significantly. These data were comparable to those observed in DPSCs cultured in a traditional 2D manner, indicating that the spontaneous assembly of cells into 3D structures did not enhance the development of a hepatic phenotype.

Evaluation of cytochrome activities in the spheroids revealed values around 25 LCU/min/million cells following treatment with DMSO for CYP 1A1 and around 100 LCU/min/million cells after treatment with PB for CYP 3A7. These values were markedly lower compared to the activities observed in freshly isolated hepatocytes.

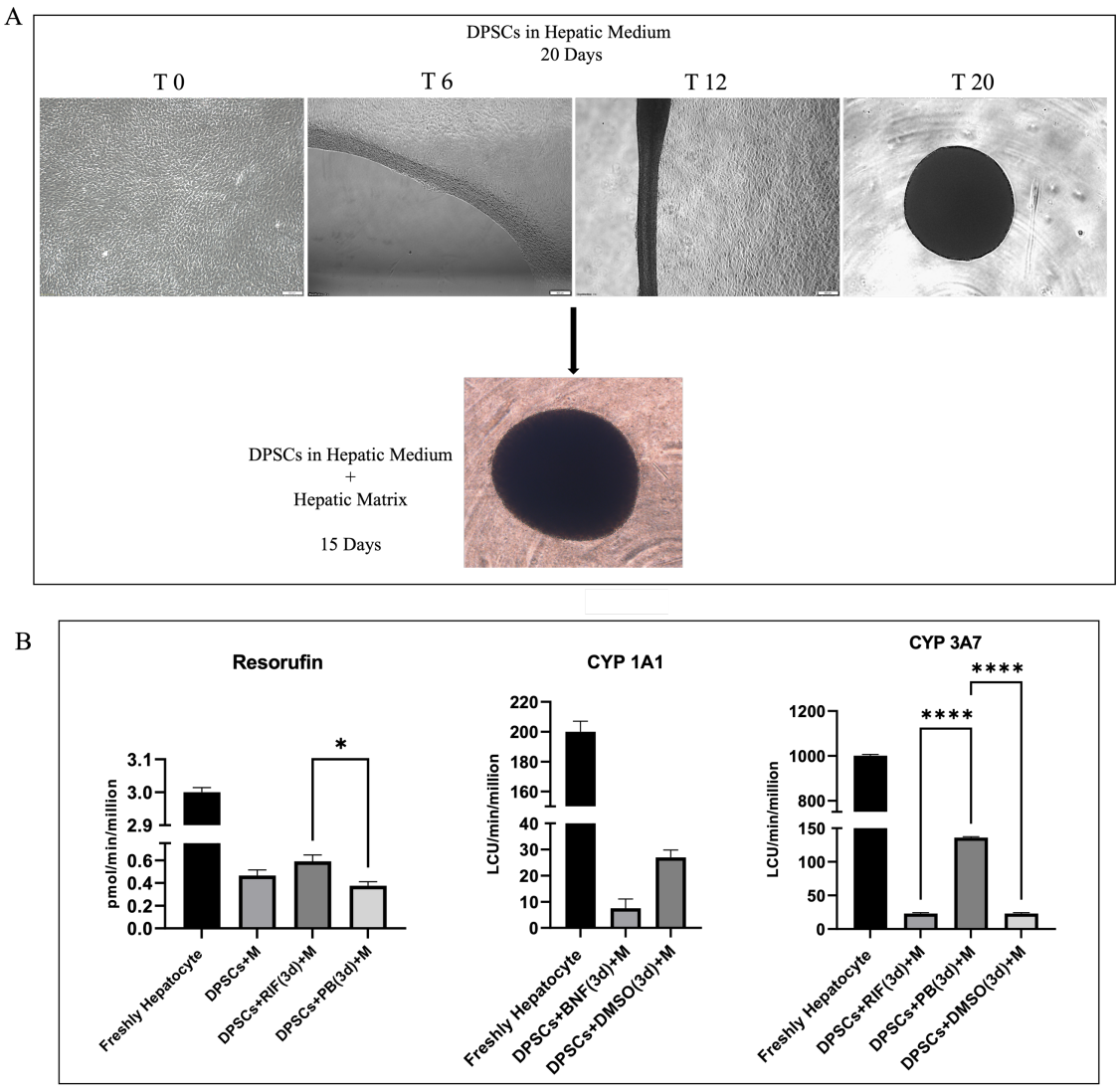


Figure 29. *Morphological analysis and functional parameters of hepatic spheroids.* ***A*** *Representative image of DPSCs cultured with hepatic differentiation medium at various times (T0, T6, T12, T20); 4X magnification.* ***B*** *Hepato-specific functional tests.*

4.7.3 Neuronal differentiation of DPSCs organoids

- ***Analysis of neurogenic differentiation-induced organoid growth over time using time-lapse imaging***

As evidenced in previous data, our laboratory has established a protocol for producing organoids from DPSCs. After forming 3D structures, these organoids were cultured in a standard medium and monitored for growth for up to T10 with the helped the Incucyte® system later, to assess their ability to differentiates a neuronal phenotype, the standard growth medium was replaced with specific differentiation media. The samples were then divided into two groups: one group continued to be cultured in DMEM Low Glucose (CTR), while the other group underwent neuronal differentiation.

Figure 30 clearly shows a noticeable difference in cell density between the control group and the group subjected to neuronal differentiation. Specifically, the organoids undergoing neuronal differentiation seemed to have a slower growth rate in cell density compared to the control group. Notably, the neurogenic differentiation-induced organoids displayed reduced density and compactness in comparison to the controls.

In addition, the data were comparable with those obtained for osteo-odontoblastic differentiation.

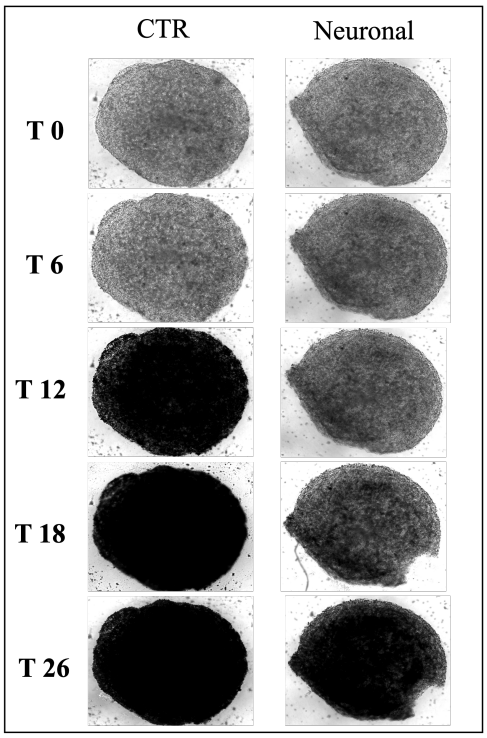


Figure 30. *Analysis of neurogenic differentiation-induced organoid growth over time. Time-lapse image acquired with the Incucyte® system from T0 to T26. 4X magnification.*

4.7.4 Peri-vascular differentiation of DPSCs organoids

- ***Analysis of perivascular induced-organoids growth over time using time-lapse imaging***

The development of 3D structures from Total DPSCs, DPSCs NG2-, and DPSCs NG2+ was monitored in time-laps using the Incucyte® system, allowing for the observation and assessment of changes in growth and cell density *(Figure 31)*. Overall, Total DPSCs exhibited an increase in both growth and cell density. On the other hand, DPSCs NG2- organoids, displayed cellular growth, with small aggregates forming, but they failed to merge into a single compact structure. In DPSCs NG2+ organoids, showed cellular aggregation from the beginning. These organoids appeared more compact compared to DPSCs NG2- organoids, however their size decreased progressively over time.

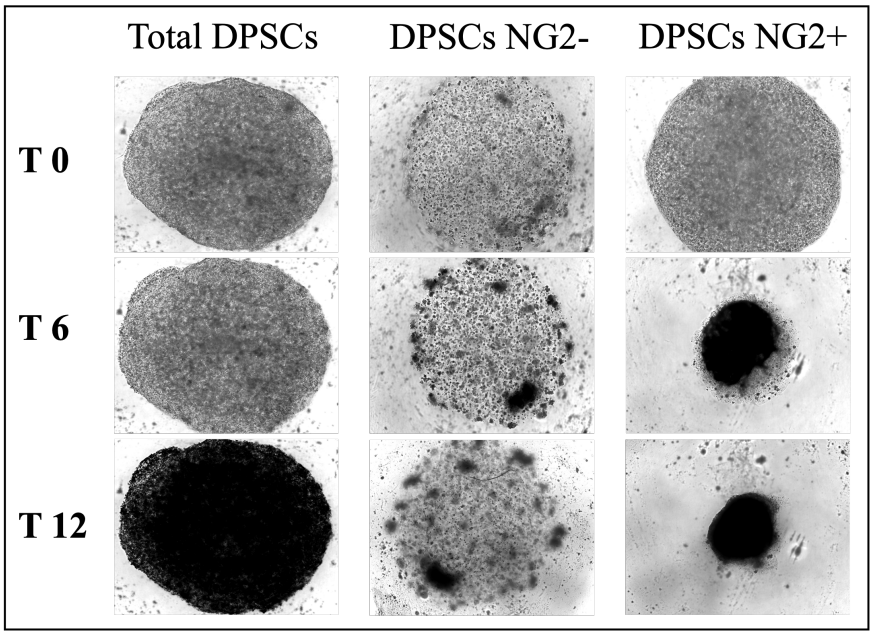


Figure 31. *Analysis of perivascular induced-organoids growth over time. Time-lapse image acquired with the Incucyte® system from T0 to T12. 4X magnification.*

5. DISCUSSION

Dental pulp derived MSCs were first identified by Gronthos et al. in 2000, highlighting the "potency" of these cells to self-renew and differentiate into various cell types; they can respond to specific environmental stimuli and generate new stem cells or activate differentiation mechanisms [Gronthos et al., 2002].

Dental pulp stem/stromal cells (DPSCs) stand out as excellent candidates for regenerative medicine. Indeed, thanks to the combination of all their characteristics, accessibility, versatility, and immunological compatibility, they make a promising choice for regenerative therapies in the field of medicine. Considering these informations, the focus of this thesis was to investigate the distinguishing capability of dental pulp stem/stromal cells. The primary objective was to assess how DPSCs could develop into osteo-odontoblastic, hepatic, neuronal, and peri-vascular phenotypes under the influence of certain conditioning factors. The aim was to utilize these differentiated cells for further research in the realm of regenerative medicine.

Indeed, the use of 3D models has been progressively increasing in recent years for potential applications as human organ-like engineered systems, although to date, there are still no well-standardized and consistently reproducible 3D models available.

In detail, dental pulp cells were isolated from young donors aged 13-19 years. These cells were then analyzed to confirm their mesenchymal properties showing a high expression of markers CD90, CD73, and CD105 (90%), and a low expression of CD19 and CD45 (less than 0.5%). Additionally, the health of the cells was evaluated by measuring intracellular ATP levels using a reliable commercial kit, which indicated strong cell viability. This study provides valuable insights into the characteristics and health of dental pulp cells in young donors. In particular, DPSCs exhibited excellent ATP production capacity within 2 days of culture (approximately 100 LCU/min/ngDNA), which remained consistent even after 16 days (approximately 100 LCU/min/ngDNA).

Currently, were focusing on studying the regenerative potential of these cells by exploring how they can transform when exposed to particular culture environments [Uccelli et al., 2008]. Numerous studies have shown that dental pulp stem cells have the ability to differentiate into odontoblasts and osteoblasts [Zhou et al., 2023]. For example, Baldión et al., demonstrated that DPSCs maintained in osteo-odontoblastic differentiating conditions exhibited time-dependent changes not only in growth but also in morphology

[Baldión et al., 2018]. Moreover, it is widely recognized that fully developed osteoblasts play a crucial role in laying down the matrix and subsequently mineralizing it [Capulli et al., 2014]. The mineralization of the matrix is a critical indicator of osteoblast differentiation in bone research and is commonly evaluated through the presence of calcium phosphate, specifically hydroxyapatite, in osteoblastic cell cultures using staining techniques like von Kossa and Alizarin Red [Moester et al., 2014; Puchtler et al., 1969]. In this study by staining with Alizarin Red, the presence of calcium phosphate deposits in the extracellular matrix were confirmed. There was a noticeable increase in these deposits at 7 and 14 days, followed by a decrease at 21 days. Despite this decrease, the cells maintained their distinct morphology, characterized by larger size and similarity to previously observed differentiated cells. These findings can be attributed to the inherent ability of odontoblasts to release matrix containing dentin and calcium phosphates [Huo et al., 2021]. Moreover, the differentiation of DPSCs into an osteo-odontogenic phenotype was further confirmed by investigating specific markers for osteoblastic differentiation, such as OSX and RUNX2, through immunofluorescence analysis. These markers are known to be highly expressed in DPSCs undergoing differentiation towards an osteo-odontoblastic phenotype [Zavatti et al., 2013]. Simultaneously, the levels of stemness markers CD44 and CD105, which are typically associated with DPSCs, decreased gradually over time. This reduction in stemness marker expression supports the idea that DPSCs are undergoing a transformation towards an osteo-odontogenic phenotype. Consistent with previous research findings, there was a notable increase in the expression of specific genes associated with the osteo-odontoblastic lineage, such as OSX, ALP, and RUNX2, in the samples that were induced for differentiation at time points T7, T14, and T21 compared to the control samples that were kept in culture for the same duration. Meanwhile, a consistent expression of the stemness gene OCT-4 was observed. This suggests the successful generation of osteo-odontoblastic cells from DPSCs, possibly due to the inherent potential of these mesenchymal/stromal cells for this specific differentiation, as indicated by their origin [Son et al., 2021].

In addition, after 21 days of culture in a differentiation medium, were noticed the emergence of aggregate cellular structures resembling spheroids, which tended to detach from the culture plate. This phenomenon is likely attributed to the presence of dexamethasone in the differentiation medium, as demonstrated by Mohammadi et al [Mohammadi et al., 2022]. Up until now, three-dimensional cell cultures have been enhancing the study and treatment of numerous neoplastic and degenerative pathologies.

Through this research, by a stationary method, were have reconstructed organoid structures using dental pulp stem cells. The findings demonstrate the potential of DPSCs to form complex three-dimensional constructs and direct these structures toward specific types of differentiation. In particular, the organoids were induced to differentiate into osteo-odontogenic cells, resulting in a slower growth rate and cell density. This decrease in proliferative activity is likely attributed to the differentiation process. The organoids undergoing differentiation were carefully analyzed in comparison to those kept in DMEM Low Glucose using the Axio Zoom V6 stereomicroscope. This advanced microscope allowed for the capture of multiple focal planes and motorized image reconstruction, resulting in clearer images. The examination revealed that even in organoids cultured in an osteo-odontogenic differentiation medium, mineralization was clearly present.

Furthermore, DPSCs possess the ability to differentiate into a multitude of cell types as a result of their origin from cranial neural crest cells during tooth development. Studies have shown that DPSCs maintain the characteristics of neural crest cells, such as specific molecules and markers, in laboratory cell cultures [Janebodin et al., 2011; Yang et al., 2017; Li et al., 2019]. These cells have the potential to differentiate into neurons and other neural crest-derived tissues. Additionally, DPSCs express both stem cell markers and neural markers, indicating their potential for neuronal differentiation. They also produce neurotrophic factors that are known to promote neurogenesis and cell survival and can be influenced by their environment to differentiate into specific neuronal cells of the nervous system [Martellucci et al., 2019; Arthur et al., 2008]. Among these conditions, oxygen levels are a critical factor influencing DPSCs differentiation. Oxygen concentration profoundly affects cellular traits and tissue remodeling processes, thus exerting a potential influence on cell fate determination [Delle Monache et al., 2016; Werle et al., 2016]. According to literature this research has shown that when dental pulp stem cells are exposed to low oxygen levels in a laboratory setting, they tend to develop into cells resembling neurons. It is intriguing to note that this transformation of DPSCs under hypoxic conditions is probably triggered by a self-regulating signaling mechanism.

The findings indicate that DPSCs undergo morphological changes when they are exposed to hypoxia. Furthermore, after 16 days of treatment these cells show a decrease in growth potential. This aligns with similar research that has demonstrated that exposing MSCs to 1% O_2 oxygen leads to reduced proliferation rates, with a cell cycle arrest occurring in the G0/G1 phase [Kumar et al., 2016].

Following exposure to 1% hypoxia, DPSCs at both time points demonstrated a shift towards a neuronal phenotype. This shift was particularly pronounced after 16 days, with a noticeable decrease in the expression of mesenchymal stem cell markers CD44, CD90, CD105, and CD73 - typically associated with DPSCs - while neuronal markers nestin, β3-tubulin, NFH, and GAP 43 showed an increase.

The commitment to a neuronal phenotype was further confirmed by analyzing the mRNA expression profile of DPSCs exposed (5H, 16H) or not (5N, 16N) to hypoxia. After 5 days, most early (nestin, GFAP, bFGF, and EFG) and late (NGF, BDNF, and GDNF) neural differentiation markers showed upregulation. Interestingly, bFGF was only upregulated after 16 days of exposure. The significant increase in nestin, GFAP, GDNF, and BDNF mRNA expression levels in DPSCs treated for 5 days confirmed their potential to be guided towards a neurogenic phenotype. This may be attributed to their origin from the neural crest, as suggested by Xiao et al. [Xiao et al., 2017]. The overexpression of EGF and bFGF mRNA levels was supported through the examination of these growth factors' secretion in conditioned media from DPSCs exposed or unexposed to hypoxia. Notably, the conditioned media from DPSCs exposed to 5H exhibited a notable increase in EGF expression. This increase was even more pronounced in the conditioned media from DPSCs treated for 16H, possibly due to the higher levels of mRNA seen after 5 days of hypoxia exposure. Moreover, there was a significant secretion of bFGF in the conditioned media from DPSCs treated for 16H, consisting with its mRNA expression pattern. Based on existing literature, the hypothesis of guiding DPSCs towards a neuronal phenotype through signals released by growth factors was further explored also in this study [Vaseenon et al., 2020; Zheng et al., 2021]. Conditioned media derived from hypoxia preconditioned DPSCs were utilized to induce differentiation in SH-SY5Y neuroblastoma cells. Immunofluorescence analysis, along with flow cytometry analysis, unveiled a pronounced expression of neuronal markers NFH and β3-tubulin in SH-SY5Y cells treated for 10 days with 16H conditioned media, reaching respective levels of 96.1% and 92.8%. These findings are consistent with studies showing that SH-SY5Y cells become more similar to primary neurons following treatment with differentiation-inducing agents [Zhang et al., 2015; Kovalevich et al., 2013]. Similarly, DPSCs treated with hypoxia-conditioned media (16H CM) experienced a significant alteration in their expression profile, shifting from a stem cell phenotype to a neural one. Interestingly, those DPSCs exposed to hypoxia-conditioned media displayed elevated levels of neuronal marker expression compared to those subjected directly to hypoxia for 16 days. This

finding indicates that DPSCs have the capacity to generate neuron-like cells primarily through the secretion of growth factors. In the end organoids generated from DPSCs were also treated with a neural differentiation medium. Similarly, to the osteo-odontogenic organoids, a slower growth pattern was observed in the 3D structures when subjected to differentiation, as evidenced by time-lapse image analysis.

As already described DPSCs can differentiate in osteoblasts, odontoblasts, and also neuron-like cells. Moreover, it has been reported that they can differentiate into the vascular endothelial lineages [Liang et al., 2021]. In particular, recent findings suggest that DPSCs can differentiate into peri-vascular cells, which play a vital role in stabilizing tubules in vitro. This process primarily occurs through a direct mechanism, as DPSCs acquire a pericyte-like phenotype. Indeed, particular attention was given to the pericyte marker NG2.

It is known that blood vessels are composed not only of endothelial cells but also of non-vascular cells derived from progenitor cells, including hematopoietic cells [Oberlin et al., 2002] and myogenic cells [Zheng et al., 2007]. Among these, pericytes, which are cells associated with endothelium, represent a key cellular population surrounding endothelial cells in capillaries and micro vessels [Hashitani et al., 2019]. Pericytes play crucial roles in various processes, including blood-brain barrier function, vascular function/stability, angiogenesis, endothelial cell proliferation/differentiation, wound healing, and maintenance of hematopoietic stem cells. Moreover, pericytes can be isolated from fetal and adult tissues and exhibit multilineage differentiation capacity like MSCs. These versatile properties make pericytes the favored cells in tissue engineering [Çelebi-Saltik et al., 2018].

Through this research, was shown that three different subpopulations of DPSCs - specifically NG2+, NG2-, and Total DPSCs - were able to successfully support the formation of tubes by Human Umbilical Vein Endothelial Cells (HUVECs) for up to 14 days post-seeding. This is in contrast to cultures containing only HUVECs, which began to break down and degenerate within 24-48 hours. Quantitative analysis of tubule retention demonstrated that DPSCs NG2+ subpopulation exhibited the highest retention rates, with 80% and 71% retention at 7 and 14 days respectively. On the other hand, DPSCs NG2- exhibited a notable decline in tubule retention over time, with only 15% retention at 14 days. These findings supported the theory that DPSCs share intrinsic traits with pericytes, and these traits are specifically influenced by the expression of the NG2 marker. To determine if DPSCs primarily contribute to tube stabilization through direct

interactions or if their impact is also mediated by paracrine signaling, were evaluated and compared the effects of conditioned media derived from the three DPSCs subpopulations on the proliferation and migration of HUVECs. Results showed that neither the CM from DPSCs NG2+ nor the CM from DPSCs NG2- significantly promoted the proliferation of HUVECs compared to CM from Total DPSCs.

Surprisingly, when exposed to conditioned media from Total DPSCs, HUVECs showed faster migration ability compared to those stimulated by conditioned media from DPSCs NG2+ and NG2-. This observation is consistent with existing literature by Hilkens et al., which demonstrated that DPSCs play a role to promote endothelial migration [Hilkens et al., 2014]. Furthermore, these results suggested that the role of DPSCs NG2+ may involve paracrine signaling. To confirm this hypothesis, the concentration of VEGF in the conditioned media of the same cell lines was evaluated. As expected, VEGF was found to be less abundant in the conditioned media derived from both DPSCs NG2+ and DPSCs NG2- compared to Total DPSCs [Dissanayaka et al., 2015]. The results showed that DPSCs NG2+ displayed a remarkable migratory capability compared to other subpopulations. Specifically, within 24 hours, DPSCs NG2+ covered 31% of the wound area, while the Total DPSCs only covered 13%. DPSCs NG2- also showed significant migratory potential, covering 27% of the wound area at the same time point. Additionally, vital staining of DPSCs allowed researchers to observe their interaction with HUVECs, confirming their ability to bind to endothelial cells and stabilize tubules effectively. Utilizing three subpopulations of DPSCs (Total, NG2-, NG2+), organoids were created. In the case of Total DPSCs, perfect spheroid formation was observed, whereas similar results were not observed with NG2- and NG2+ cells. Instead, with NG2- cells, only small aggregates were formed by T12, while with NG2+ cells, the spheres decreased in size over time (T12). These events described may be linked to the differentiation process towards perivascular cells, highlighted by the selection of a distinct cellular population.

In recent years, researchers have been exploring the use of MSCs in liver regeneration [Ishkitiev et al., 2012]. The liver is a vital organ responsible for detoxification, metabolism, and nutrient storage. Liver diseases, such as cirrhosis and hepatitis, can lead to liver failure and the need for transplantation. Research has shown that DPSCs, when grown in a specialized environment to encourage liver cell development, can transform into cells resembling hepatocytes [Hara et al., 2020]. These cells are capable of blending into liver tissue and aiding in regeneration [Kumar et al., 2017]. While the exact process is still unclear, it is believed that DPSCs release growth factors and cytokines that support

tissue healing and regulate the immune system, ultimately shielding the liver from additional harm [Kumar et al., 2022; Kumar et al., 2017]. Liver regeneration is a normal occurrence after liver injury, but in some cases, this ability may be hindered or restricted. Liver regeneration is a natural process that occurs following liver damage; however, in certain conditions, this regenerative capacity may be compromised or inhibited. Consequently, alternative strategies need to be developed, with cell-based approaches emerging as promising avenues for the treatment of congenital and chronic liver diseases. The administration of donor cells has been explored to support liver regeneration by directly replacing functional cells, such as mature allogeneic hepatocytes or hepatocyte-like cells derived from pluripotent or multipotent cells. The current study revealed that treating DPSCs with a specialized differentiation protocol, even in the presence of a hepatic matrix, failed to elicit functional activity to consider their potential application in regenerative medicine. Specifically, when compared to fresh hepatocytes, their phase I and II activity was notably lower. Despite treatment with specific inducers (RIF, PB, BNF, DMSO), only in some conditions was there an increase in activity observed (RIF 6d for resorufin, and PB 6d for cytochrome 3A7 activity), yet it remained insufficient. From the results obtained, it is evident that DPSCs do not differentiate into functional hepatocytes. However, their paracrine capacity has not been investigated yet, nor has the impact of conditioned medium on tissue regeneration or to induce differentiation of stem cells or hepatic precursors. Interestingly, it was observed that when DPSCs were cultured in a hepatic differentiation medium, after 6 days of culture, the cells spontaneously detached from the plate, and rolled up upon themselves. By day 20, a spheroid formation was evident. In this case, as previously explained, the effect was likely due to dexamethasone, which alters cellular adhesion [Mohammadi et al., 2022]. Performing hepato-specific functional analysis revealed low activity of conjugated resorufin and cytochromes 1A1 and 3A7, similar to the 2D culture case. Therefore, in these spontaneously formed spheroids, there hadn't been sufficient differentiation to warrant consideration for further analysis.

6. CONCLUSION

In conclusion, this study demonstrates the ability of dental pulp stem/stromal cells to differentiate into osteo-odontoblastic and neuronal phenotypes when exposed to specific factors, such as appropriate medium or hypoxia (1% O_2). This capacity holds whether the cells are cultured in 2D or 3D environments. Furthermore, DPSCs exhibit pericyte-like behavior, contributing to vessel stabilization *in vitro*. Moreover, these treatments have been shown to prompt DPSCs to release multiple growth factors, which could promote differentiation, immunomodulatory properties, and promote tissue regeneration. These findings underscore the potential of DPSCs as promising candidates for stem cell therapy in future therapeutic approaches, particularly in treating neurological diseases, as well as aiding in bone and tissue regeneration. Additionally, the study suggests that not only differentiated DPSCs but also conditioned medium derived from DPSCs may hold clinical utility. This conditioned medium could be administered *in vivo*, yielding therapeutic effects in diverse disease models. Importantly, implementing such a strategy could prove advantageous due to the cell-free nature of the product, making it more manageable as a medicinal product.

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