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PhD Thesis

Age-related regenerative potential of human Dental Pulp Stem/Stromal Cells (hDPSCs): possible consequences in an ageing society

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1. INTRODUCTION

Stem cells are certain biological cells found in all multicellular organisms, with the capability to self-renew and differentiate to various types of cells, thus to construct tissues and organs (Hui et al. 2011). Different types of stem cells vary in their degree of plasticity or developmental versatility and they can be classified according to their plasticity and sources (Tab.1).

Classification	***Characteristics***	
Sources/types	Embryonic stem cells (ESCs)	Pluripotent stem cells derived from the inner cell mass of the blastocyst, an early-stage embryo.
	Adult stem cells	Endodermal origin: Pulmonary Epithelial SCs, Gastrointestinal Tract SCs, Pancreatic SCs, Hepatic Oval Cells, Mammary and Prostatic Gland SCs, Ovarian and Testicular SCs.
		Mesodermal origin: Hematopoietic SCs, Mesenchymal Stromal SCs, Mesenchymal SCs, Mesenchymal Precursor SCs; Multipotent Adult Progenitor Cells, Bone Marrow SCs, Foetal Somatic SCs, Unrestricted Somatic Cardiac SCs, Satellite cells of muscle.
		Ectodermal origin: Neural SCs, Skin SCs, Ocular SCs.
	Cancer stem cells	They have been identified in almost all cancer/tumour.
	Induced pluripotent stem cells (iPSCs)	Type of pluripotent stem cell artificially derived from a non-pluripotent cell, typically an adult somatic cell, by inducing a "forced" expression of specific genes.
Cell potency	Totipotent cells	Zygote, Spore, Morula. Potential to give rise to any and all human cells, such as brain, liver, blood, heart cells. They can even generate from an entire functional organism.
	Pluripotent cells	Embryonic stem cells, Callus. They can give rise to all tissue types, but cannot form an entire organism.
	Multipotent cells	Progenitor cell, such as hematopoietic stem cell and mesenchymal stem/stromal cell. They give rise to a limited range of cells within a tissue type.
	Unipotent cells	Precursor cells.

Table 1. Classification of stem cells (SCs).

Stem cell biology has become an important field for the understanding of tissue regeneration and the implementation of regenerative medicine strategies. Since the discovery and characterization of multipotent mesenchymal stem/stromal cells (MSCs) from bone marrow, MSC-like populations from other tissues have now been characterized based on the "gold standard" criteria established for BM-MSCs (Friedenstein, Gorskaja, and Kulagina 1976).
Nowadays, research is focused on adult stem cells because they are easily accessible and free from ethical issues (Moore, Mills, and Thornton 2006). Adult stem cells are a group of stem cells taken from mature tissue. Because of their stage of development, these cells have limited potential compared to those derived from embryos and foetuses. Most adult stem cells are lineage-restricted (multipotent) and are generally referred to by their tissue origin (mesenchymal stem cell, adipose-derived stem cell, endothelial stem cell, dental pulp stem cell, etc.) (Gronthos et al. 2000; Wagner et al. 2005; Yoder 2012).

1.1 Mesenchymal stem/stromal cells (MSCs)

In the last decade, MSCs have generated considerable excitement for their multiple properties, including self-renewal, colony formation, phenotypic expression pattern and differentiation potential (Pittenger et al. 1999). It has also been shown that the MSCs are involved in several physiological and pathological processes, including tissue homeostasis, aging, tissue damage and inflammatory diseases (Uccelli, Moretta, and Pistoia 2008; Shi et al. 2010).
Friedenstein first described MSCs in the bone marrow as fibroblast-like colony forming units in late 1960 (Friedenstein, Gorskaja, and Kulagina 1976). Later, these plastic-adhering, extensively replicating clonogenic somatic cells were found to have a multipotent character both under *in vitro* and *in vivo* settings (Gronthos et al. 2000; Spath et al. 2010).
To date, MSCs are not unique feature of the bone marrow, as they also have been found in a wide range of adult tissues such as lipo-aspirated adipose, dental pulp, periosteum, tendons, synovial membrane and skeletal muscle (T. A. Mitsiadis et al. 2011; Hatakeyama et al. 2017; Minteer, Marra, and Rubin 2013; J. C. Chen and Goldhamer 2003; Ferretti et al. 2015). They can be isolated from the umbilical cord blood, the amniotic fluid, and from placental tissues as well (Wagner et al. 2005; Malek and Bersinger 2011). In 2006, the International Society for Cellular Therapy (ISCT) described in a position article the minimal criteria for defining MSCs,

to diminish the ambiguity in their identification. These criteria are based on adherence to plastic in standard culture, expression of a specific surface antigen set (must express CD 105, CD 73 and CD 90 and must lack expression of CD 45, CD34, CD 14, CD19 and HLA-DR), and the trilineage mesoderm (osteogenic, chondrogenic, and adipogenic) differentiation capability *in vitro* (Dominici et al. 2006) (Fig.1).

It is well known that MSCs also have the ability to differentiate into non-mesodermal tissues (Jeon et al. 2015). Besides, the exact lineage and the set of surface antigen markers defining MSCs are not in concurrence, a feature largely depends on tissue source, as well as to donor age and gender, passage number and seeding density, etc. (S. Huang et al. 2011). However, these standard parameters represent a beneficial guide to investigators evaluating the extent of plasticity of the heterogeneous fibroblast-like cell population.

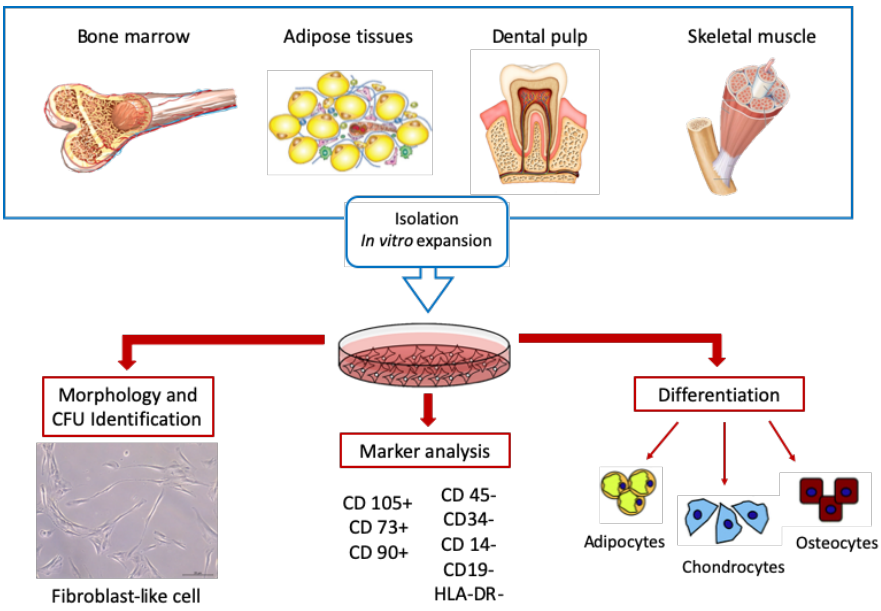

Figure 1. MSCs isolated from various adult tissues. After *in vitro* expansion, MSCs can be authenticated as per the guidelines laid down by International Society of Cell Therapy (ISCT).

Currently, investigations on the definition of MSCs gave an overview of the recent advances concerning their profiling. In particular, gene expression pattern has fundamentally enhanced the understanding and the approach to characterizing MSCs identifying potential biomarkers, or determining key molecules regulating biological processes (S. Huang et al. 2011). MicroRNAs (miRNAs) are likely to be involved in gene regulation during the early stages of differentiation, which may play an important role in determining cell fate (Tab. 2). Although the detailed role of each miRNA in stem cell biology remains to be defined, understanding how miRNAs affect stem cell behaviour will advance MSC definition (Collino et al. 2011).

Functionality	***MicroRNAs***	***Publications***
Neural differentiation	miR-9, miR-10, miR-30	(Kulcenty et al. 2019)
Adipogenic differentiation	miR-30 family, -642a-3p miR-143, -24, -31, -30c miR-21	(Zaragosi et al. 2011) (Kang and Hata 2015) (Kim et al. 2012)
Osteogenic differentiation	miR-27a miR-21 miR-217 miR-26a miR-148a	(Gu et al. 2016) (Yang et al. 2019) (Dai et al. 2019) (Z. Liu et al. 2018) (H. Liu et al. 2018)
Chondrogenic differentiation	miR-410 miR-146b miR-214 miR-30a	(Y. Zhang, Huang, and Yuan 2017) (Budd et al. 2017) (Roberto et al. 2018) (Tian et al. 2016)

Table 2. MicroRNAs reported to be involved in the regulation of MSC fates.

1.1.1 MSCs in regenerative medicine

MSCs have emerged as an attractive candidate for cell-based therapy of various conditions, such as cardiovascular diseases, diabetic nephropathy, and brain injuries including stroke (Faiella and Atoui 2016; Y. Liu and Tang 2016; Hsuan et al. 2016). Among various stem cell types, MSCs afford several advantages for clinical use, such as availability and ease of harvesting; multilinear differentiation potential; immunosuppressive effects; safety, without any risk of malignant transformation after infusion of allogeneic cells, which is common in the case of ESCs and iPSCs; and the lack of ethical issues that occur with the application of human ESCs.

Regarding cellular therapy clinical research, it is very important to understand their immunomodulatory capabilities. Typical *in vitro* modulatory functions of MSCs are the inhibition of T and B cell proliferation, as well as dendritic cell differentiation (Ren, Roberts, and Shi 2011). MSCs also regulate immune responses by secreting several factors including Interleukin (IL) -10, indoleamine 2, 3-dioxygenase (IDO), Vascular Endothelial Growth Factor (VEGF), Chemokine (C-C motif) ligand (CCL)-5, prostaglandin E2 (PGE2), and nitric oxide (NO) (Uccelli, Moretta, and Pistoia 2008; Samsonraj et al. 2017) (Fig. 2). MSC-to-T cell contact induces IL-10 secretion, which attenuates T cell proliferation, and stimulates leukocyte antigen-G molecules (HLA-G5) secretion, which in turn inhibits activated T cells and NK-cell cytotoxicity (Selmani et al. 2008; Zhao et al. 2008; Nasef et al. 2008). *In vivo*, systemic administration of MSCs facilitates immunosuppression in graft-versus-host-disease (GVHD) models (Ren et al. 2008), multiple sclerosis, diabetes (Lee et al. 2006) as well as cardiomyopathies (Anzalone et al. 2010; Corrao et al. 2013). Following the successful outcomes from animal models, clinical trials for Crohn's disease, acute GVHD, and severe osteogenesis imperfecta by allogenic biomaterials have been conducted. Trials are ongoing for acute myocardial infarction, aplastic anaemia, osteoarthritis, diabetes, and other conditions (Squillaro, Peluso, and Galderisi 2016). MSC therapy appears to be promising for treating immunological disorders, therefore the punctual characterization of MSC immunosuppressive functions will provide an important indicator for *in vivo* efficacy of MSCs. It is also crucial to note that MSCs from different sources may differ in their mechanisms and capacities for immunomodulation (Mattar and Bieback 2015).

Because of their trophic and immunomodulatory functions, MSCs are generally considered to possess greater advantages in cell-based regenerative medicine. However, these cells can either support or suppress tumorigenesis (Klopp et al. 2007). In contrast to their anti-apoptotic and anti-inflammatory functions, MSCs have been shown to interact with tumour cells via paracrine signalling and possibly increase the risk for metastasis (Costanza et al. 2017). This less desirable effect requires some caution in their use in circumstances of pre-existing tumour conditions.

To date, much of the interest in the therapeutic application of MSCs to various disease settings can be linked to their immunosuppressive and anti-inflammatory properties. One of the key mechanisms of these effects is the secretion of soluble factors with paracrine actions. Recently, it has emerged that the paracrine functions of MSCs could be mediated by extracellular vesicles (EVs). EVs are predominantly released from the endosomal compartment and carry a complex cargo that includes nucleic acids, proteins and lipids from their cells of origin (Rani et al. 2015). MSC-EVs were first studied in 2010 by Lai et al. in a mouse model of myocardial ischemia-perfusion injury and were thereafter investigated in several disease models (Lai et al. 2010). Further animal model-based studies have reported that MSC-EVs have significant potential as a novel alternative cell-free therapy for several pathological conditions, including cardiovascular, kidney, liver and neurological diseases (Jarmalavičiūtė and Pivoriūnas 2016; Z. Zhang et al. 2016; He et al. 2012; Sung, Kim, and Jung 2018).

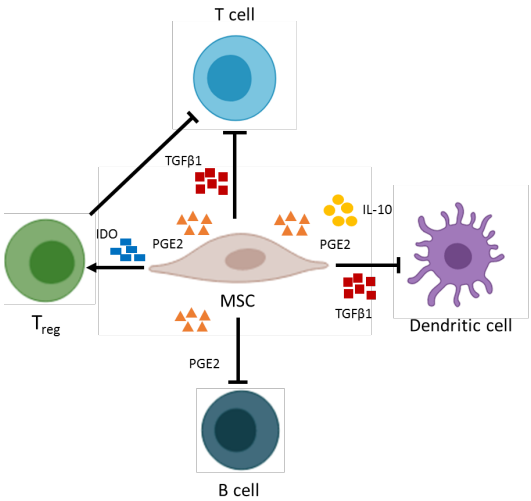

Figure 2. Interactions of MSC with immune cells. The secretion of specific secrete soluble molecules factors suppresses the proliferation and/or activity of a variety of immune cells, including T cells, B cells, Natural Killer cells, and dendritic cells, as well as activated regulatory T cells (T_{reg}). Modified by Samsonraj et al., 2017.

1.1.2 Age-related changes in MSCs

Despite positive outcomes derived from MSC clinical research, their properties and functionalities can be influenced by several intrinsic factors including ageing (Tümpel and Rudolph 2019). Ageing is manifested as an overall decline in organs' functional capacity, which normally maintains tissue homeostasis and physiological reactions (Hennrich et al. 2018). Age-related regressions are often gradual, mild in middle-aged patients, and become preeminent later in life, particularly under stressful conditions that require regenerative responses. The age-associated decrease in tissue cellularity and the consequent inadequate tissue reparative reactions are intimately linked to weak immune responses and impaired wound healing (Franceschi et al. 2018). MSCs extracted from young and elder individuals have been shown to have diverse properties. Upon isolation, MSC numbers obtained by bone marrow aspiration decline with donor age (A. Stolzing et al. 2008). Also, a reduction in colony forming efficiency, proliferative capacity and osteogenic potential has been observed in aged MSCs in comparison with juvenile cells (Zhou et al. 2008).

Senescence constitutes an active mechanism that provides cellular homeostasis by blocking the proliferation of aberrant cells that are under stressful conditions, including aging (Fridlyanskaya, Alekseenko, and Nikolsky 2015; McHugh and Gil 2018). Accumulation of senescent cells in aged tissues may create a favourable environment for the onset and progression of various age-related diseases (Childs et al. 2017; Ermolaeva et al. 2018; Aramillo Irizar et al. 2018).

The response to a gradual loss of genomic, proteomic and metabolic integrity in ageing MSCs is triggered and controlled by two main tumour suppressor pathways: p53-$p21^{WAF1/CIP1}$-retinoblastoma (RB) and $p16^{ink4a}$-RB proteins (Zhou et al. 2008). Furthermore, reactive oxygen species and nitric oxide are thought to be involved in the ageing process, whose levels have been determined to be significantly higher in aged MSCs (A. Stolzing et al. 2008) and the induced oxidative stress can potentially impair normal biological functions of organisms (Alexandra Stolzing, Sethe, and Scutt 2006). The activation of chronic DNA damage response, telomere shortening, and upregulation of lysosomal protein levels are other typical signs of ageing cells (Hernandez-Segura, Nehme, and Demaria 2018). The activity of the specific lysosomal enzyme senescence-associated beta-galactosidase (SA-β-Gal) is widely used as

marker for the augmented lysosomal content of aged MSCs (Choudhery et al. 2014). Accumulating evidence also indicates that ageing is associated with a decrease in autophagy (López-Otín et al. 2013). Autophagy is a highly conserved pathway that removes redundant or defective organelles and protein aggregates. Its decrease in older cells has been associated with several age-dependent pathologies, spanning from neurodegenerative diseases to metabolic disorders (Martinez-Lopez, Athonvarangkul, and Singh 2015).

Aged MSCs significantly affect their microenvironment, as they secrete pro-inflammatory and matrix-degrading molecules, a process known as senescence-associated secretory phenotype (SASP) (Childs et al. 2015). SASP includes a variety of soluble signalling factors (*e.g.* interleukins, chemokines and growth factors), secreted proteases, and insoluble extracellular matrix components. These molecules affect neighbouring cells by activating different cell-surface receptors and the corresponding signal transduction pathways (Coppé et al. 2010).

Increasing evidence suggests a role for exosomes in the establishment of age-dependent tissue alterations (Xu and Tahara 2013). Exosomes are nanovesicles (30-120 nm) secreted by all cell types and found in most of the body fluids. Along with trans-membrane proteins, they contain RNA material (mRNA and miRNA) and cytosolic proteins. Their active release from aged cells could influence the cellular microenvironment and lead to tissue degeneration and age-related diseases (Isola and Chen 2017). MiRNAs are non-coding RNA molecules, composed of about 22 nucleotides, which function in RNA silencing and interference-mediated post-transcriptional gene regulation. They are considered essential to normal cellular physiology and provide regulation of gene expression at the post-transcriptional level (Bartel 2018). Also miRNAs can directly contribute to age-related senescence by deregulating the cell cycle and modulating cytoskeletal dynamics (Maes et al. 2008; Li et al. 2011) (Fig. 3).

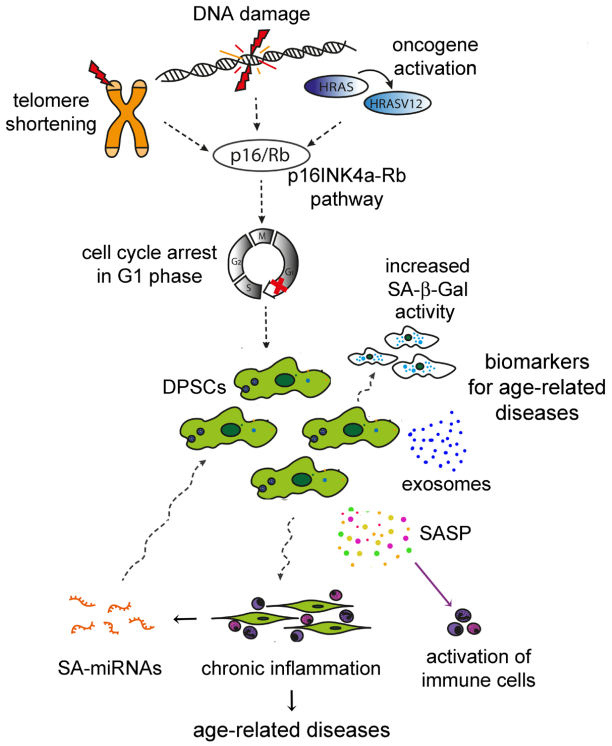

Figure 3. Age-related changes in MSCs. Telomere shortening, DNA damage, and oncogene activation are primary drivers of pathology in ageing. This process activates RB/p16 pathways to block cell cycle and sustain growth arrest, leading to age-related cell senescence. Senescent cells (SNCs) are positive for SA-β-gal, indicating lysosomal content augmentation, and are characterised by specific senescence-associated secretory phenotype (SASP). SASP recruit immune cells to phagocytose and remove SNCs. Impairment of miRNAs may impact the chronic condition of several tissues. MSCs-derived exosomes are involved in ageing processes and may constitute novel therapeutic tools in age-related diseases.

1.2 Tooth morphogenesis

The development of craniofacial tissues is a unique process which occurs stepwise and is part of human prenatal development. During the fourth week, craniofacial development begins and requires many complex biological mechanisms. An important role during this process is played by the neural crest cells, whose migration is crucial for the development of the face and the teeth in craniofacial context. Tooth development progress through a series of morphological stages that necessitate sequential and reciprocal interactions between the oral epithelium and the underlying cranial neural crest-derived mesenchyme (Miletich and Sharpe 2004).

1.2.1 Stages of tooth development

After the sixth embryonic week a band of epithelial tissue arises in the more medial or buccal margin of the developing gingiva of both jaws, which is known as the dental lamina (Som and Miletich 2018). At specific intervals all along the dental lamina, the dental placodes are induced. They include thickened epithelium and underlying neural crest-derived ectomesenchyme; their reciprocal signalling will promote the development of the future tooth. During physiological conditions, dental placodes give rise to ten spherical tooth buds (*bud stage*) that penetrate the ectomesenchyme of both jaws thus forming deciduous teeth (Thesleff 2014). By the eighth week, the ectomesenchyme below the tooth bud starts to condense and the ectodermal tooth bud extends partially around these condensed cells resulting in a cap-shaped dental organ (*cap stage*). Afterword, it is possible to appreciate a clear division in two regions: the dental papilla and enamel organ. The ectomesenchymal tissue is the dental papilla, which will arise to most of the proper tooth, including dentine and dental pulp (Tziafas and Kodonas 2010). Instead, the enamel organ consists of inner and outer enamel epithelium. The first represents the ameloblastic layer, the second along with the stellate reticulum will form the future enamel. Moreover, between both epithelia there is the stratum intermedium, whose flat shaped cells may help orchestrate the progression of odontogenesis. During this stage the tooth acquires a bell shape, and it is referred to the *bell stage* (Balic and Thesleff 2015). At the bell stage, two mesenchymal cell populations can be distinguished: the dental follicle and dental pulp. Pulp cells adjacent to the dental epithelium differentiate into odontoblasts, while epithelial cells juxtaposing the dental pulp differentiate into ameloblasts (Thimios A. Mitsiadis and Graf 2009) (Fig 4).

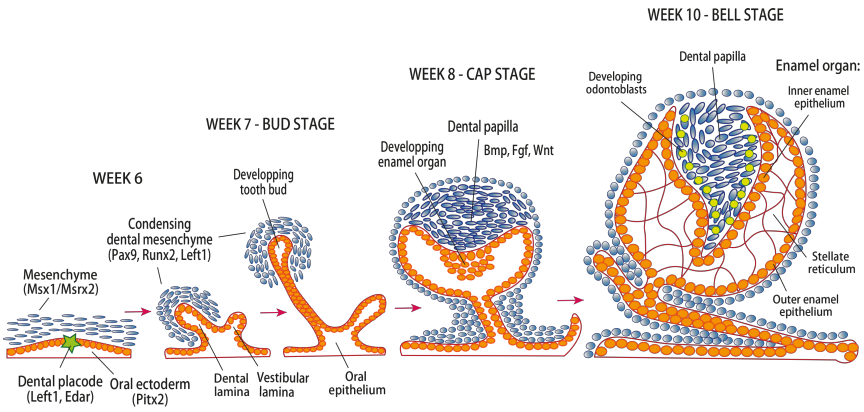

Figure 4. Morphological stages of human tooth development (dental lamina, bud stage, cap stage, bell stage).

1.2.2 Maturation of tooth cells

Odontoblasts are polarized columnar cells with processes formed at their distal part. These processes penetrate the dentine and participate in the secretion of the dentine matrix, consisting of hydroxyapatite and organic matrix. The majority of organic dentine matrix is composed of collagen (90%) and non-collagenous proteins (NCPs), such as dentine matrix protein 1 (DMP1) that represent 10%, and dentine sialophosphoprotein (DSPP). NCPs play a role in transforming predentine to dentine and some of them promote the growth of hydroxyapatite crystals (Fig. 5). Also, several extracellular matrix components, including proteins and lipids, are involved in the control of mineralization (Chaussain et al. 2013). Apatite minerals are deposited on the matrix by odontoblast during their maturation stage, forming the mature calcified dentine. Following initial dentine deposition, the ameloblasts polarize and start to secrete the enamel matrix along the dentine-enamel junction. The major components of enamel matrix proteins are amelogenins, a family of hydrophobic proteins accounting for more than 90% of the total proteins. Amelogenins self-assemble into supramolecular aggregates that form an insoluble extracellular matrix complex and operate to control the ultrastructural organization of the developing enamel crystallites (Lyngstadaas et al. 2009). Other hydrophobic proteins found in the enamel matrix include enamelin, ameloblastin (also called amelin or sheathlin), amelotin, apin and various proteinases as well. Although these proteins are expressed in fewer quantities, recent investigation confirmed their valuable roles in various aspects of periodontal regeneration (Miron et al. 2016).

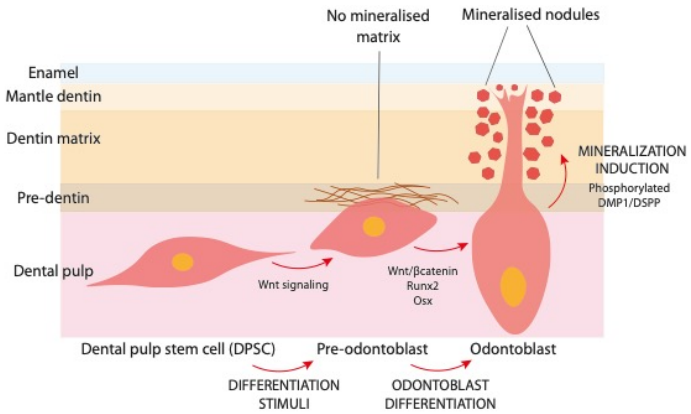

Figure 5. Mineralization process of odontoblasts. Dentineogenesis is initiated by the inductive influence of the enamel organ involving molecular signalling pathways, such as Wnt/ βcatenin, and Runx-2.

1.3 Human dental mesenchymal stem/stromal cells

Dental tissues are specialized tissues that do not undergo continuous remodelling as shown in bone tissue. Therefore, differences have been noticed between the dental stem/stromal cell populations and BM-MSCs, since they appear to be more committed to odontogenic rather than osteogenic development. On the other hand, due to their embryological origin, dental stem/stromal cells possess characteristics akin to those of neural crest cells. These cells display multidifferentiative potential, with the capacity to give rise to several cell lineages, including osteo/odontogenic, adipogenic, chondrogenic and neurogenic (G.T.-J. Huang, Gronthos, and Shi 2009b). Postnatal dental pulp stem cells (DPSCs) were the first human dental MSCs to be identified. Gradually, other dental MSC-like populations, such as stem cells from human exfoliated deciduous teeth (SHED), periodontal ligament stem cells (PDLSCs), dental follicle progenitor cells (DFPCs), and stem cells from apical papilla (SCAP), were also reported (Egusa et al. 2012) (Fig. 6).

1.3.1 Dental Pulp Stem Cells

DPSCs were first isolated by enzymatic digestion from dental pulp tissue and found to express several surface markers, such as CD73, CD90, and CD105, but not CD14, CD34, or CD45 (Gronthos et al. 2000; 2002). These cells showed similarities to BMSCs. In fact, they have a fast population doubling time, possess immunosuppressive properties, and are prone to forming mineralised tissue (Peng, Ye, and Zhou 2009). Interestingly, it has been demonstrated that transplantation of expanded DPSC formed a dentine-pulp complex and transplantation of expanded BMMSCs formed ectopic bone (G. T.-J. Huang, Gronthos, and Shi 2009a; Tatullo et al. 2015). The tissue regeneration capability of BMMSCs and DPSCs was further examined by transplantation using human dentine as a carrier (Batouli et al. 2003). BMMSCs failed to form mineralized tissue on the surface of dentine or pulp-like tissue. On the other hand, DPSCs generated a reparative dentine-like structure in contact with the human dentine, indicating the possibility of using DPSCs in tooth repair.

In addition, DPSCs can differentiate *in vitro* into adipocytes, osteoblasts, chondrocytes, and myocytes, as well as neural cells, as confirmed by evaluating the expression of specific gene markers (d'Aquino et al. 2007; Arthur et al. 2008; Patil et al. 2014).

One important feature of DPSCs is their odontoblastic differentiation potential. Human pulp cells can be induced *in vitro* to differentiate into cells of odontoblastic phenotype, characterized by polarized cell bodies and accumulation of mineralized nodules (Couble et al. 2000). If seeded onto dentine, some DPSCs convert into odontoblast-like cells with a polarized cell body and a cell process extending into the existing dentinal tubules (George T.-J. Huang et al. 2006). In addition, a valid *in vivo* regenerative and immunomodulatory potential have been assessed. In fact, transplanted *ex vivo* expanded DPSCs mixed with hydroxyapatite/tricalcium phosphate (HA/TCP) has been demonstrated to form ectopic pulp-dentine-like tissue complexes in immunocompromised mice (Batouli et al. 2003).

1.3.2 Stem Cells from Human Exfoliated Teeth

The transition from deciduous teeth to adult permanent teeth is a unique and dynamic process in which the development and eruption of permanent teeth are coordinated with resorption of the deciduous teeth roots (Parner et al. 2002). A distinct population of multipotent stem cells can be isolated from the remnant pulp of exfoliated deciduous teeth and expanded *ex vivo*, thereby unexpectedly providing a unique and accessible tissue source of MSCs (Miura et al. 2003). SHED are distinct from DPSCs due to their higher proliferation rate, increased cell population doublings time, and ability to form sphere-like cell cluster. SHED display surface markers that conform to the minimal criteria for MSCs proposed by ISCT, and they also express the embryonic stem cell markers Oct4 and Nanog, the neural stem cell marker Nestin, and the stage-specific embryonic antigens SSEA-3 and SSEA-4 (Miura et al. 2003).

1.3.3 Periodontal Ligament Stem Cells

The periodontal ligament (PDL) is a soft connective tissue embedded between the cementum and the alveolar bone socket. Early evidence showed that PDL not only plays an important role in supporting teeth, but it also contributes to tooth nutrition, homeostasis, and the regeneration of periodontal tissue (Bartold et al. 2000). Explant cultures or enzyme digestion treatment of

the PDL released a population of PDLSCs, postnatal multipotent stem cells that could be readily expanded *in vitro* to generate a cementum/PDL-like complex. Additionally, PDLSCs are highly proliferative cells and express STRO-1 and other MSC surface markers that are also present on DPSCs (Seo et al. 2004). PDLSCs express a higher level of the tendon-specific marker scleraxis than do DPSCs, suggesting that PDLSCs might form a unique population of postnatal MSCs (Wada et al. 2009).

1.3.4 Dental Follicle Stem Cells

The dental follicle is an ectomesenchymal tissue that surrounds the developing tooth germ prior to eruption. This tissue is thought to contain stem cells and lineage committed progenitor cells for cementoblasts, periodontal ligament cells, and osteoblasts. Progenitor cells have typically been isolated from the dental follicle of human third molars (Morsczeck et al. 2005). Like other dental stem cells, DFPCs have an extensive proliferative ability, express similar cell surface antigens, and demonstrate osteogenic differentiation capacity *in vitro* after induction (G.T.-J. Huang, Gronthos, and Shi 2009). Moreover, they differentially express osteocalcin (OCN) and bone sialoprotein (BS) after transplantation in immunocompromised mice (Morsczeck et al. 2005).

1.3.5 Stem Cells from Apical Papilla

The apical papilla is the soft tissue found at the apices of developing permanent teeth. In developing teeth, root formation begins with the apical proliferation of epithelial cells from the cervical loop. The dental papilla contributes to tooth formation and is eventually converted into pulp tissue, and an apical cell-rich zone lies between the apical papilla and the pulp (Sonoyama et al. 2008). A unique population of MSCs referred as SCAPs was discovered in the apical papilla of human immature permanent teeth. SCAPs show a higher proliferation rate and mineralization potential than DPSCs, and they express typical MSC markers, including STRO-1, CD73, CD90, and CD105 (Ding et al. 2010). Similarly to DFPCs, SCAPs represent a population of cells from a developing tissue and might thus exhibit greater plasticity than other dental stem cells (DSCs).

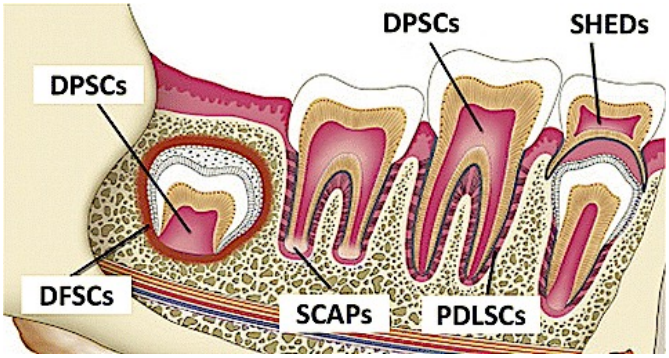

Figure 6. Sources of adult stem cells in the oral region. DPSCs: dental pulp stem cells; SHED: stem cells from human exfoliated deciduous teeth; PDLSCs: periodontal ligament stem cells; DFSCs: dental follicle stem cells; TGPCSs: tooth germ progenitor cells; SCAP: stem cells from the apical papilla. Modified by Egusa et al., 2012.

1.4 DPSCs clinical potential and possible limits

It has long been established that dental pulp possesses remarkable regenerative abilities. Upon tooth injury, odontoblasts, the pulp-derived cells responsible for dentine production, degenerate and are replaced by undifferentiated mesenchymal cells that migrate to the affected site from the deeper regions of the pulp (T.A. Mitsiadis and Rahiotis 2004; T. A. Mitsiadis, Orsini, and Jimenez-Rojo 2015). These cells differentiate into new odontoblast-like cells and produce reactionary/reparative dentine (Mitsiadis and Rahiotis, 2004; Mitsiadis *et al*., 2015; Orsini *et al*., 2018) (Fig. 7).

To date, DPSCs have been used for tissue-engineering studies to assess their potential in pre-clinical applications. Tissue-engineering has been defined as interdisciplinary field that applies the principles of life science and engineering toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ (Langer and Vacanti 1993). In this sense, the creation of a medical device requires accessible sources of cells and biocompatible materials that can also carry signalling molecules. DPSCs represent a specific cell type that may contribute to the regeneration of several tissues due to the peculiar development pathway that gives rise to the dental pulp; therefore, it could be applied in numerous medical fields (Anitua, Troya, and Zalduendo 2018).

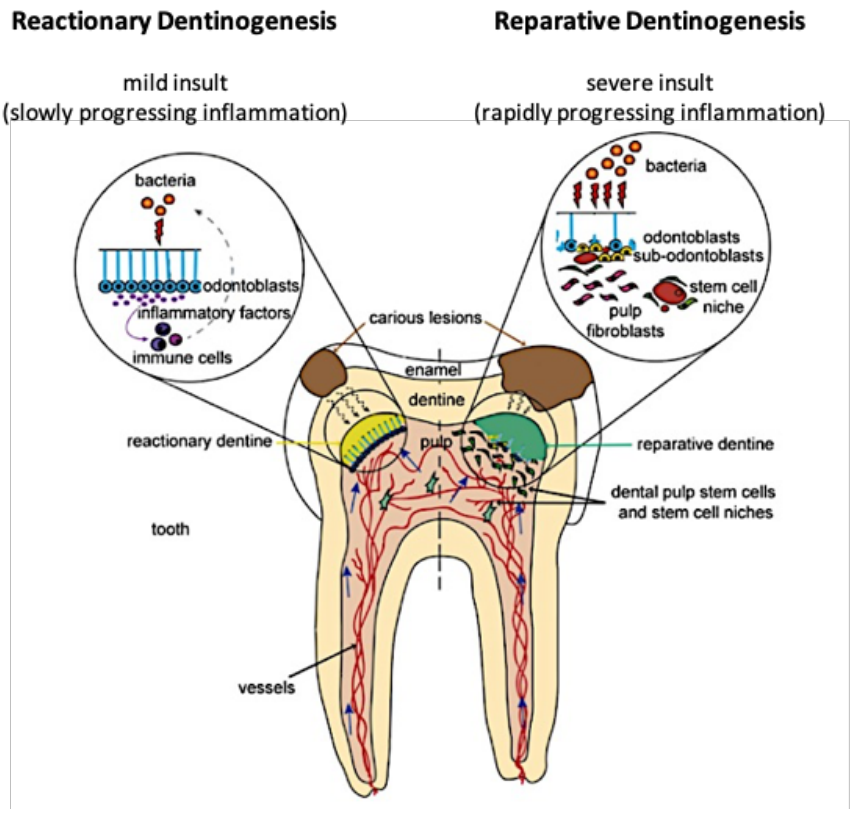

Figure 7. Schematic representation of a tooth indicating the regenerative capacity of the dentine-pulp complex upon a carious lesion. Reactionary dentine is deposited by odontoblasts in response to a mild insult, potentially including proinflammatory mediators. These molecules are in charge to activate the immune response against the pathogens. Reparative dentineogenesis is a more complex mechanism and requires the generation of new odontoblast-like cells from stem/progenitor pulp.

1.4.1 Regeneration of oral and maxillofacial defects

Preservation of dental pulp function is crucial for the teeth homeostasis, thus root canal therapy, that is necessary after the infection of pulp by caries or trauma, could lead to the loss of the dental piece. In early studies, Gronthos *et al*. demonstrated that transplantation of *in vitro* expanded DPSCs formed a dentine-pulp complex (Gronthos et al. 2002). Other results showed the possibility of using DPSCs in tooth repair by *in vivo* transplantation of stem cells or *in vitro* culture on biodegradable scaffolds and subsequent transplantation *in vivo*. In fact, the tissue regeneration capability of DPSCs was investigated using human dentine as a carrier where cells generated a reparative dentine-like structure directly on the surface of human dentine

(Batouli et al. 2003). In addition, the complete *in situ* pulp regeneration with neurogenesis and vasculogenesis in an adult canine model of pulpectomy after autotrasplantation of DPSCs has been demonstrated (Iohara et al. 2011). In case of entire pulp tissue lost, different material platforms have been tested, such as PEGylated fibrin, silica bioactive glass-induced, collagen/chitosan hydrogel, and so on (Galler et al. 2011; Qu and Liu 2013; Ravindran et al. 2013). Several investigations have demonstrated the importance of direct vascular supply to achieve *de novo* pulp regeneration. For example, porcine DPSCs were seeded on organic or synthetic scaffold and implanted as hybrid root implants in the jaw bone of minipigs. Even though newly formed organic matrix of pre-dentine was deposited on the root canal walls, necrotic tissue at the central area of some implant was observed (Kodonas et al. 2012).

Successful reconstruction of bony defect is a difficult task in craniofacial surgery. The gold standard treatment consists of the autogenous bone alone or combined with other autologous biomaterials (Elsalanty and Genecov 2009). However, the ectomesenchymal origin of DPSCs gives them an osteogenic differentiation profile resulting in an emerging alternative option. A fully compact bone with high matrix dentistry was found in regenerated bone tissue after a grafting intervention with DPSCs seeded onto a collagen sponge scaffold (Giuliani et al. 2013). Other constructs of DPSCs and scaffolds have been assayed for calvaria defects in animal models with successful generation of bone (Chamieh et al. 2016; Wongsupa et al. 2017). Consequently, it could be stated that an alternative option for bone tissue engineering has emerged.

1.4.2 Regeneration of other tissues

Numerous neurological disorders are characterized by the degeneration of neurons and consequently loss of their function, including Alzheimer, Parkinson, neurodegenerative disease, stroke, spinal cord injury, and peripheral nerve injury. DPSCs derive from the neural crest; therefore, they exhibit a high expression of several neural crest-related genes. Recently, several authors have demonstrated the *in vitro* neural differentiation potential of DPSCs using different approaches and methods (Arthur et al. 2008). The neuroregenerative capability of these cells has been also validated by *in vivo* works, especially for treating local sciatic damage, peripheral nerve lesion or spinal cord injuries (Askari et al. 2014; J. Zhang et al. 2016).

Vasculogenesis is a potential therapy for ischemic disease. Vessel development consists of complex and dynamic processes, such as ECM degradation, endothelial cell proliferation and migration, and tube formation and maturation into functional blood vessels. The ability of DPSCs for the treatment of myocardial infarction and ischemia has been evaluated in numerous studies (Bronckaers et al. 2013; Shen et al. 2015). Moreover, recent *in vitro* and *in vivo* experiments proved the regenerative potential of DPSCs on corneal and retinal degenerative disease. They prevent retinal ganglion cells from death and protected their axons, thus preserving their function (Mead et al. 2016). The efficacy of DPSCs transplantation for the treatment of diabetes has also been demonstrated. Currently, the expression of genes related to pancreatic β-cell development and function were reported after 7 days of differentiation. Also, the capacity of these cells to convert into insulin-producing cells was confirmed (Kanafi et al. 2013). Furthermore, DPSCs may be a promising cell resource for liver regenerative medicine. Their differentiation potential into hepatocyte-like cells has been proven (Ishkitiev et al. 2012). A recent *in vivo* investigation showed that the combination of melatonin and hDPSCs significantly suppressed liver fibrosis and restored transaminase and ammonia levels in murine model (Cho et al. 2015). Additionally, the *in vitro* ability of hDPSCs to differentiate into bladder smooth muscle cells has been reported (Song et al. 2016). Regeneration of new end bulbs and the creation of multiple differentiated hair fibbers have also been described after DPSC transplantation into surgically inactivated hair follicles (Reynolds and Jahoda 2004). Finally, the formation of bone, cartilage and adipose tissue, along with tendon-like tissue have been revealed after transplant of DPSCs cultured with basic fibroblastic growth factor in immunocompromised mice (Morito et al. 2009; Y.-Y. Chen et al. 2016). All these studies explore the potential of DPSCs as a therapy for several biomedical applications.

2 AIM OF THE THESIS

General aim

The overall aim of the work in this thesis was to deepen our knowledge about human DPSCs (hDPSCs) behaviour considering their clinical application in dentistry. To this aim three different *in vitro* approaches were used.

Specific aims

Study I

Examination of senescence features of hDPSCs and their possible role in the impairment of multipotency and differentiation capability.

Study II

To study the impact of an age-related microenvironment on the biological performance of hDPSCs isolated from young and old subjects.

Study III

To investigate the effects of commonly used restorative nanofilled composites on hDPSCs in terms of cell proliferation, apoptosis and differentiation potential, as well as in matrix mineralization.

3 MATERIALS AND METHODS

3.1 Dental pulp collection and DPSC culture

In collaboration with Odontostomatological and Special Surgery Unit, Ospedali Riuniti of Ancona, the dental pulp was obtained from third molars of 21 individuals gender matching, following approved guidelines set by the Local Ethics Committee. All patients were aware of the voluntariness of their participation and provided informed consent in the form of verbal authorization.

Extracted teeth were stored in Dulbecco's Modified Eagle's Medium F12 (DMEM/F12) supplemented with 10% Foetal Bovine Serum (FBS), 2% penicillin/streptomycin (p/s) until the removal of the pulp, within 24 hours. Before pulp extraction, the tooth was cleaned with a tissue soaked in 70% ethanol and attached tissues were removed with a scalpel. The cleaned tooth was wrapped into tinfoil, previously sterilized and broken in a screw clamp. After mechanical fracturing, the pulp was gently removed with a dentinal excavator and minced. The small pieces were placed for 1h at 37°C in a T25 flask with the digestion solution of 3.0 mg/ml type I collagenase and 4.0-mg/ml dispase for 1h. Then, the solution was filtered with a 70 μm cell strainer to remove non-digested tissue pieces and centrifuged at 1500 rpm for 10 min. Cells were transferred into T25 flask with complete culture medium DMEM/F12 added with 10% FBS and 1% p/s. The cultures were maintained in a 5% CO_2 humidified incubator at 37°C.

DPSCs were observed under Nikon Eclipse E600 light microscope to evaluate their morphologic appearance.

3.1.1 First study

In the first study 12 individuals gender matching (mean age, 43 years; range 20-64 years) were divided into three age groups: group Y (21 years; 20-23 years), group M (43 years; 42-45 years) and group O (64 years; 62-66 years).

3.1.2 Second study

In the second investigation 6 subjects gender matching (mean age, 43 years; range 20-64) were enrolled into two age groups: group Y (21 years; 20-23) and group O (64 years; 62-66 years). The dental pulp cell-conditioned medium (DPC-CM) was collected as described by Ma et al., (Ma et al. 2009). Briefly, the culture medium of primary dental pulp cells at 70% confluence was changed every 24 hours until full confluence, then the supernatant was collected every day and filtered through a 0.22 μm Millipore strainer (TPP, Trasadingen, Switzerland), followed by centrifugation at 2000g for 10 minutes. The supernatant was mixed with equal volume of fresh complete medium (dil. 1:1) and used as DPS-CM for DPSC culture. The complete medium was used as control medium (group Y ctrl, group O ctrl). DPSCs of group Y were treated with group O DPSC-CM (group Y-test) and DPSCs of group O were treated with group Y DPSC-CM (group O-test). The average time period for culture was 7 days.

3.1.3 Third study

In the third research 3 individuals gender matching (mean age, 22 years; range, 20-24 years) have been involved and samples were divided into three groups: group A (DPSCs in culture with dentine pieces), group B (DPSCs in culture with composite Supreme Filtek XTE, 3M) and control group (DPSCs cultured on tissue culture plates, TCPs).

Extracted third molars were chosen, stored in ethanol 70%, and fragmented accurately selecting only dentine. The composite pieces (1 cm^2 each) were light-cured (40 s). 12 pieces of dentine, as well as of composite were autoclaved. $2{,}5 \times 10^3$ cells/cm^2 were seeded in 6-well plates and 3 ml of basal medium were added. After 24 h a tooth piece was added to 12 wells (group A) and a composite piece to another 12 wells (group B), the remaining 12 wells representing the control. Cells were cultured for three weeks and medium was changed every 3 days.

3.2 Flow cytometric analysis

For immunophenotyping 2.5×10^4 cells underwent flow cytometric analysis according to the International Society for Cellular Therapy (ISCT) for the identification of human mesenchymal stem cells (hMSCs; Dominici et al. 2006).

hDPSCs were stained for 25 min at RT with anti-CD90, CD105, CD34, CD73, and HLA-DR (all from Immuno Tools, Friesoythe, Germany) for 30 min at 4°C. For isotype controls, the primary antibodies were used instead of FITC- or PE-coupled nonspecific mouse immunoglobulin G. Cell fluorescence was evaluated in a FACSCalibur flow cytometry system (BD Italia, Milan, Italy) and data were analysed using FCS Express 6 Plus software (De Novo Software, Glendale, CA). In addition, physical parameters such as cell size (forward scatter [FSC]) and granularity (side scatter [SSC]) were analysed.

3.3 Senescence-associated-β galactosidase assay (SA-β-Gal)

The SA-β-Gal activity was determined using a SIGMA kit (Sigma-Aldrich, Milan, Italy) according to the manufacturer's instructions. In brief, hDPSCs were cultured overnight on two-well Lab-Tek® Chamber Slides (Sigma-Aldrich) at a density of 2×10^4 cells/cm^2 and fixed with 4% paraformaldehyde (PFA). After overnight incubation with SA-β-Gal, cells were washed with phosphate-buffered saline (PBS), counterstained with hematoxylin and then observed under Nikon Eclipse 600 light microscope. Images were captured with a Nikon DSVi1 digital camera and processed with NIS Elements BR 3.22 imaging software (all from Nikon Instruments, Florence, Italy). The senescent cells, blue-stained, were counted in at least five randomly selected fields per sample and quantified as a percentage of the total counted cells. Mean ± SD was considered.

3.4 Immunocytochemistry

For ICC staining, 15×10^4 cells per well hDPSCs were cultured on two-well Lab-Tek® Chamber Slide, fixed in 4% PFA in 0.1M sodium phosphate buffer pH 7.4 for 20 min at room temperature (RT) and then permeabilized in 0.1% Triton X-100 in PBS for 10 min. Following overnight incubation at 4°C with mouse anti-human p16^{ink4a} monoclonal antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA), hDPSCs were immunostained using the streptavidin-biotin peroxidase technique (Envision Peroxidase Kit; DakoCytomation, Darmstat, Germany). After the incubation with 0.05% 3,3′-diaminobenzidine (Sigma-Aldrich) in 0.05 M Tris buffer, pH 7.6, with 0.01% hydrogen peroxide, cells were counterstained with hematoxylin, dehydrated in ethanol, and cover-slipped with Eukitt mounting medium (Electron Microscopy

Sciences, Hatfield, PA). For negative controls, the primary antibody was replaced with nonimmune serum. The immunocytochemical expression of $p16^{ink4a}$ was evaluated under Nikon Eclipse E600 light microscope by two independent observers. Images were captured and processed as above.

3.5 Population doubling time

hDPSCs were seeded at an initial density of 5×10^4 cells/well in six-well tissue culture plates (VWR®, VWR International Srl, Milan, Italy). Cells were detached by trypsinization every 4 days, counted and reseeded at the same density. To examine the time required by cells of each population to double, PDT was calculated according to the formula:

$$PDT = T \log 2/(\log N_f - \log N_0),$$

where T represents the duration of cell culture (in days) and N_f and N_0 the final and the initial concentration, respectively (Roth V. 2006).

3.6 Telomere length determination

Telomere length was assessed by Relative Human Telomere Length Quantification qPCR Assay Kit (ScienCellTM, Carlsbad, CA; 92011) according to the manufacturer's instruction. The average telomere lengths of samples designed were directly compared by telomere primer set that recognized and amplified telomere sequences.

3.7 *In vitro* cell differentiation

For adipogenic, osteogenic, and chondrogenic differentiation, commercial kits from Life Technologies Corporation (Carlsbad, CA) were used according to the manufacturer's instruction. For adipogenic differentiation 10^4 cells/cm^2 were seeded in two-well Lab-Tek® Chamber Slide and treated with the appropriate medium (STEM- PRO® Adipogenesis Kit, Gibco life technologies, Grand Island, NY) for 14 days, changing the medium twice a week. Differentiation was assessed by Oil red staining. Briefly, cells fixed in 4% PFA were exposed to Oil Red O solution (0.5% in 100% isopropyl alcohol) for 30 min at RT, cleared with isopropanol 60%, washed in dH_2O, and counterstained with hematoxylin. Cells were immediately observed under a light microscope to avoid stain decay and counted.

For osteogenesis, cells were seeded at a density of 1.5×10^3 cells/cm^2 in two-well Lab-Tek® II Chamber Slide and treated with STEMPRO® osteogenesis medium for 3 weeks, changing the medium twice a week. Alkaline phosphatase (ALP) staining was performed after 1 week by incubating cells with a solution of 5- bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) alkaline-phosphate substrate (Sigma-Aldrich) in 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 10 mM $MgCl_2$ buffer for 1 hr at RT, and rinsed in dH_2O. After 3 weeks, mineralization was assessed by Alizarin Red S (ARS) staining (Sigma-Aldrich). Briefly, cells were fixed with 4% PFA in PBS for 10 min, incubated with ARS for 30 min at RT and finally washed in dH_2O. The reaction was observed under Nikon Eclipse 600 light microscope. To quantitatively determine calcium mineral deposits, ARS was extracted with 10% cetylpyridinium chloride in 10 mM sodium phosphate for 60 min at RT. Concentration was quantified spectrophotometrically at 540 nm (Secoman, Anthelie light, version 3.8; Contardi, Cesano Maderno, Italy).

For chondrogenesis, hDPSCs were cultured in a pellet culture system. For the preparation of each pellet, aliquots of 10^6 cells in 1 ml of STEMPRO® Chondrogenesis Kit (STEMPRO® Chondrogenesis Kit. Gibco life technologies, Grand Island, NY) were spun down at 1200 rpm for 5 min. Pellets were cultured for 14 days, changing the medium twice a week. Pellets were then fixed in 4% PFA, paraffin-embedded, and sectioned. Sections were exposed to a solution of Alcian Blue (pH 1; Bio-Optica, Milano, Italy) for 30 min at RT and observed under Nikon Eclipse 600 light microscope.

Odontogenic differentiation was obtained by seeding 2.5×10^3 cells/cm^2 into six-well plates (VWR®) and cultured for 3 weeks in α-minimum essential medium (α-MEM) supplemented with 10% FBS, 1% penicillin/streptomycin, 10 mM/L β-glycerophosphate, 10 nM/L dexamethasone and 50 μg/ml ascorbic acid (all from Sigma-Aldrich). Differentiation was assessed by the detection of dentine matrix acidic phosphoprotein 1 (DMP1) and dentine sialophosphoprotein (DSPP).

For cytoplasmic DMP1 detection, cell cultures underwent digestion with type I collagenase.

Neurogenic differentiation was generated by seeding hDPSCs at a density of 2.5×10^3 cells/cm^2 into six-well plates (VWR®) in Neurobasal Medium (Gibco®, Life Technologies, Carlsbad, CA) supplemented with 1% penicillin/streptomycin, 1% B27 (Gibco®, Life Technologies),

40ng/ml fibroblast growth factor 2, and 20 ng/ml epidermal growth factor (both from Immuno Tools). A medium was changed every 3–4 days. Differentiation was assessed by the detection of nestin and β-tubulin III after 2 weeks.

Control cultures were represented by cells maintained in an appropriate medium without differentiating factors for the same time.

3.8 Quantitative polymerase chain reaction (qRT-PCR)

qRT-PCR reactions were carried out in triplicate. Threshold cycle values were quantified, and the expression of each gene was normalized relative to that of reference genes.

Real-time assays were performed by the Mastercycler RealPlex2 (Eppendorf GmbH) using SsoFast^{TM} EvaGreen Supermix 1x, in a final volume of 10 ml. All PCRs contained 1 µl of complementary DNA. Each PCR assay was performed in the white plate and comprised at 95°C for 30 s for enzyme activation and 40 cycles of denaturation at 95°C for 5 s, annealing, and extension at 60°C for 20 s. Every primer was used at 10 µM final concentration. Primer sequences were designed by Primer 3 web (Primer3Web version 4.1.0, https:// primer3.ut.ee/) (and their specificity was tested by BLAST to avoid any appreciable homology to pseudogenes or other unexpected targets). In each assay, the messenger RNA (mRNA) of both reference genes and each gene of interest were measured simultaneously under identical conditions. Primers showed the same amplification efficiency. The specificity of the PCR reactions was confirmed by melt curve analysis: for each amplicon, the detected melting temperature was the expected one.

Each assay was performed in triplicate and threshold Cycle (C_t) values for reference genes were used to normalize cellular mRNA data. In this instance, normalization involved the ratio of mRNA concentrations for specific genes of interest (as mentioned above) to that corresponding to C_t medium values for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-glucuronidase (GUSB; Ragni et al. 2013). The amount of each mRNA was calculated using the comparative C_t method with $\Delta C_t = C_t$ (mRNA) − C_t (GAPDH/GUSB), and data were expressed as gene relative expression ($2^{-\Delta Ct}$). Moreover, to highlight the effect of aging or material on cell behaviour, $\Delta\Delta C_t$ method for the evaluation of fold-change ($2^{-\Delta\Delta Ct}$) was used comparing values obtained in cells of test groups with those of control groups (Livak and

Schmittgen 2001). The qPCR efficiency in all our experiments was more than 90%. The difference between the actual and theoretical (100%) efficiencies would result in an underestimation of the mRNA concentration of all the analysed samples. Oligonucleotide sequences for target genes are reported in Table 3.

3.9 Western blot (WB) analysis

The expression of specific proteins was quantified by WB. In particular, in the first study DMP1, Nestin, and β-tubulin III were analysed in hDPSCs after odontogenic and neurogenic differentiation. hDPSC total proteins were extracted at the fourth passage of subculture in basal cultures (i.e., cells grown in DMEM/F12) and after cell differentiation toward odontoblast and neuron phenotypes. Moreover, to ascertain the possible natural hDPSCs differentiation a further control was represented by cells cultured in appropriate media without differentiating cues. In the third investigation DMP1 protein expression was quantified evaluating the mineralization capability between different biomaterials.

Radioimmunoprecipitation assay buffer (RIPA Lysis Buffer System; Santa Cruz Biotechnology) was used for protein extraction. Protein concentration was determined using Bradford reagent (Sigma-Aldrich). Electroblotting was performed using Wet Blotting System (XCell II Blot Module; InvitrogenTM). Membranes were incubated overnight with primary antibodies anti-DMP1 (1:200, Santa Cruz Biotechnology), anti-Nestin and β-tubulin III (1:500; Santa Cruz Biotechnology) followed by incubation with a secondary antibody conjugated to horseradish peroxidase (1:1000; Santa Cruz Biotechnology). The signals were captured using an Alliance Mini system (UVITEC, Cambridge, UK); the DMP1, Nestin, and β-tubulin III bands were quantified with UVITEC software and their intensity was normalized by comparison to the GAPDH housekeeping, used as a loading control. The intensity of each band was then compared with the negative controls and any change was expressed as a ratio.

Genes	Primer Forward (5'->3')	Primer Reverse (5'->3')
Sox2	ACACCAATCCCATCCACACT	GCAAACTTCCTGCAAAGCTC
Oct4	AGCGAACCAGTATCGAGAAC	GCCTCAAAATCCTCTCGTTG
Nanog	TGAACCTCAGCTACAAACAG	CTGGATGTTCTGGGTCTGGT
Klf4	CCCACACAGGTGAGAAACCT	ATGTGTAAGGCGAGGTGGTC
Bmp2	CCAGCCGAGCCAACACTGTGC	TCTCCGGGTTGTTTTCCCACTCG
Runx2	CTCGTCCGCACCGACAGCC	TACCTCTCCGAGGGCTACCACC
β-catenin	CCAATGGCTTGGAATGAGAC	GTTCCATCATGGGGTCCATA
Bglap	GACTGTGACGAGTTGGCTGA	GCCCACAGATTCCTCTTCTG
DMP1	AGGAAGACAGTGACTCCAGC	GAATGGCTTTCCTCGCTCTG
DSPP	AGCAAACCACTGGAGAGACA	CCCTTCTCCCTTGTGACCAT
Nestin	CAATCCCTGCAAAAGGAGAA	TGGAGATCTCAGTGGCTCTT
β-tubulin III	AGGAAGACAGTGACTCCAG	CAGGCAGTCGCAGTTTTCA
CD 105	AGTCTTGCAGAAACAGTCCA	TGGACTTCAAGGATGGCATT
CD 90	AATACCAGCAGTTCACCCAT	GCTAGTGAAGGCGGATAAGT
CD 73	AGCTTACGATTTTGCACACC	ATCTGCTGAACCTTGGTGAA
Gusb*	AAACGATTGCAGGGTTTCAC	TCTCGTCGGTGACTGTTCA
Gapdh*	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC

Sox2=Sex-determining region Y (SRY)-Box2); Oct4=octamer-binding transcription factor 4; Kfl4=Kruppel-like factor 4; Bmp2=bone morphogenetic protein 2; Runx2=runt-related transcription factor 2; Bglap= osteocalcin; DMP1=Dentin matrix acidic phosphoprotein 1; DSPP=Dentin sialophosphoprotein; Gusb= beta glucuronidase; Gapdh= glyceraldehyde-3-phosphate dehydrogenase. * reference genes

Table 3. Analysed gene description.

3.10 Immunofluorescence

In the first study, hDPSCs were seeded at the density of 2.5×10^4 cells per well on two-well Lab-Tek® Chamber Slides and treated with neurogenic differentiating media for 2 weeks. For the detection of Nestin and βtubulin III localization IF was performed. After differentiation cells were fixed in 4% PFA and then permeabilised in 0.1% Triton X-100 in PBS. For immunofluorescence morphological analysis, samples were blocked with a 2% bovine serum albumin (BSA) solution in PBS for 30 min. Cells were then incubated overnight at 4°C with mouse anti-human-nestin or anti-human-β tubulin III (1:500; Santa Cruz Biotechnology). After overnight incubation, cells were stained with a goat anti-mouse FITC-conjugated secondary antibody (Bethyl Laboratories Inc., Montgomery, Alabama; dilution, 1:100).

In the second investigation, to measure cell death, hDPSCs were subjected to TUNEL

immunofluorescence assay using the In Situ Cell Death Detection Kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol. To detect cell proliferation Ki67 antibody (Abcam, Cambridge, MA) was used on hDPSCs and countered with goat anti-mouse FITC-conjugated secondary antibody (Bethyl Laboratories Inc., Montgomery, Alabama; dilution, 1:100) to locate the primary Ab. The quantification of the TUNEL and Ki67 data were performed by using ImageJ 2.0.0 software on images captured at 40X magnification which counts the nuclei (DAPI staining) and the TUNEL and Ki67 positive cells and calculates the percentage of positive cells to the total number of cells.

In all studies nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and samples were mounted using Vectashield® mounting medium (Vector Laboratories Inc, Burlingame, CA). For negative controls, the primary antibody was replaced with nonimmune serum. Cells were observed with a Nikon E600 fluorescence microscope.

3.11 Statistical analysis

Statistical analysis was performed by Prisma 4 Software (GraphPad Software, La Jolla, CA). Mean and SD of three different experiments were reported. Data were analysed by analysis of variance and Bonferroni's T-test. Statistical significance was tested at $p < 0.05$.

4 RESULTS

Study I				
	Group Y	**Group M**	**Group O**	
	Young hDPSCs	Middle-aged hDPSCs	Aged hDPSCs	
	Group Y, M, O post	**Group Y, M, O ctrl**		
	Group Y, M, O after 3 weeks in odontogenic medium	Group Y, M, O after 3 weeks in complete medium		
Study II				
	Group Y ctrl	**Group O ctrl**	**Group Y test**	**Group O test**
	Young hDPSCs	Aged hDPSCs	Young hDPSCs + aged DPC-CM	Aged hDPSCs + young DPC-CM
Study III				
	CTRL	**Group A**	**Group B**	
	hDPSCs	hDPSCs + dentin	Young hDPSCs + composite	

hDPSCs=human dental pulp stem cells; DPC-CM=dental pulp cell conditioned medium.

Table 4. Group abbreviations.

4.1 First study

This study evaluated the senescence features of hDPSCs and their possible effects in the impairment of multipotency and differentiation capability of cells.

4.1.1 Senescent features in hDPSCs

To ascertain the presence of age-related changes in cells derived from the three groups, we analysed the expression of SA-β-Gal and $p16^{ink4a}$, SSC, and FSC cytofluorimetric parameters, as well as changes in telomere length. The positive rate of SA-β-Gal and $p16^{ink4a}$ was remarkably higher in hDPSCs of group O in comparison with cells harvested from younger subjects (Figure 4.1a, b). As far as $p16^{ink4a}$ is concerned, we detected a significantly ($p < 0.05$) higher number of positive cells in groups M (81 ± 4%) and O (97 ± 5%) in comparison to group Y (56 ± 3%). Flow cytometry analysis confirmed SA-β-Gal observations, evidencing an increase of SSC values with aging. On the contrary, no changes in cell size were observed (Figure 4.1c). PDT showed no differences until passage 13 of subculture. After then, cells from

groups M and O started to slow down and at P17 hDPSCs of group O stopped their proliferation (Figure 4.1d).

qRT-PCR analysis displayed changes in the expression of stemness genes in relation to age (Table 4.1a). Comparing gene expression of hDPSCs from groups M and O with those of group Y, a slight upregulation of sex-determining region Y (SRY)-Box 2 (Sox2) mRNA expression and downregulation of both octamer-binding transcription factor 4 (Oct4) and Nanog were showed. On the contrary, the expression of Kruppel-like factor 4 (Klf4) appeared downregulated in cells from group M and almost unchanged in hDPSCs from group O (Figure 4.1e).

At last, the length average of telomere of group Y was 1.1- and 1.2-fold longer than that of groups M and O, respectively, strengthening the features of age-related changes in our hDPSCs (Table 4.1b).

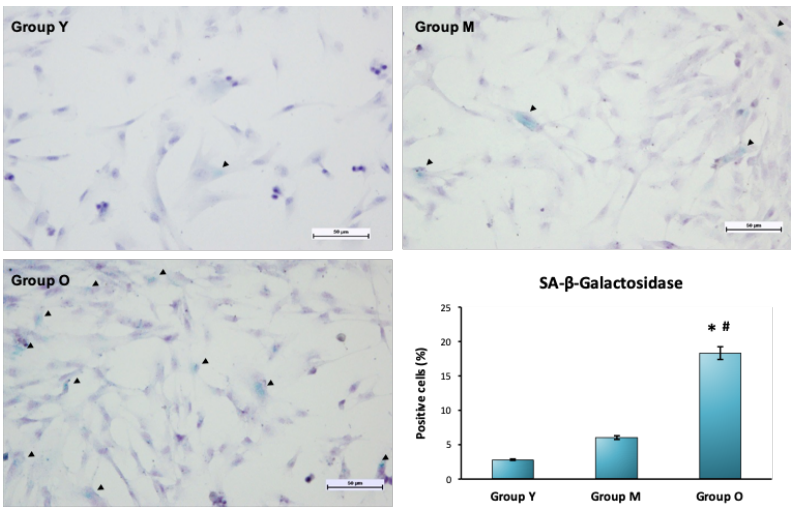

Figure 4.1a. Detection of SA-β-Gal (arrowhead) in hDPSCs isolated from three different age groups. *$p < 0.05$ versus group Y, $^{\#}p < 0.05$ versus group M.

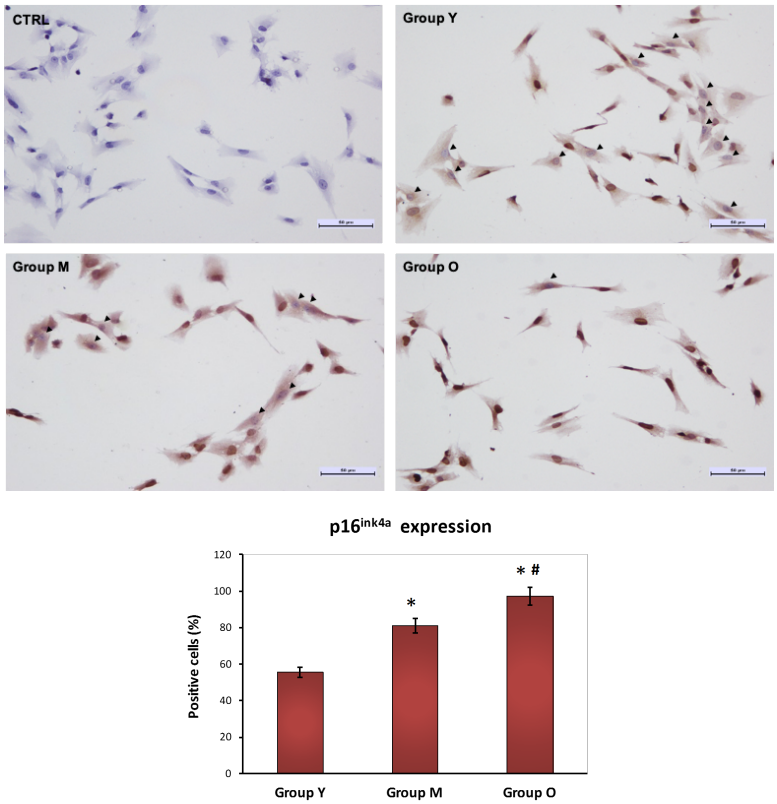

Figure 4.1b. Immunocytochemical analysis of $p16^{ink4a}$ in the differently aged hDPSCs. *$p < 0.05$ versus group Y, $^{\#}p < 0.05$ versus group M. Arrowheads show negative nuclei.

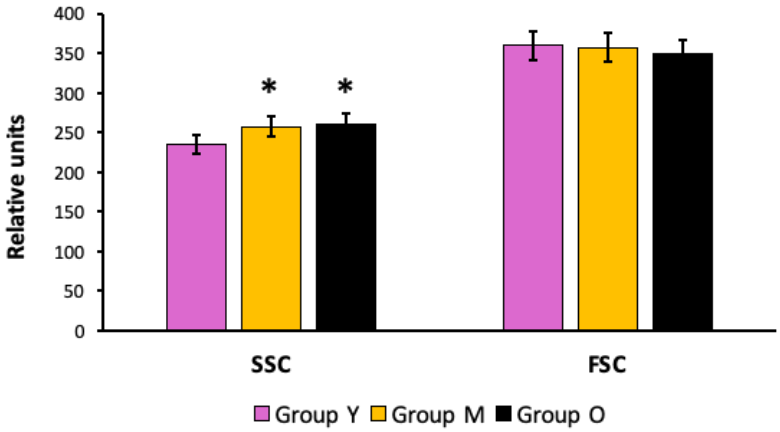

Figure 4.1c. Histograms of cytofluorimetric detection of changes in cell internal complexity (SSC) and size (FSC). *$p < 0.05$ versus group Y.

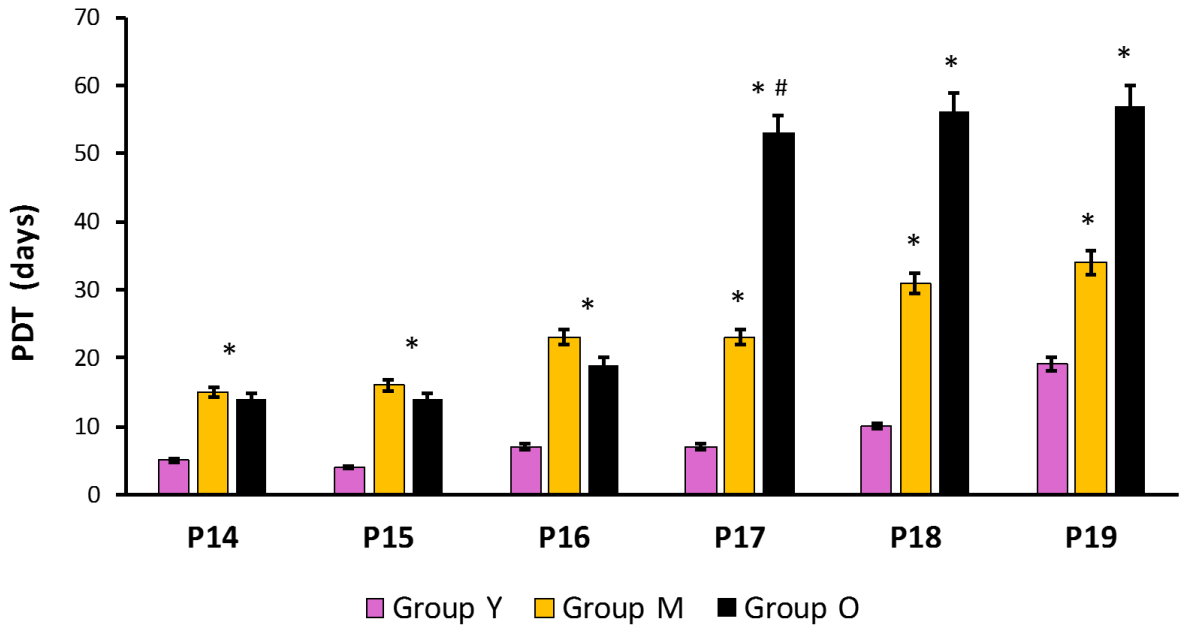

Figure 4.1d. Histograms of the evaluation of PDT during subculturing of hDPSCs isolated from three different age groups. *p < 0.05 versus group Y, #p < 0.05 versus group M.

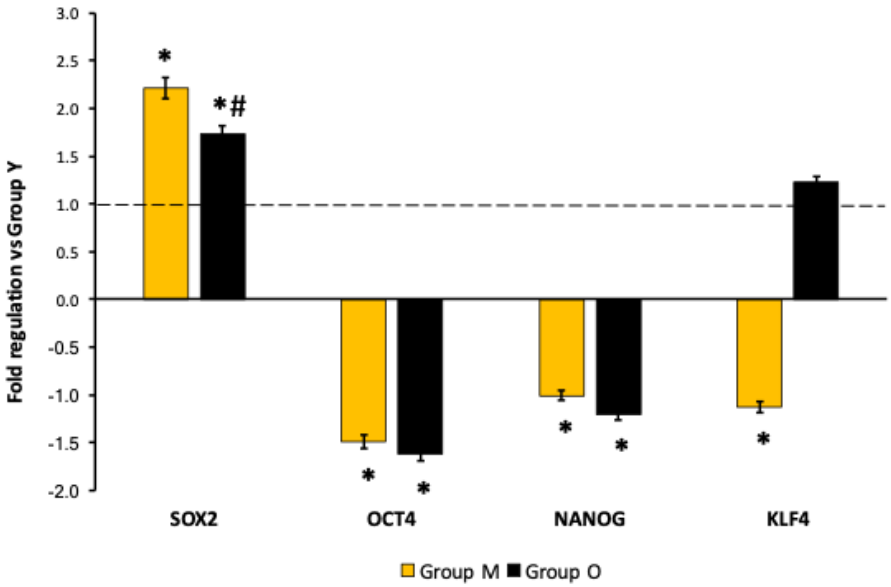

Figure 4.1e. Histograms depict changes in stemness gene mRNA expression in the differently aged hDPSCs. Data are expressed as fold-regulation ($2^{-\Delta\Delta Ct}$), the dotted black line indicates the mRNA expression of group Y; *p < 0.05 versus group Y, #p < 0.05 versus group M.

	Group Y		Group M		Group O		Bonferroni's T test
	Mean	SD	Mean	SD	Mean	SD	
Sox2	9.66E-06	4.83E-07	2.13E-05	2.13E-05	1.66E-05	1.66E-05	*Group O vs Groups Y and M; Group M vs Group Y*
Oct4	4.69E-04	2.34E-05	3.15E-04	1.58E-05	2.90E-04	1.45E-05	*n.s.*
Nanog	8.93E-05	4.46E-06	8.83E-05	4.42E-06	1.66E-05	8.30E-07	*Group O vs Groups Y and M*
Klf4	3.43E-02	1.71E-03	3.04E-02	1.52E-03	4.21E-02	2.11E-03	*Group O vs Groups Y and M*
Bmp2	3.48E-04	1.74E-05	2.98E-03	1.49E-04	1.81E-03	9.04E-05	*Group O vs Groups Y and M*
Runx2	6.96E-03	3.48E-04	2.09E-03	1.05E-04	3.08E-03	1.54E-04	*Group O vs Groups Y and M; Group M vs Group Y*
β-catenin	2.37E-02	1.19E-03	1.51E-02	7.57E-04	2.50E-02	1.25E-03	*Group M vs Groups Y and C*
Bglap	1.41E-04	7.03E-06	1.14E-05	5.70E-07	3.30E-05	1.65E-06	*Group O vs Groups Y and M*
DMP1	4.69E-05	2.35E-06	6.79E-05	3.40E-06	5.73E-05	2.87E-06	*Group O vs Group Y and M; Group M vs Group Y*
DSPP	2.16E-05	1.08E-06	3.30E-05	1.65E-06	2.59E-05	1.30E-06	*Group O vs Group Y and M*
Nestin	1.01E-02	5.04E-04	4.52E-03	2.26E-04	8.75E-03	4.38E-04	*Group O vs Group Y and M; Group M vs Group Y*
β-tubulin III	1.36E-03	6.78E-05	1.71E-03	8.55E-05	1.71E-03	8.57E-05	*n.s.*

Sox2=Sex-determining region Y (SRY)-Box2); Oct4=octamer-binding transcription factor 4; Kfl4=Kruppel-like factor 4; Bmp2=bone morphogenetic protein 2; Runx2=runt-related transcription factor 2; DMP1=Dentin matrix acidic phosphoprotein 1; DSPP=Dentin sialophosphoprotein; n.s.=not significant

Table 4.1a. mRNA relative expression in native cells.

	Group Y (Cycle value)	Group M (Cycle value)	Group O (Cycle value)
TEL	13.2	13.4	14
SCR	22.1	22.1	22.6

	Group Y vs M	Group Y vs O	Group M vs O
Fold regulation	1.1	1.2	1.1

TEL= genomic DNA telomere; SCR= genomic DNA single copy (reference)

Table 4.1b. qPCR quantification of telomere length in the three different age groups.

4.1.2 *hDPSCs characterization and mesenchymal tissues differentiation*

hDPSCs isolated from the three groups showed no differences in their ability to adhere to plastic, in morphology (Figure 4.1f) and in their phenotypic profile according to ISCT criteria: Our cells were positive for CD73, CD90, and CD105 and negative for CD45, HLA-DR, and CD14 (Figure 4.1g). The analysis of their differentiation capability exhibited a reduction of the adipogenic and osteogenic potential in cells from group O in comparison with both groups Y and M, as evidenced by Oil red, ALP and ARS stainings. On the contrary, no changes in chondrogenic potential were observed (Figure 4.1h). Age-related modifications of genes involved in osteogenic differentiation were detected, with a decrease in runt-related transcription factor 2 (Runx2), β-catenin, and bone γ-carboxy glutamic acid-containing protein (BGLAP) expression (Table 4.1a). It was interesting to observe that, after 3 weeks in odontogenic media, the expression of mRNA for Bmp2, markedly upregulated before differentiation, appeared downregulated in groups M and O in comparison with group Y. On the contrary, no significant changes were detected for the other osteogenic genes except for BGLAP (Figure 4.1i and Table 4.1 c).

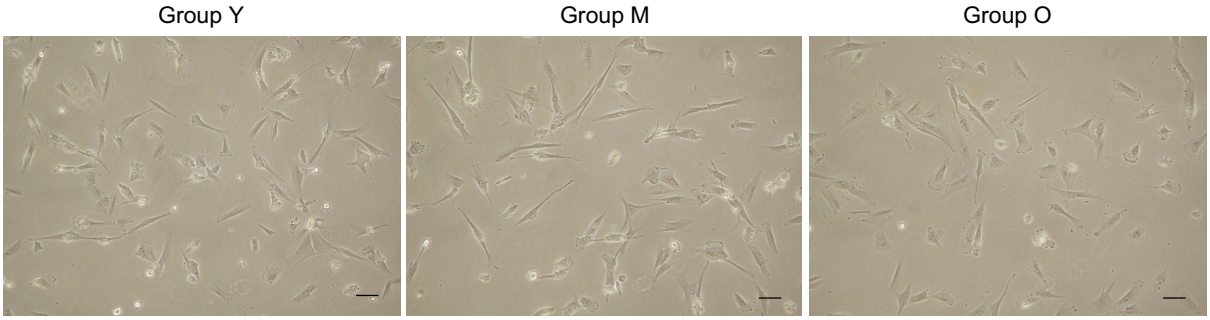

Figure 4.1f. Representative phase-contrast images of hDPSCs isolated from the three different age groups (scale bars = 10 μm).

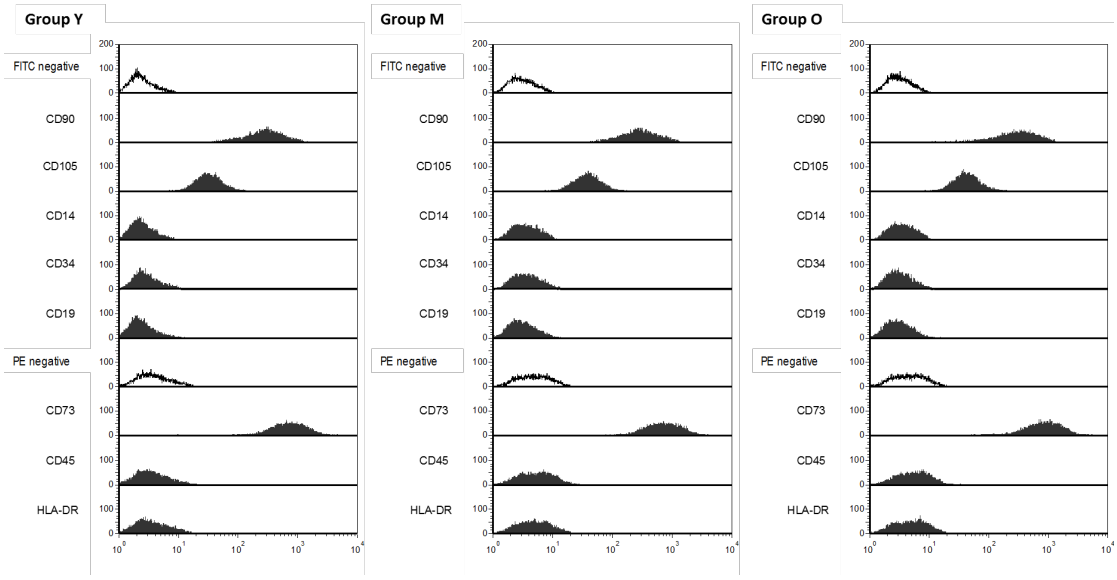

Figure 4.1g. Cytofluorimetric analysis of the detection of MSC surface markers in hDPSCs isolated from the three groups (white plots indicate FITC and PE negative controls).

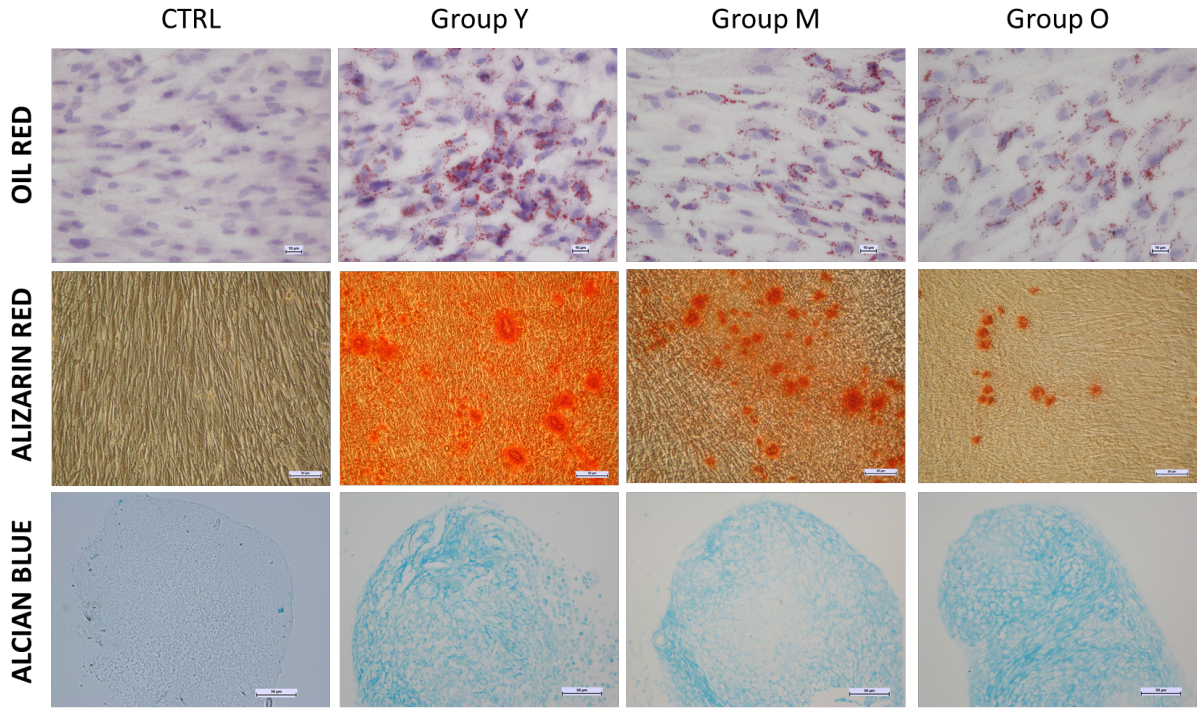

Figure 4.1h. Differentiation of hDPSCs isolated from the differently aged groups toward adipocytes (Oil red staining; scale bars = 10 µm); osteoblasts (ARS and ALP stainings; scale bars = 50 µm), and chondrocytes (Alcian blue staining; scale bars = 50 µm).

	Group Y		Group M		Group O		Bonferroni's T test
	Mean	SD	Mean	SD	Mean	SD	
Bmp2	3.32E-03	1.74E-05	2.02E-03	1.49E-04	1.32E-03	9.04E-05	*Group O vs Groups Y and M*
Runx2	2.90E-03	3.48E-04	3.00E-03	1.05E-04	3.87E-03	1.54E-04	*n.s*
β-catenin	4.63E-02	1.19E-03	4.89E-02	7.57E-04	5.38E-02	1.25E-03	*n.s*
Bglap	1.33E-06	7.03E-06	3.81E-06	5.70E-07	1.84E-06	1.65E-06	*Group O vs Groups Y and M Group M vs Group Y*
DMP1	5.04E-05	8.44E-07	1.52E-05	7.10E-07	1.53E-05	8.17E-07	*Group Y vs Groups M and O*
DSPP	2.11E-06	2.60E-07	1.56E-06	7.62E-08	1.90E-06	8.67E-08	*Group Y vs Groups M and O*

Sox2=Sex-determining region Y (SRY)-Box2); Oct4=octamer-binding transcription factor 4; Kfl4=Kruppel-like factor 4; Bmp2=bone morphogenetic protein 2; Runx2=runt-related transcription factor 2; DMP1=Dentin matrix acidic phosphoprotein 1; DSPP=Dentin sialophosphoprotein

Table 4.1c. Relative expression after odontogenic differentiation.

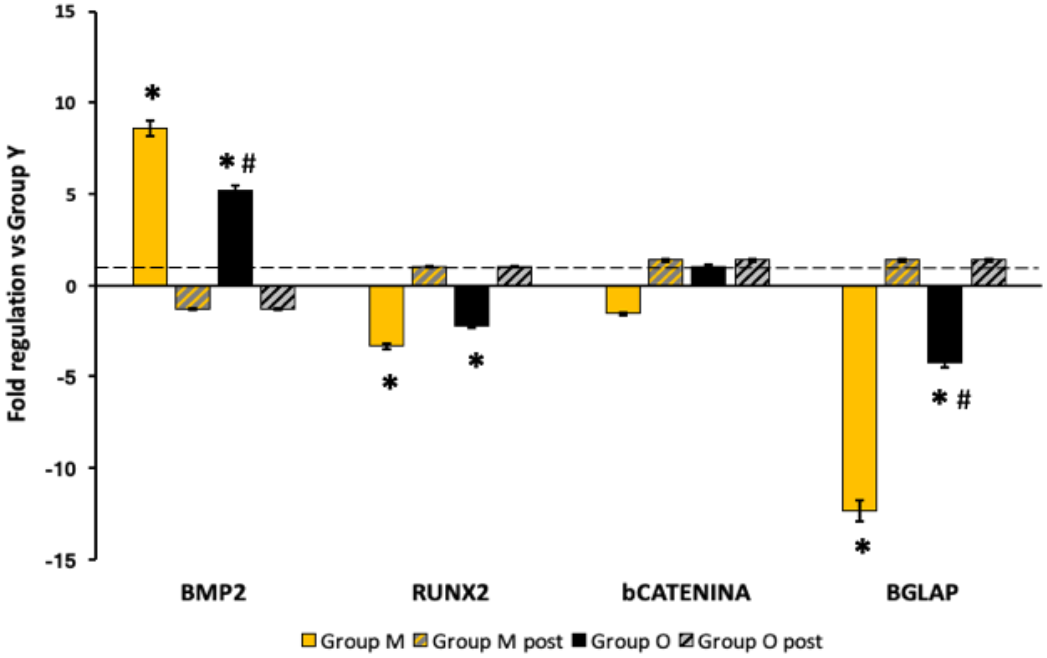

Figure 4.1i. Histograms depict changes in mRNA expression of genes involved in osteogenic differentiation in the differently aged DPSCs before and after (post) odontogenic differentiation.
Data are expressed as fold-regulation ($2^{-\Delta\Delta Ct}$), the dotted black line indicates the mRNA expression of group Y; *$p < 0.05$ versus group Y, $^{\#}p < 0.05$ versus group M.

4.1.3 Odontogenic differentiation of hDPSCs

As odontogenic differentiation is concerned, even if no significant morphological age-related changes were observed, ARS showed a marked increase in cells of groups M and O in comparison with hDPSCs of group Y (Figure 4.1j). This was sustained by the changes of odontogenic gene expression and DMP1 protein. After differentiation, a decrease in the expression of DMP1 and DSPP mRNAs was observed in hDPSCs of groups M and O in comparison to cells of group Y (Figure 4.1k and Table 4.1c). WB analysis showed a faint expression of DMP1 fragments in undifferentiated cells, except for the N-terminal one in cells from group M. The expression of cytoplasmic DMP1 increased in cells undergoing odontogenic differentiation as well as in hDPSCs cultured without an odontogenic media (Figure 4.1l). In hDPSCs treated with odontogenic medium, the majority of DMP1 was in the form of 105 kDa, while in cells cultured, without odontogenic cues, it was in the size of 37 kDa fragments. Protein aggregates (120 kDa), accumulated on top of the sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, were also present, especially in cells of group O cultured for 3 weeks without odontogenic induction. Mineralization nodules were detected only in cells treated with differentiating media, indicating that DMP1 promoted mineralization (Figures 4.1j, m). Moreover, ARS quantization evidenced age-related variations in the mineralization capability of hDPSCs after osteogenic or odontogenic stimuli (Figure 4.1n).

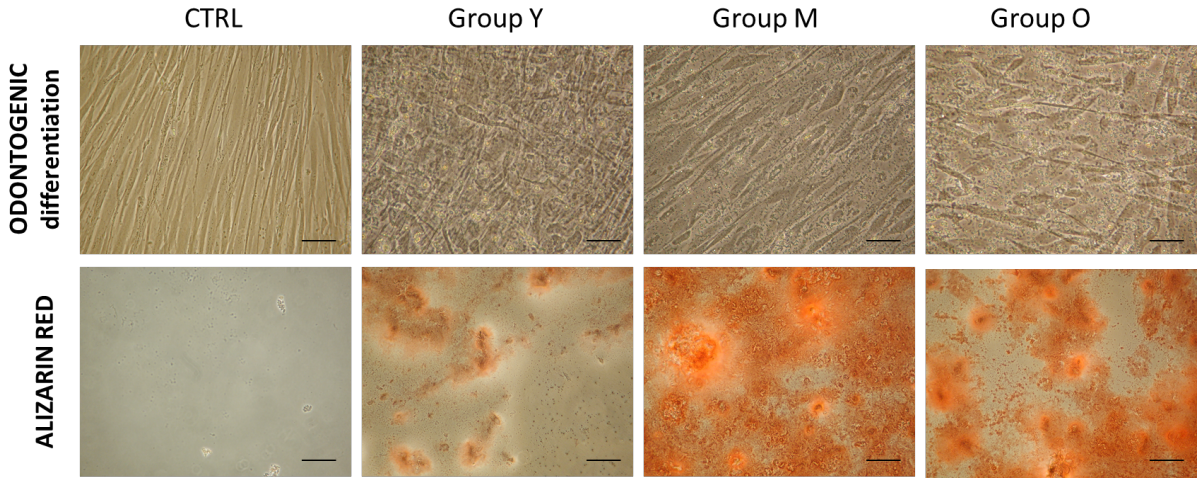

Figure 4.1j. Representative images displaying the different morphology and the formation of mineralized nodules in hDPSCs after 3-weeks culture in odontogenic media (scale bars = 50 µm).

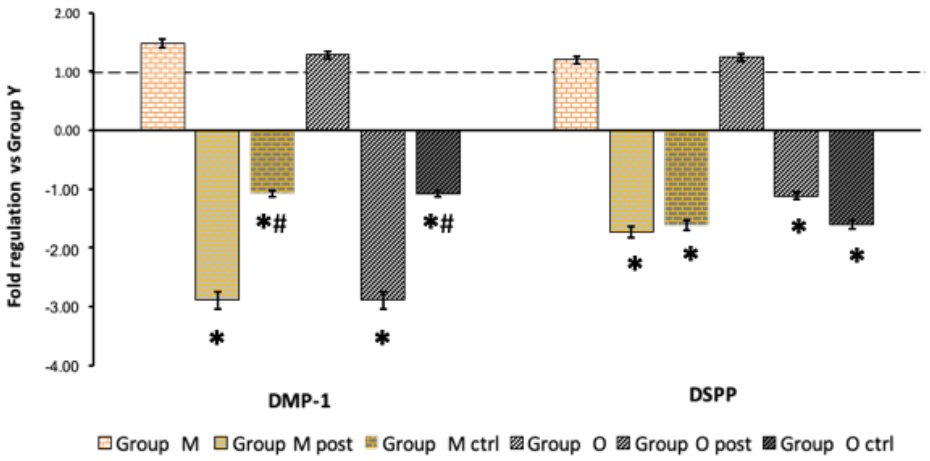

Figure 4.1k. Histograms depict changes in the expression of DMP1 and DSPP mRNAs in the differently aged hDPSCs before and after (post) odontogenic differentiation and in DPSCs maintained for 3 weeks in control media. Data are expressed as fold-regulation ($2^{-\Delta\Delta Ct}$), the dotted black line indicates the mRNA expression of group Y.

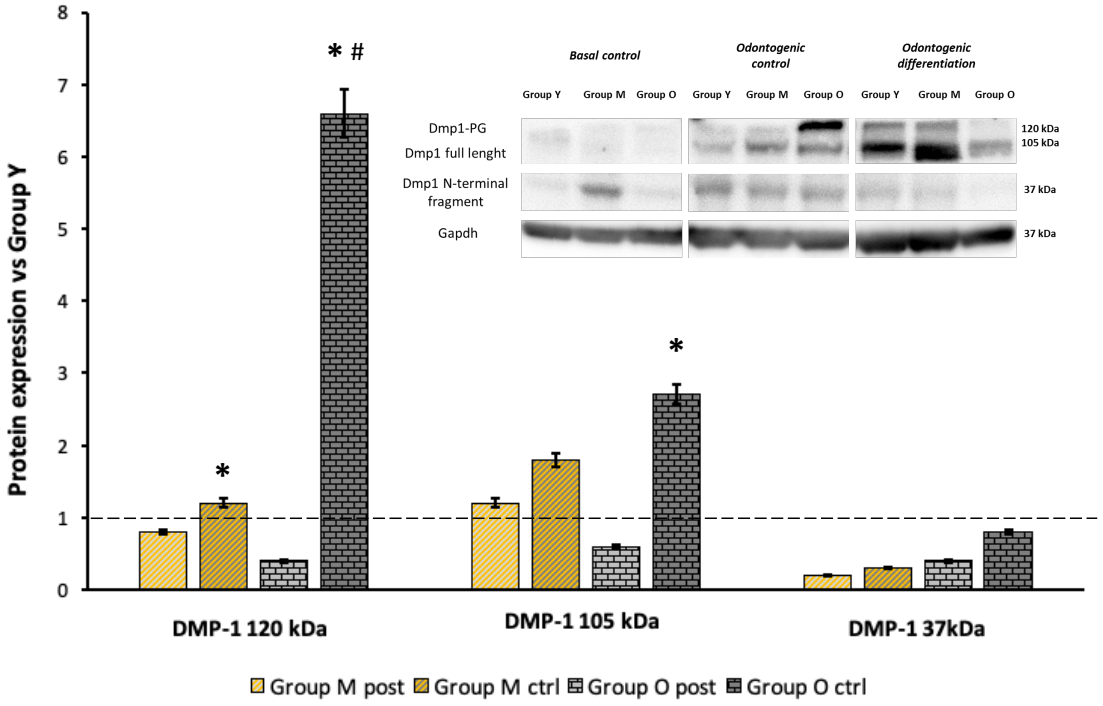

Figure 4.1l. Western blot analysis of DMP1 protein expression; histogram depicts changes in differently aged hDPSCs before and after (post) odontogenic differentiation and in DPSCs maintained for 3 weeks in control media in comparison to group Y (dotted black line).

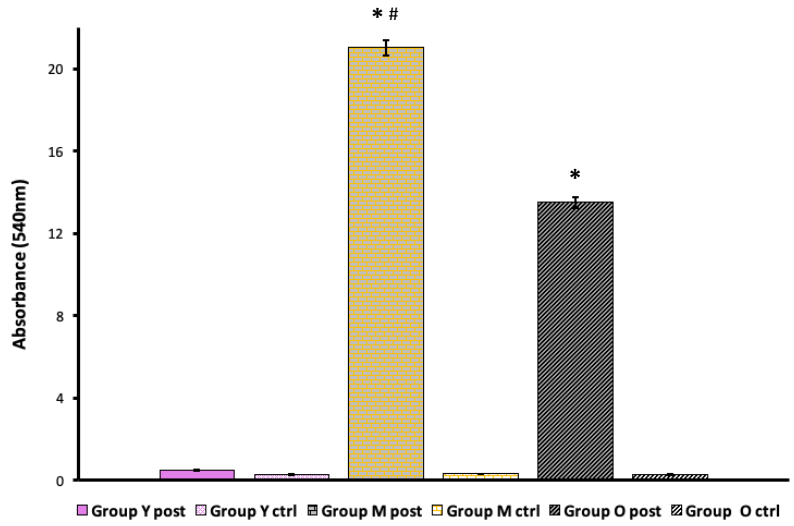

Figure 4.1m. Histogram of Alizarin red quantization in differently aged hDPSCs in (post) odontogenic differentiation and in hDPSCs maintained for 3 weeks in control media.

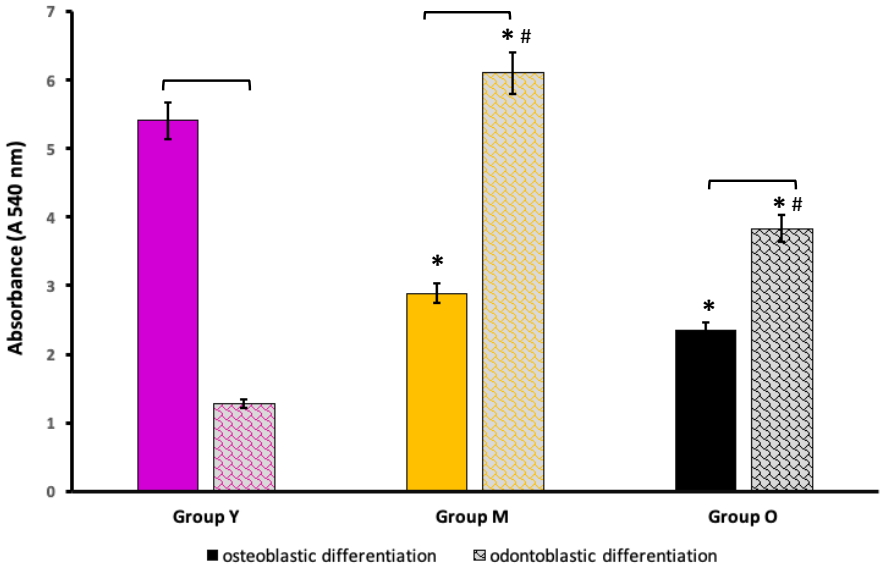

Figure 4.1n. Histogram of Alizarin red quantization in differently aged hDPSCs after osteogenic or odontogenic differentiation. *$p < 0.05$ versus group Y, $^{\#}p < 0.05$ versus group Y.

4.1.4 hDPSCs neurogenic differentiation

Following 2 weeks of induction with neurogenic medium, hDPSCs decreased in proliferation and acquired a stellate morphology with no differences among the groups (Figure 4.1o). qRT-PCR analysis indicated that Nestin and β-tubulin III mRNA expression profile decreased with aging (Figure 4.1p). WB protein expression patterns of early (Nestin) and intermediate (β-tubulin III) neuronal markers showed an increase of Nestin 90 kDa in hDPSCs with age, whilst no changes were detected for Nestin 120 kDa and β-tubulin III (Figure 4.1q). Nestin and β-tubulin III IF detection evidenced age-related differences in their intensity and localization (Figure 4.1o).

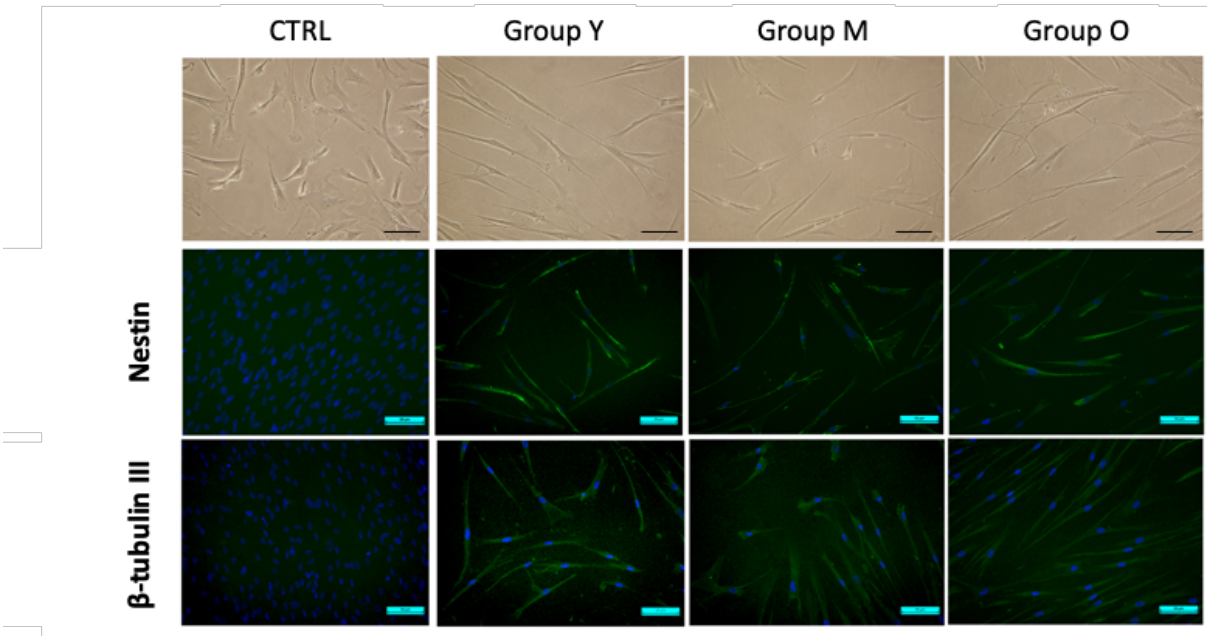

Figure 4.1o. Representative images displaying the morphology of hDPSCs after culturing in neurogenic media and the immunofluorescence detection of Nestin and β-tubulin III (scale bars = 50 μm).

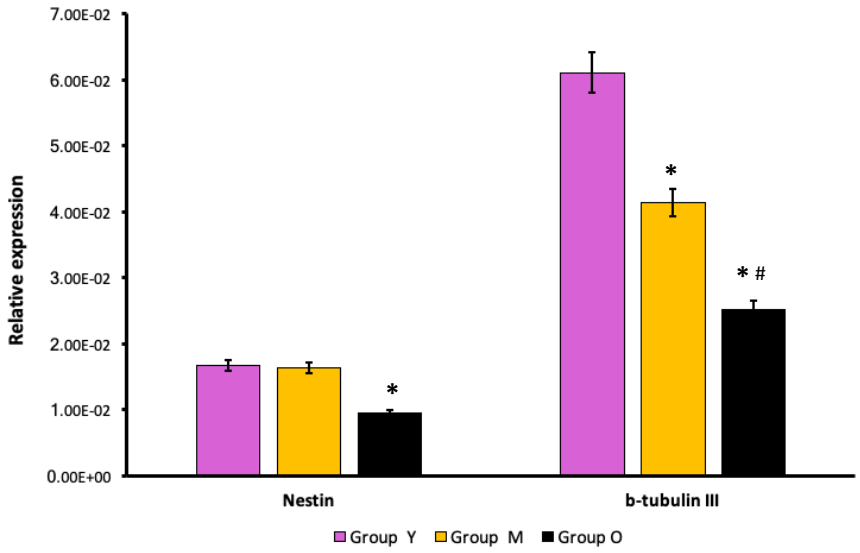

Figure 4.1p. Histogram depicts changes in the expression of Nestin and β-tubulin III mRNAs in the differently aged hDPSCs after neurogenic differentiation. Data are expressed as relative expression ($2^{-\Delta Ct}$).

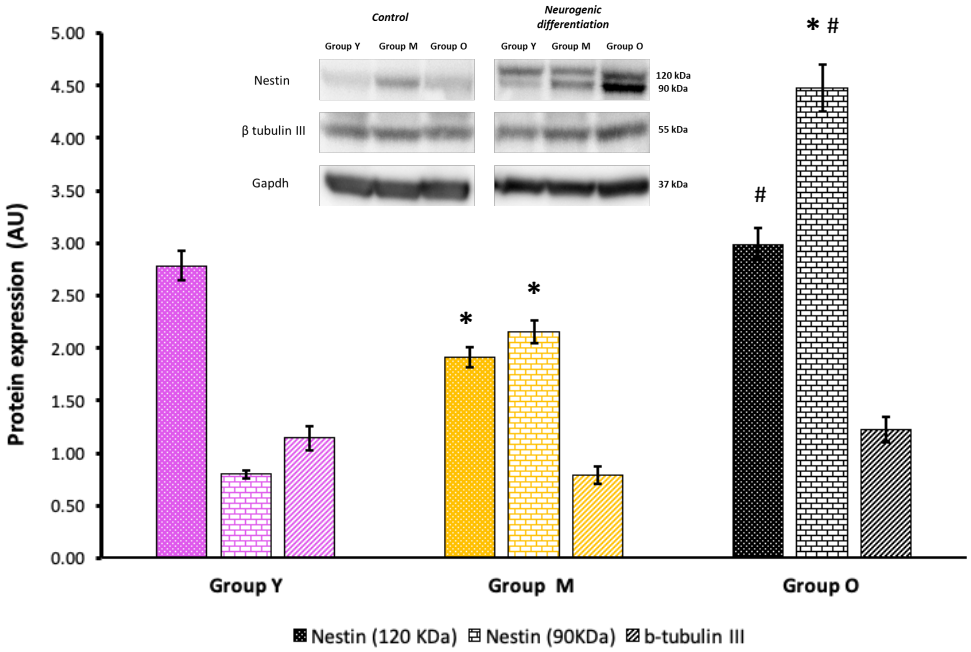

Figure 4.1q. Western blot analysis of Nestin and β-tubulin III protein expression; *$p < 0.05$ versus group Y, $^{\#}p < 0.05$ versus group M.

4.2 Second study

In this study, we evaluated the effect of an age-related microenvironment on the biological performances of young hDPSCs and vice versa.

4.2.1 hDPSCs Proliferative capacity and apoptosis

To ascertain the effect of the microenvironment on differently aged hDPSC, we examined the density and morphological aspect of cells, as well as the proliferation and apoptosis, under a Nikon Eclipse E600 light microscope. Changes in hDPSCs size were observed. Cells of group O showed an enlarged shape in comparison to cells of group Y. No change in morphology was found in cells of test groups (Figure 4.2a).

Ki67 immunofluorescence and TUNEL staining showed that the age-related microenvironment affected the differently aged hDPSC proliferative ability, as well as their apoptosis (Figure 4.2b). After the treatment with the reversed conditioned media, the cells of group Y-test have declined in their number, on the contrary those of group O-test grew considerably (Figure 4.2c).

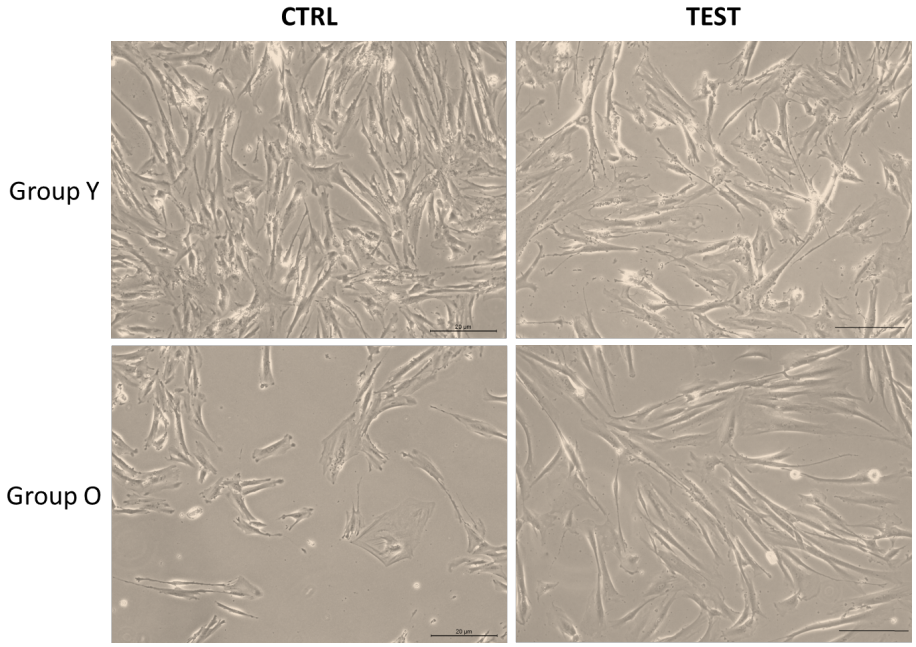

Figure 4.2a. Phase contrast representative images displaying the density and the morphology of hDPSCs isolated from the two different aged groups before (ctrl) and after (test) culturing in DPC-CM of group Y and DPC-CM of group O (scale bars = 20 μm).

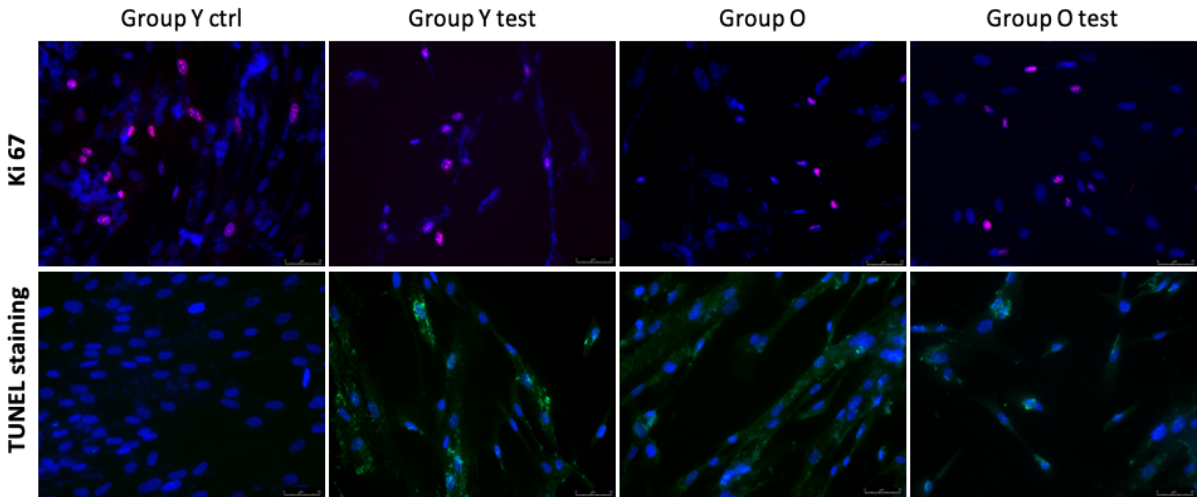

Figure 4.2b. Representative immunofluorescence images showing proliferation (Ki67) and apoptosis (TUNEL staining) in differently aged hDPSCs from control and test groups (scale bars = 25 μm).

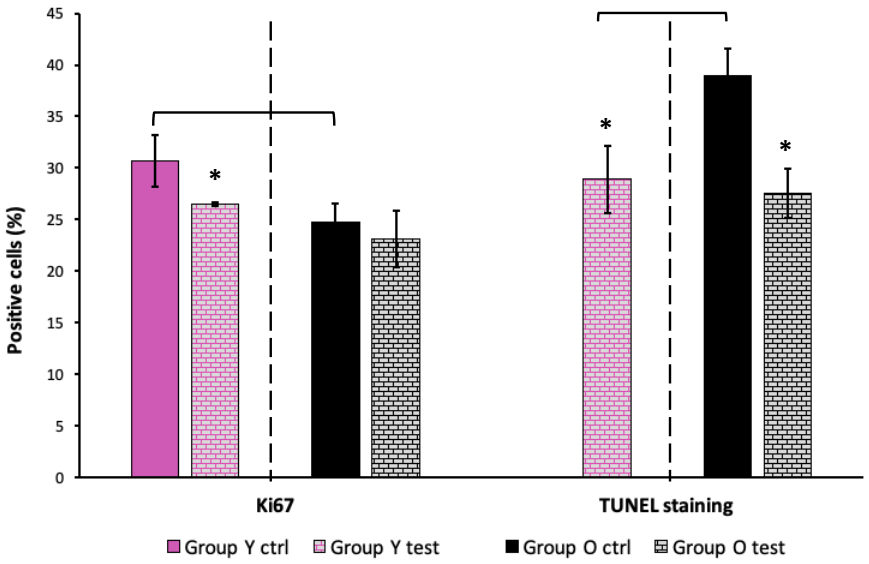

Figure 4.2c. Histograms depict changes in hDPSC proliferation (Ki67) and apoptosis (TUNEL staining) rate. *$p < 0.05$ versus group ctrl.

4.2.2 hDPSC stemness and senescence: gene expression changes

qRT-PCR analysis displayed modifications in the expression of MSC-specific genes in relation to age and microenvironment (Table 4.2). Comparing gene expression of hDPSCs from group Y with those of group O, downregulation of CD90, CD105, CD73 mRNA levels was shown. The expression of CD90 and CD105 appeared upregulated in cells of group O-test compared to its control group (Figure 4.2d). Also, the level of Notch3 mRNA in the cells of group Y-test was detected higher than in the hDPSCs of group Y ctrl (Figure 4.2e). No significant difference in self-renewal genes (Nanog, Oct4 and Notch2) gene expression was observed.

As concerns senescence-specific genes, qRT-PCR analysis indicated that $p16^{ink4a}$ and p21 mRNA expression profile enhanced with ageing and in group O-test compared to its control group (Figure 4.2f). No changes were found in p53 mRNA levels.

	Group Y ctrl		Group Y test		Group O ctrl		Group O test	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
CD90	7.7E-06	1.2E-06	9.5E-06	2.1E-06	2.2E-05	1.8E-06	5.4E-05	1.3E-05
CD105	8.4E-06	3.3E-06	1.1E-05	2.1E-06	2.4E-05	4.4E-06	4.1E-05	7.0E-06
CD73	3.4E-05	1.0E-05	5.6E-05	2.0E-06	1.2E-04	3.3E-05	1.4E-04	2.1E-05
Oct4	1.6E-07	3.7E-08	2.5E-07	4.4E-08	4.1E-07	1.7E-07	3.0E-07	4.7E-08
Nanog	1.0E-07	1.3E-08	1.2E-07	5.4E-08	2.0E-07	7.0E-08	2.4E-07	6.1E-08
Notch2	2.5E-04	2.9E-05	2.0E-04	3.4E-05	3.0E-04	4.2E-05	2.6E-04	7.5E-05
Notch3	2.9E-05	5.0E-06	5.3E-05	2.2E-05	1.0E-04	5.4E-05	1.2E-04	2.8E-05
p16	4.3E-07	2.0E-07	5.6E-07	6.0E-08	9.9E-07	1.9E-07	1.3E-06	4.8E-08
p21	3.4E-06	6.2E-07	2.9E-06	3.4E-08	6.3E-06	1.2E-06	9.4E-06	6.4E-07
p53	7.5E-05	7.6E-06	8.0E-05	1.9E-05	1.0E-04	3.3E-05	1.1E-04	2.8E-05

Nanog=Homeobox protein Nanog; Notch2=notch homolog 2, translocation-associated (Drosophila); Notch3=notch homolog 2, translocation-associated (Drosophila); Oct4=octamer-binding transcription factor 4; n.s.=not significant

Table 4.2. mRNA relative expression in hDPSCs of control and test groups.

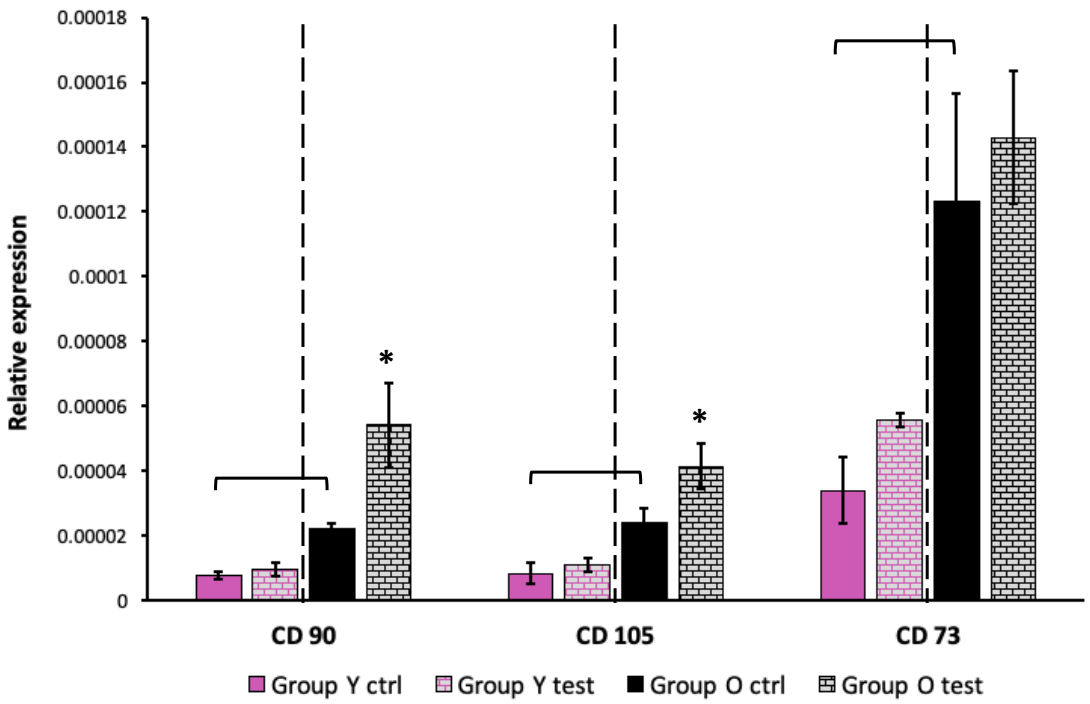

Figure 4.2d. Histograms depict changes in the expression of MSC-specific genes in the control and test groups of differently aged hDPSCs. Data are expressed as relative expression ($2^{-\Delta Ct}$); *p < 0.05 versus group ctrl.

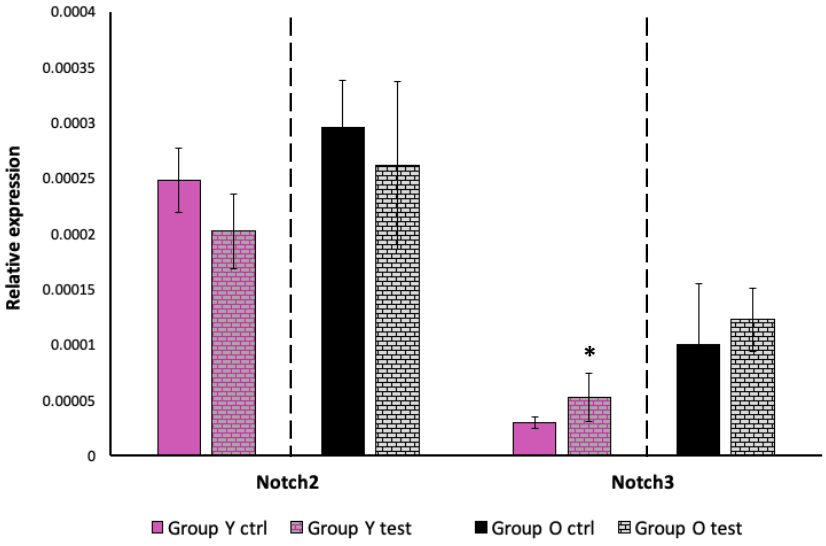

Figure 4.2e. Histograms depict changes in the expression of Notch2 and Notch3 mRNAs in the control and test groups of differently aged hDPSCs. Data are expressed as relative expression ($2^{-\Delta Ct}$); *p < 0.05 versus group ctrl.

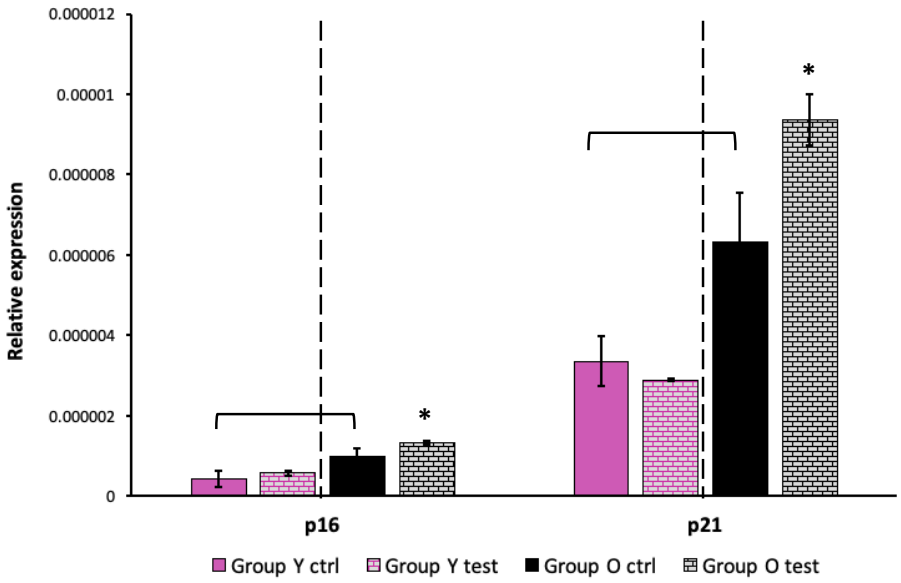

Figure 4.2f. Histograms depict changes in senescence gene mRNA expression in the control and test groups of differently aged hDPSCs. Data are expressed as relative expression ($2^{-\Delta C_t}$); *p < 0.05 versus group ctrl.

4.3 Third study

In the last study, we investigated the effects of commonly used nanofilled restorative composites on hDPSCs proliferation, apoptosis and differentiation potential, as well as in matrix mineralization.

4.3.1 hDPSCs proliferation and apoptosis

To assess the biocompatibility of commonly used nanofilled restorative composite (Filtek Supreme XTE, 3M) on hDPSCs, we evaluated cell morphology, proliferation and apoptosis after three weeks of *in vitro* culture (group B). Then, we compared results with two control groups: hDPSCs cultured in tissue culture plates (TCPs) (ctrl group) and presence of dentine (group A) for the same time. hDPSCs from the three groups were spindle-shaped and those of group A displayed abundance in ECM in comparison with the other two groups (Figure 4.3a). MTT assay evidenced that the cells of all groups possessed valid proliferation capacity.

In particular, the proliferation decreased in hDPSCs of groups A and B in comparison to those of ctrl group, during the first two weeks. In the third week, the growth rate of cells from group

B remained slightly lower than those of group A (Figure 4.3b). Moreover, they showed several brighter nuclei suggesting the presence of apoptotic bodies (Figure 4.3a).

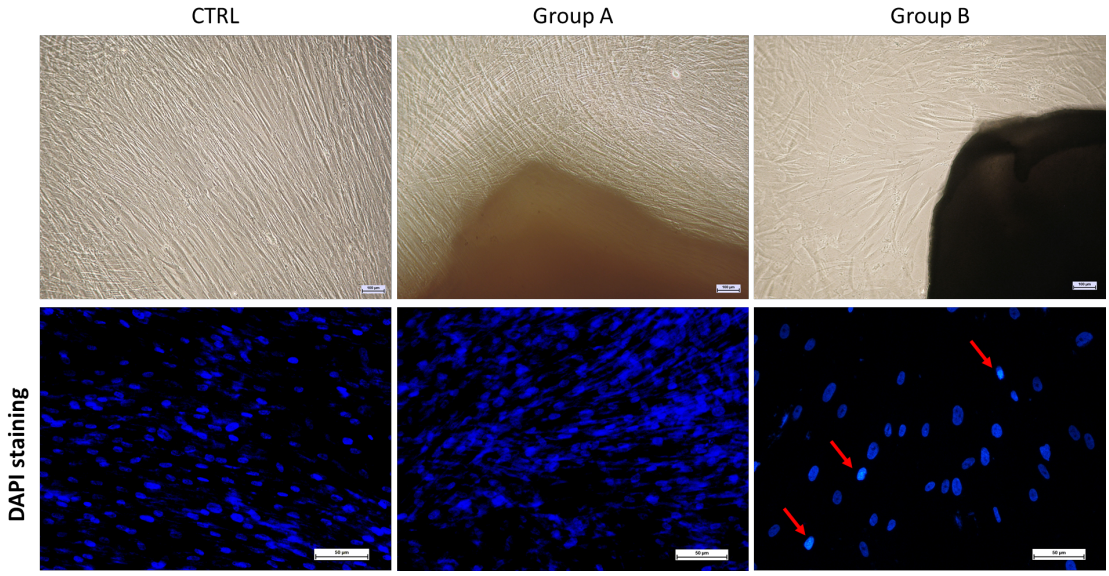

Figure 4.3a. hDPSCs morphology after 3 weeks of culture in tissue culture plates (TCPs) (ctrl group), presence of dentine (group A) or composite (group B) (scale bars = 100 µm). Apoptosis detection through DAPI staining after 3 weeks of hDPSC cultures (scale bars = 50 µm).

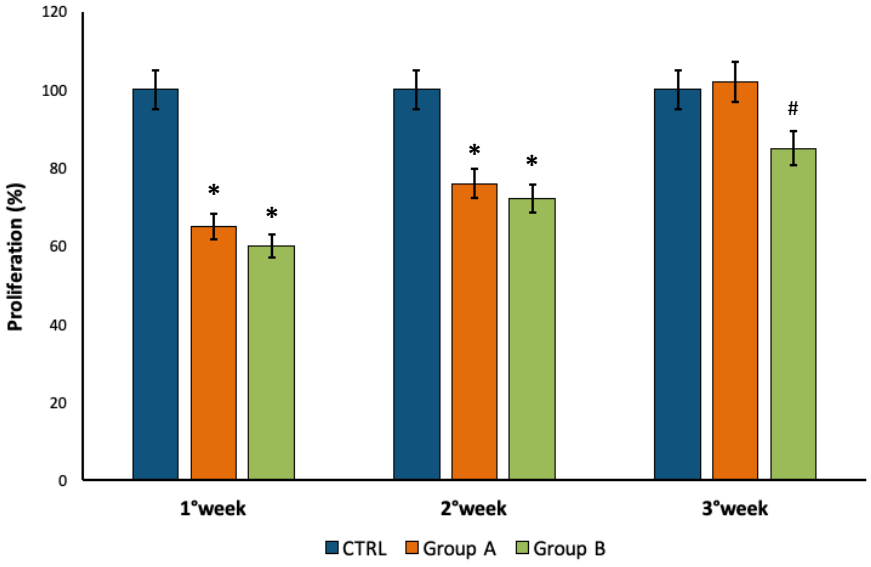

Figure 4.3b. MTT viability assay. Histograms depict modifications in the proliferation capacity of hDPSCs in the three groups, during the 3 weeks of culture. *$p < 0.05$ versus ctrl group, $^{\#}p < 0.05$ versus group A.

4.3.2 Odontogenic differentiation in hDPSCs

It was interesting to observe that, after three weeks of culture with complete culture medium DMEM/F12, hDPSCs expressed odontogenic differentiation-related genes, such as DMP1 and DSPP (Table 4.3). Notably, downregulation of DSPP gene expression was found in cells of group B in comparison to those of the other two groups. On the other hand, no significant changes were detected for DMP1 mRNA levels (Figure 4.3c).

Moreover, WB analysis revealed a pronounced expression of DMP1 protein aggregates (120 kDa) in all groups. However, in contrast with cells of ctrl and A groups, hDPSCs cultured in presence of Filtek Supreme XTE composite showed no DMP1 full-length form (105 kDa) (Figure 4.3d).

	CTRL		Group A		Group B	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
DMP1	4.44E-07	1.18E-07	5.02E-07	7.49E-08	5.42E-07	6.97E-08
DSPP	1.23E-07	1.40E-08	1.26E-07	1.78E-08	3.16E-08	7.24E-09

DMP1=Dentin matrix acidic phosphoprotein 1; DSPP=Dentin sialophosphoprotein; n.s.=not significant

Table 4.3. mRNA relative expression in hDPSCs after 3 weeks of culture.

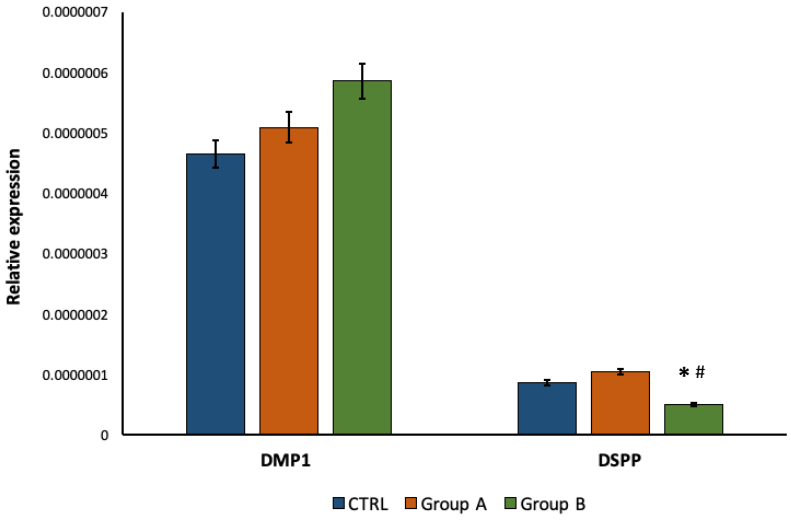

Figure 4.3c. Histograms depict changes in the expression of DMP1 and DSPP mRNAs in hDPSC of the three different groups. Data are expressed as relative expression ($2^{-\Delta Ct}$); *p < 0.05 versus ctrl group, $^{\#}$p < 0.05 versus group A.

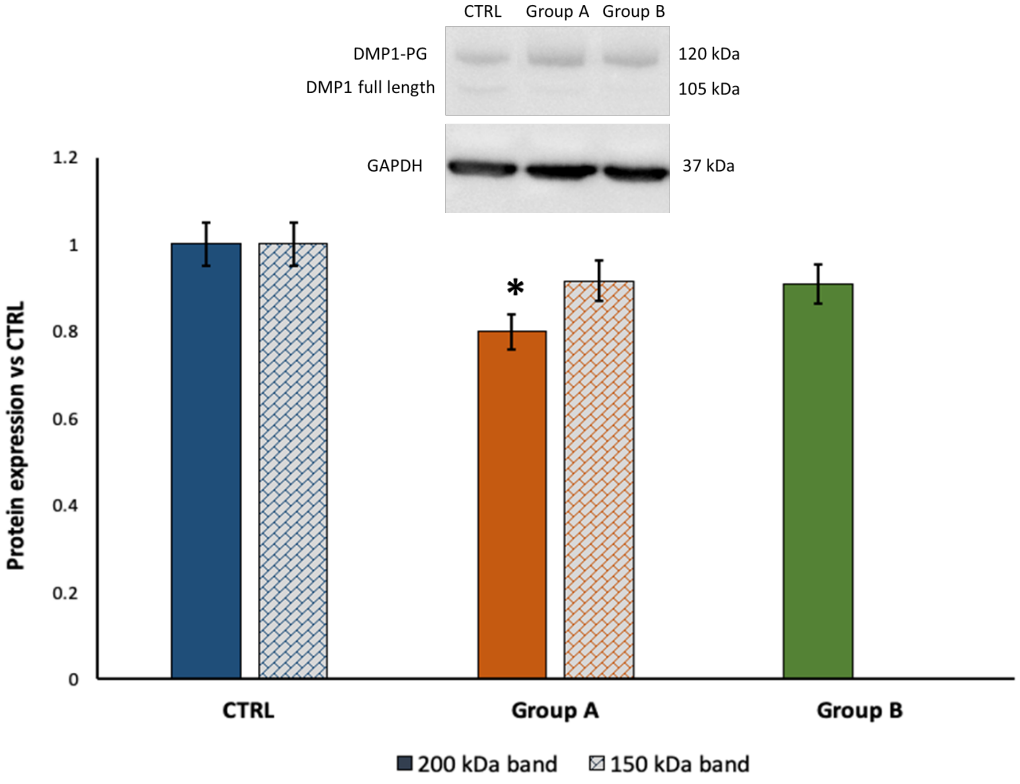

Figure 4.3d. Western Blotting bands and histogram depict DMP1 protein expression changes in hDPSCs cultured in three different conditions after three weeks. *p < 0.05 versus ctrl group.

4.3.3 hDPSCs mineralization capability

ARS staining was performed to examine the impact of the current biomaterial on hDPSCs mineralization. Interestingly, ARS quantization evidenced a decline in mineralization capability of hDPSCs from group B (Figure 4.3e).

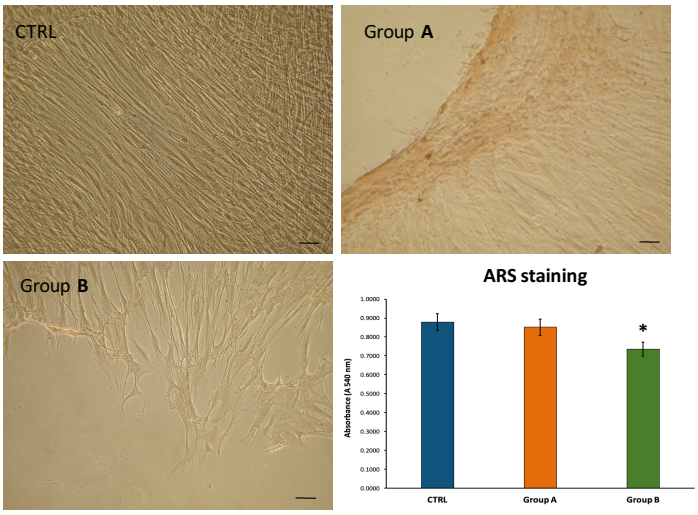

Figure 4.3e. Alizarin Red (ARS) staining showing hDPSC mineralization capability (scale bars = 100 µm). Histograms of ARS quantization in hDPSCs cultured in three different conditions after three weeks. *$p < 0.05$ versus ctrl group.

5 DISCUSSION

5.1 First study

Although great progress has been made in dentistry, the treatment of pulpitis and periodontitis continues to have postoperative complications. Nowadays regenerative approaches, as well as biomaterials commonly used in dentistry, have been considered for patients without a specific age target. The world's population is ageing, and it has been appraised that by 2050 the number of people 65 years of age and older will reach 1.5 billion (Richard and John 2011). Hence, the concept of customized therapies must meet this contingency. Like others, dental pulp tissue undergoes age-depended changes and the decline of its regenerative capacity might be related to stem cell senescence (Chu 2013). Up to now, few and conflicting data are available on possible age-related changes in the regenerative potential of DPSCs (Chu 2013; Yi et al. 2017). These cells possess a great self-expansion and differentiation capabilities toward osteogenic (Teti et al. 2015), odontogenic (Gronthos et al. 2002) and neurogenic (Gervois et al. 2015) phenotypes, making them an interesting cell source for regenerating pulp and periodontal tissues, and in the treatment of neurodegenerative diseases.

Based on these considerations, we investigated changes in morphology, proliferation, and *in vitro* differentiation potential in hDPSCs isolated from differently aged donors. Cells were split into juvenile (group Y), middle-age (group M), and aged (group O) clusters based on the donors' age (Bernick and Nedelman 1975). No significant age-related morphological differences were detected. On the contrary, we noticed a significant increase of SA-β-Gal and $p16^{ink4a}$ nuclear expression in group O compared with both groups Y and M. SA-β-Gal is a lysosomal enzyme highly correlated with cellular senescence in cultured cells and in tissues from various animals (Dimri et al. 1995). $P16^{ink4a}$ is a protein involved in cell cycle progression (X. Feng et al., 2014), and along with SA-β-Gal activity, is an established biomarker of senescent cells (Dimri et al. 1995). These changes were in accordance with the cytofluorimetric detection of an augmented internal complexity (i.e., SSC values) with ageing. We additionally investigated if hDPSCs progressively modify their self-renewal gene expression during ageing, undergoing a dysregulation of proliferative activities and declining functional capacity as described for other MSCs (Oh, Lee, and Wagers 2014).

The evaluation of the gene expression profile of Sox2, Oct4, Nanog, and Klf4 transcription factors experienced changes in all age groups. These molecules are critical to preserving stem cell phenotype and, therefore, their impairment could be responsible for the loss of stemness properties and pluripotency commitment of DPSCs (Gronthos et al. 2000; C.-E. Huang et al. 2014). The upregulation of Klf4 detected during ageing (i.e. in cells of group O) could be at least in part due to its role in the odontoblastic differentiation and inhibition of hDPSC proliferation (Lin et al. 2011), as evidenced by our results. Further confirmation of age-related changes in our hDPSCs was represented by the shortening of telomere length in both groups M and O in comparison with cells of group Y (Mokry et al. 2010).

No changes in the expression pattern of the common ISCT markers (Dominici et al. 2006; Teti et al. 2015) were detected independently from the age of donors. On the contrary impaired osteogenic and adipogenic potentials were found in hDPSCs of groups M and O in comparison with group Y, noticing a marked decline in group O, as suggested by previous work (Yi et al. 2017). Concerning odontoblastic differentiation, qRT-PCR showed a marked reduction of DMP1 and DSSP mRNAs in cells from aged subjects in comparison to young ones. The decrease in DMP1 and DSSP expression was less marked in cells cultured without odontogenic media in comparison to differentiated hDPSCs. DMP1 is an acidic no collagenous protein naturally present in bone and dentine extracellular matrix (ECM) as proteolytically processed fragments (Chunlin Qin et al. 2003, 20). DMP1 occurs predominantly as a C-terminal (57 kDa) and an N-terminal (37kDa) fragments, both promoters of HA formation and growth. Further aggregates occur as DMP1 proteoglycan fragment (DMP1-PG; C. Qin, D'Souza, and Feng 2007), an inhibitor of mineralization (Gericke et al. 2010). DMP1 is implicated in a complex biological process: It is initially localized in the nucleus, where it acts as transcriptional factor, during cell maturation DMP1 is in the cytoplasm and, further, Ca^{2+} is responsible for its export into the ECM, where it regulates the nucleation of HA and the formation of calcified tissue (Narayanan et al. 2003). The WB of cytoplasmic DMP1 expression, showed an increase in DMP1 full-length form (105 kDa) and N-terminal fragment in hDPSCs after three weeks of culture without differentiating medium, with a marked amount in cells from group O. This suggests that *in vitro* our cells possess a spontaneous odontogenic potential, albeit mineralized nodules were not evident as suggested by ARS.

After hDPSC odontoblastic differentiation, 37 kDa fragment of DMP1 decreased in the cytoplasm in support of ECM mineralization (Maciejewska et al. 2009).

The decrease of differentiation potential toward mineralized tissues (i.e., bone and dentine) with age was also sustained by the changes in the expression of Bmp2, β-catenin, Runx2, and BGLAP (osteocalcin) after odontogenic differentiation (Ferretti et al. 2015). In hDPSCs, β-catenin is upregulated during *in vitro* odontogenic differentiation (Han et al. 2014) and its overexpression is linked to the suppression of mineralization (Scheller, Chang, and Wang 2008). On the contrary, Runx2 expression must be downregulated in odontoblast to reach full differentiation for a correct dentineogenesis (S. Chen et al. 2005). In this regard, the increase of mineralized nodules in hDPSCs of group B treated with odontogenic medium may be connected to the diffusing calcific degeneration of pulp, a pathologic condition occurring as a response to ageing (Piattelli and Trisi 1993).

A vast body of evidence indicates that neural crest-derived DPSCs express specific markers of the neuronal cells (Arthur et al. 2008; Chai et al. 2000). Consequently, they might provide a promising source of stem cells for therapeutic application in neurodegenerative diseases. The comparison of our biological and morphological analyses evidenced not only an age-related decrease of markers of neurogenic differentiation (Feng et al. 2013), but also an incorrect localization of β-tubulin III (Martens et al. 2012).

5.2 Second study

HDPSCs undergo a decline in stemness and differentiation potential with ageing, as we observed in the previous study. In particular, significative differences exist between cells isolated from young and aged donors, in terms of proliferation and mineralization ability (Yi et al. 2017). These findings are of clinical relevance since the less cellularity of pulp tissue, along with the altered immune functioning that accompanies increasing age (Preshaw et al. 2017), can make the tooth of old individuals more susceptible to traumatic and carious lesions (Kawasaki et al. 2000). Moreover, it is well known that aged cells significantly affect their microenvironment, as they secrete pro-inflammatory and matrix-degrading molecules, called senescence-associated secretory phenotype (SASP) (Childs et al. 2015). In this proposal, the impact of these factors on MSCs isolated from young individuals and those of young

microenvironment on aged cells could be interesting to examine, for better understanding the biological mechanisms behind hDPSCs senescence processes.

Based on these concerns, the second study was designed to reveal *in vitro* the effects of an age-related microenvironment on the biological performance of hDPSCs isolated from young and old subjects. Harvested cells were divided into juvenile (group Y ctrl) and aged (group O ctrl) groups based on the donors' age. Then, DPSCs of group Y were treated with DPSC-CM of group O (group Y-test) and vice versa (group O-test). From morphological analysis significant differences were detected among the two control groups, as well as in test groups compared to their controls. Also, we noticed a significant decrease in Ki67 expression in cells of group O and Y-test in comparison with group Y. The Ki67 protein is a cellular marker for proliferation and it is strictly associated with cell grown (Scholzen and Gerdes 2000). Ki67 antigen can be exclusively detected within the cell nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosome (Cuylen et al. 2016). The variability of proliferation appears to reflect differences in hDPSCs apoptosis rate, using TUNEL assay. TUNEL staining is a method used to identify and quantify apoptotic cells by enzymatic catalysis (Kyrylkova et al. 2012). Taken together, Ki67 and TUNEL results stated that proliferation ability underwent a decline with age in hDPSCs in favour of apoptosis, as confirmed by previous studies (Yi et al. 2017). Moreover, both age-related DPC-CMs seemed to have a certain influence on the modulation of cell death in the two groups. Similarly to recent results on murine DPSCs, we also provided evidence that the aged DPC-CM negatively affect the juvenile hDPSC grown (Ma et al. 2009). This phenomenon could be due to the whole set of pro-inflammatory and matrix-degrading molecules (SASP), along with exosomes and miRNAs existing in the aged MSC secretome (Childs et al. 2015; Coppé et al. 2010; Xu and Tahara 2013).

We additionally verified if hDPSCs progressively lose their mesenchymal potential during ageing, otherwise after the treatment with the differently aged DPC-CMs. The hDPSCs of all groups expressed comparable levels of MSC-specific genes (CD90, CD105, CD73), although many differences existed. Despite it is well known that ageing leads to decline of hDPSCs properties (Iezzi et al. 2019), CD90, CD105, and CD90 mRNA levels were upregulated in cells of group O in comparison to those of group Y. These changes may be associated with the post-translational gene silencing by specific miRNAs (i.e. miR-195), rather than by epigenetic factors (Cakouros and Gronthos 2019; Ermolaeva et al. 2018).

Recently, the role of miR-195 in stem cell ageing has been studied. In particular, the abrogation of this miRNA appears to rejuvenate MSCs from aged subjects by telomere re-lengthening and anti-ageing markers reactivation (Okada et al. 2016). Interestingly, the different regulation of Notch3 mRNA levels could be tied to the decline in cell proliferation of group Y-test. In fact, the expression of Notch3 has been found to activate senescence process in human cells (Cui et al. 2013). On the other hand, the evaluation of the self-renewal gene (Nanog, Oct4 and Notch2) expression showed no significant differences.

Further confirmation of age-related changes in hDPSCs from different groups was represented by qRT-PCR detection of senescence-related genes. In fact, both $p16^{ink4a}$ and p21 mRNA levels displayed an increment in cells of group O in comparison to group Y. The $p16^{ink4a}$ and p21 cyclin-dependent kinase (CDK) inhibitors are known to play key roles in the onset of cellular senescence. Their simultaneous induction cooperatively blocks the activation of both cyclin D kinase (CDK4/6) and cyclin E kinase (CDK2), allowing the accumulation of the retinoblastoma tumour suppressor protein (pRb) and thereby causing permanent cell cycle arrest (Takahashi et al. 2006; Takeuchi et al. 2010).

Finally, variations in mesenchymal potential, as well as in the expression of senescence-related genes in group O-test compared to its control, demonstrated that there is a positive impact of soluble factors secreted by the young DPC-CM on aged cells; however, the cells of group O-test maintain the hallmark features of senescent cells.

5.3 Third study

During the Ph.D. abroad, in collaboration with the Institute of Oral Biology, University of Zurich, my research activity was focused on the behave of hDPSCs in presence of composite for dental clinical use.

Resin-based dental composites have been considered as a biological and functional acceptable surrogate to mercury-containing silver amalgam filling in clinical practice (Sunnegårdh-Grönberg et al. 2009). Despite their growing use in restorative dentistry in the past decades, resin composites are thought to release cytotoxic components due to their degradation or erosion over time, such as resin monomers and ions (Moharamzadeh et al. 2007; Saxena et al. 2012). To date, ground-breaking modifications of classic resin-based composites have been

performed, with the aim of minimizing polymerization shrinkage, improve marginal integrity, and reduce adverse biocompatibility (Schubert et al. 2019). Nanocomposites represent an example of new generation of resin composite and contain nanofillers ranging from 1 to 100 nm^2. Due to reduced particle size, these materials exhibit very good resistance to wear and fracture, along with advances in optical properties (Pecho et al. 2016; Scribante et al. 2019). However, there are few and conflicting knowledge about the toxic effect of these recent composites on dentine-pulp complex (Putzeys et al. 2019; Schubert et al. 2019). Considering the key role of pulp cells in dentine regeneration processes after traumatic or carious lesions (Sloan 2015), we analysed the effects of a commonly used restorative nanofilled composite (Filtek Supreme XTE, 3M) on DPSC viability, proliferation, and mineralization ability.

Cells were cultured on TCPs (ctrl group), presence of dentine (Group A) or composite (Group B) during three weeks, time required for the *in vitro* DPSC odontogenic differentiation (Teti et al. 2015; Paduano et al. 2016). We considered group A as a further control, since comparing our biomaterial with dentine, the natural structure in which dental pulp cells depose the new mineralized matrix (Arana-Chavez and Massa 2004), could result crucial.

As concern proliferation, results appeared comparable among the three clusters, albeit cells of group B showed a slight decline during the third week of culture. This could be due, at least in part, to the increase of apoptotic activity of hDPSCs in contact with the dental composite. Therefore, Filtek Supreme XTE has no biotoxicity on hDPSCs, but it might act as environmental stressor, perhaps for containing resin monomers (i.e. Bis-GMA, TEGMA, UDMA) (Krifka et al. 2013; Ausiello et al. 2013).

DPSCs produce human dentine *in vitro* (About et al. 2000). In addition, it is well known that dentine creates an *in vivo* favourable microenvironment for ECM production from odontoblasts (Arana-Chavez and Massa 2004). Our results are in line with these observations, as we detected an increase in cell number and matrix deposition in group A in comparison with cells cultured with nanofilled material. Moreover, this finding is in accordance with a previous study on deciduous teeth stem cells, in which low concentration of HEMA and TEGMA in dental resin composite can severely disrupt the normal differentiation toward odontogenic lineage (Bakopoulou et al. 2011). The delay in the mineralized matrix deposition was also accompanied by significant downregulation of DSPP gene expression.

Despite the fact that DSPP has been found in trace amounts in bone, it is considered a representative marker of odontoblastic differentiation, having an active role in the mineralization of dentine matrix (Chunlin Qin et al. 2002). No changes in mRNA levels of DMP1 were detected, although DMP1 protein expression was less marked in hDPSCs of group B. As we mentioned in the first study, this acidic NCP protein is naturally present in bone and dentine ECM as proteolytically processed fragments, C-terminal (57 kDa) and N-terminal (37 kDa) (Chunlin Qin et al. 2003). Additional aggregates occur as proteoglycan fragment (DMP1-PG) ranging around 200 kDa, an inhibitor of mineralization (Gericke et al. 2010). WB bands of cytoplasmic DMP1 indicated the absence of DMP1 full-length form (105 kDa) after three weeks of hDPSCs cultured in presence of composite. Together with ARS analysis, these data suggest that a three-week exposure of hDPSC cultures to the dental nanofilled composite could significantly delay the physiological differentiation and mineralization processes of these cells.

6 CONCLUSION AND FUTURE PERSPECTIVES

Overall our results confirmed that hDPSCs biological properties, as well as stemness and differentiation potential, could be differently impaired by ageing. Moreover, there are local factors modulating the activity of hDPSCs. In fact, cell-conditioned media of the age-related extrinsic microenvironment have been able to modify the biological performance of hDPSCs isolated from young and old subjects. These observations must be considered for the development of customized strategies based on the possibility to encourage osteogenesis, pulpogenesis, and dentineogenesis as well as for application in cell therapy to treat neurological disorders. DPSCs of young donors may provide an ideal source of stem cells that could extend their therapeutic application in oral (i.e., alveolar bone, dental pulp, and dentine regeneration) and neurodegenerative (i.e., Alzheimer and Parkinson) diseases. Aged DPSCs could be used as *in vitro* tool for the study of biomaterials solving the challenges of an ageing population.
Considering the immunomodulative properties of MSCs, additional *in vivo* investigation also assessing this phenomenon in DPSCs during ageing, will be hugely important, making them potential novel immunotherapeutic resources in inflammatory age-related diseases. Furthermore, it will be of great interest to better identify the specific roles of senescence-

associated factors that have such influence on supporting hDPSCs age-related changes or responses.

Lastly, the experiments of our third investigation provide evidence that long-term exposure to dental restorative nanofilled composite is able to significantly delay and even disrupt the physiological migration, odontogenic differentiation and mineralization process of hDPSCs, when compared to both control groups, where the dentine-slice is added in culture as well as in TCPs.

This observation is of outmost importance for clinical applications since in traumatic or carious lesions the pulp cells will have a decreased capability to regenerate the already vulnerable dental tissues. Additional studies are needed to validate safety and efficiency of the new composites as the golden standard in dental restorations. Finally, to further understand mechanisms behind the impairment of hDPSC mineralization process might be the key factor for developing a new generation of bioactive dental composites.

References

About, Imad, Marie-José Bottero, Philippe de Denato, Jean Camps, Jean-Claude Franquin, and Thimios A. Mitsiadis. 2000. "Human Dentin Production in Vitro." *Experimental Cell Research* 258 (1): 33–41. https://doi.org/10.1006/excr.2000.4909.

Anitua, Eduardo, María Troya, and Mar Zalduendo. 2018. "Progress in the Use of Dental Pulp Stem Cells in Regenerative Medicine." *Cytotherapy* 20 (4): 479–98. https://doi.org/10.1016/j.jcyt.2017.12.011.

Anzalone, Rita, Melania Lo Iacono, Simona Corrao, Francesca Magno, Tiziana Loria, Francesco Cappello, Giovanni Zummo, Felicia Farina, and Giampiero La Rocca. 2010. "New Emerging Potentials for Human Wharton's Jelly Mesenchymal Stem Cells: Immunological Features and Hepatocyte-Like Differentiative Capacity." *Stem Cells and Development* 19 (4): 423–38. https://doi.org/10.1089/scd.2009.0299.

Aquino, R. d', A. Graziano, M. Sampaolesi, G. Laino, G. Pirozzi, A. De Rosa, and G. Papaccio. 2007. "Human Postnatal Dental Pulp Cells Co-Differentiate into Osteoblasts and Endotheliocytes: A Pivotal Synergy Leading to Adult Bone Tissue Formation." *Cell Death and Differentiation* 14 (6): 1162–71. https://doi.org/10.1038/sj.cdd.4402121.

Aramillo Irizar, Peer, Sascha Schäuble, Daniela Esser, Marco Groth, Christiane Frahm, Steffen Priebe, Mario Baumgart, et al. 2018. "Transcriptomic Alterations during Ageing Reflect the Shift from Cancer to Degenerative Diseases in the Elderly." *Nature Communications* 9 (1): 327. https://doi.org/10.1038/s41467-017-02395-2.

Arana-Chavez, Victor E, and Luciana F Massa. 2004. "Odontoblasts: The Cells Forming and Maintaining Dentine." *The International Journal of Biochemistry & Cell Biology* 36 (8): 1367–73. https://doi.org/10.1016/j.biocel.2004.01.006.

Arthur, Agnes, Grigori Rychkov, Songtao Shi, Simon Andrea Koblar, and Stan Gronthos. 2008. "Adult Human Dental Pulp Stem Cells Differentiate toward Functionally Active Neurons under Appropriate Environmental Cues." *Stem Cells (Dayton, Ohio)* 26 (7): 1787–95. https://doi.org/10.1634/stemcells.2007-0979.

Askari, Nahid, Mohammad Mehdi Yaghoobi, Mehdi Shamsara, and Saeed Esmaeili-Mahani. 2014. "Human Dental Pulp Stem Cells Differentiate into Oligodendrocyte Progenitors Using the Expression of Olig2 Transcription Factor." *Cells Tissues Organs* 200 (2): 93–103. https://doi.org/10.1159/000381668.

Ausiello, Pietro, Angela Cassese, Claudia Miele, Francesco Beguinot, Franklin Garcia-Godoy, Bruno Di Jeso, and Luca Ulianich. 2013. "Cytotoxicity of Dental Resin Composites: An in Vitro Evaluation." *Journal of Applied Toxicology* 33 (6): 451–57. https://doi.org/10.1002/jat.1765.

Bakopoulou, Athina, Gabriele Leyhausen, Joachim Volk, Asterios Tsiftsoglou, Pavlos Garefis, Petros Koidis, and Werner Geurtsen. 2011. "Effects of HEMA and TEDGMA on the in Vitro Odontogenic Differentiation Potential of Human Pulp Stem/Progenitor Cells Derived from Deciduous Teeth." *Dental Materials* 27 (6): 608–17. https://doi.org/10.1016/j.dental.2011.03.002.

Balic, Anamaria, and Irma Thesleff. 2015. "Tissue Interactions Regulating Tooth Development and Renewal." In *Current Topics in Developmental Biology*, 115:157–86. Elsevier. https://doi.org/10.1016/bs.ctdb.2015.07.006.

Bartel, David P. 2018. “Metazoan MicroRNAs.” *Cell* 173 (1): 20–51. https://doi.org/10.1016/j.cell.2018.03.006.

Bartold, P. Mark, Christopher a. G. Mcculloch, A. Sampath Narayanan, and Sandu Pitaru. 2000. “Tissue Engineering: A New Paradigm for Periodontal Regeneration Based on Molecular and Cell Biology.” *Periodontology 2000* 24 (1): 253–69. https://doi.org/10.1034/j.1600-0757.2000.2240113.x.

Batouli, S., M. Miura, J. Brahim, T.W. Tsutsui, L.W. Fisher, S. Gronthos, P. Gehron Robey, and S. Shi. 2003. “Comparison of Stem-Cell-Mediated Osteogenesis and Dentinogenesis.” *Journal of Dental Research* 82 (12): 976–81. https://doi.org/10.1177/154405910308201208.

Bernick, Sol, and Charles Nedelman. 1975. “Effect of Aging on the Human Pulp.” *Journal of Endodontics* 1 (3): 88–94. https://doi.org/10.1016/S0099-2399(75)80024-0.

Bronckaers, Annelies, Petra Hilkens, Yanick Fanton, Tom Struys, Pascal Gervois, Constantinus Politis, Wendy Martens, and Ivo Lambrichts. 2013. “Angiogenic Properties of Human Dental Pulp Stem Cells.” *PLOS ONE* 8 (8): e71104. https://doi.org/10.1371/journal.pone.0071104.

Budd, Emma, María C. de Andrés, Tilman Sanchez-Elsner, and Richard O. C. Oreffo. 2017. “MiR-146b Is down-Regulated during the Chondrogenic Differentiation of Human Bone Marrow Derived Skeletal Stem Cells and up-Regulated in Osteoarthritis.” *Scientific Reports* 7 (1): 1–11. https://doi.org/10.1038/srep46704.

Cakouros, Dimitrios, and Stan Gronthos. 2019. “Epigenetic Regulation of Bone Marrow Stem Cell Aging: Revealing Epigenetic Signatures Associated with Hematopoietic and Mesenchymal Stem Cell Aging.” *Aging and Disease* 10 (1): 174–89. https://doi.org/10.14336/AD.2017.1213.

Chai, Y., X. Jiang, Y. Ito, P. Bringas, J. Han, D. H. Rowitch, P. Soriano, A. P. McMahon, and H. M. Sucov. 2000. “Fate of the Mammalian Cranial Neural Crest during Tooth and Mandibular Morphogenesis.” *Development (Cambridge, England)* 127 (8): 1671–79.

Chamieh, Frédéric, Anne-Margaux Collignon, Benjamin R. Coyac, Julie Lesieur, Sandy Ribes, Jérémy Sadoine, Annie Llorens, et al. 2016. “Accelerated Craniofacial Bone Regeneration through Dense Collagen Gel Scaffolds Seeded with Dental Pulp Stem Cells.” *Scientific Reports* 6 (December): 38814. https://doi.org/10.1038/srep38814.

Chaussain, Catherine, Tchilalo Boukpessi, Mayssam Khaddam, Leo Tjaderhane, Anne George, and Suzanne Menashi. 2013. “Dentin Matrix Degradation by Host Matrix Metalloproteinases: Inhibition and Clinical Perspectives toward Regeneration.” *Frontiers in Physiology* 4. https://doi.org/10.3389/fphys.2013.00308.

Chen, Jennifer CJ, and David J Goldhamer. 2003. “Skeletal Muscle Stem Cells.” *Reproductive Biology and Endocrinology : RB&E* 1 (November): 101. https://doi.org/10.1186/1477-7827-1-101.

Chen, Shuo, Sheela Rani, Yimin Wu, Aaron Unterbrink, Ting Ting Gu, Jelica Gluhak-Heinrich, Hui-Hsiu Chuang, and Mary Macdougall. 2005. “Differential Regulation of Dentin Sialophosphoprotein Expression by Runx2 during Odontoblast Cytodifferentiation.” *The Journal of Biological Chemistry* 280 (33): 29717–27. https://doi.org/10.1074/jbc.M502929200.

Chen, Yu-Ying, Sheng-Teng He, Fu-Hua Yan, Peng-Fei Zhou, Kai Luo, Yan-Ding Zhang, Yin Xiao, and Min-Kui Lin. 2016. “Dental Pulp Stem Cells Express Tendon Markers under Mechanical Loading and Are a Potential Cell Source for Tissue Engineering of Tendon-

like Tissue." *International Journal of Oral Science* 8 (4): 213–22. https://doi.org/10.1038/ijos.2016.33.

Childs, Bennett G, Matej Durik, Darren J Baker, and Jan M van Deursen. 2015. "Cellular Senescence in Aging and Age-Related Disease: From Mechanisms to Therapy." *Nature Medicine* 21 (12): 1424–35. https://doi.org/10.1038/nm.4000.

Childs, Bennett G., Martina Gluscevic, Darren J. Baker, Remi-Martin Laberge, Dan Marquess, Jamie Dananberg, and Jan M. van Deursen. 2017. "Senescent Cells: An Emerging Target for Diseases of Ageing." *Nature Reviews. Drug Discovery* 16 (10): 718–35. https://doi.org/10.1038/nrd.2017.116.

Cho, Young-Ah, Kwantae Noh, Seong-Suk Jue, So-Youn Lee, and Eun-Cheol Kim. 2015. "Melatonin Promotes Hepatic Differentiation of Human Dental Pulp Stem Cells: Clinical Implications for the Prevention of Liver Fibrosis." *Journal of Pineal Research* 58 (1): 127–35. https://doi.org/10.1111/jpi.12198.

Choudhery, Mahmood S., Michael Badowski, Angela Muise, John Pierce, and David T. Harris. 2014. "Donor Age Negatively Impacts Adipose Tissue-Derived Mesenchymal Stem Cell Expansion and Differentiation." *Journal of Translational Medicine* 12 (1): 8. https://doi.org/10.1186/1479-5876-12-8.

Chu, Rene. 2013. "Age and Sex-related Differences in Dental Pulp Stem Cells." University of Iowa.

Collino, Federica, Stefania Bruno, Maria Chiara Deregibus, Ciro Tetta, and Giovanni Camussi. 2011. "MicroRNAs and Mesenchymal Stem Cells." *Vitamins and Hormones* 87: 291–320. https://doi.org/10.1016/B978-0-12-386015-6.00033-0.

Coppé, Jean-Philippe, Pierre-Yves Desprez, Ana Krtolica, and Judith Campisi. 2010. "The Senescence-Associated Secretory Phenotype: The Dark Side of Tumor Suppression." *Annual Review of Pathology* 5: 99–118. https://doi.org/10.1146/annurev-pathol-121808-102144.

Corrao, Simona, Giampiero La Rocca, Melania Lo Iacono, Giovanni Zummo, Aldo Gerbino, Felicia Farina, and Rita Anzalone. 2013. "New Frontiers in Regenerative Medicine in Cardiology: The Potential of Wharton's Jelly Mesenchymal Stem Cells." *Current Stem Cell Research & Therapy* 8 (1): 39–45. https://doi.org/10.2174/1574888X11308010006.

Costanza, Brunella, Ijeoma Adaku Umelo, Justine Bellier, Vincent Castronovo, and Andrei Turtoi. 2017. "Stromal Modulators of TGF-β in Cancer." *Journal of Clinical Medicine* 6 (1): 7. https://doi.org/10.3390/jcm6010007.

Couble, M. L., J. C. Farges, F. Bleicher, B. Perrat-Mabillon, M. Boudeulle, and H. Magloire. 2000. "Odontoblast Differentiation of Human Dental Pulp Cells in Explant Cultures." *Calcified Tissue International* 66 (2): 129–38.

Cui, Hang, Yahui Kong, Mei Xu, and Hong Zhang. 2013. "Notch3 Functions as a Tumor Suppressor by Controlling Cellular Senescence." *Cancer Research* 73 (11): 3451–59. https://doi.org/10.1158/0008-5472.CAN-12-3902.

Cuylen, Sara, Claudia Blaukopf, Antonio Z. Politi, Thomas Müller-Reichert, Beate Neumann, Ina Poser, Jan Ellenberg, Anthony A. Hyman, and Daniel W. Gerlich. 2016. "Ki-67 Acts as a Biological Surfactant to Disperse Mitotic Chromosomes." *Nature* 535 (7611): 308–12. https://doi.org/10.1038/nature18610.

Dai, Zhipeng, Yi Jin, Jia Zheng, Ke Liu, Jiajun Zhao, Shanfeng Zhang, Fei Wu, and Zhibo Sun. 2019. "MiR-217 Promotes Cell Proliferation and Osteogenic Differentiation of

BMSCs by Targeting DKK1 in Steroid-Associated Osteonecrosis." *Biomedicine & Pharmacotherapy* 109: 1112–19. https://doi.org/10.1016/j.biopha.2018.10.166.

Dimri, G P, X Lee, G Basile, M Acosta, G Scott, C Roskelley, E E Medrano, M Linskens, I Rubelj, and O Pereira-Smith. 1995. "A Biomarker That Identifies Senescent Human Cells in Culture and in Aging Skin in Vivo." *Proceedings of the National Academy of Sciences of the United States of America* 92 (20): 9363–67.

Ding, Gang, Wei Wang, Yi Liu, Yunqing An, Chunmei Zhang, Songtao Shi, and Songlin Wang. 2010. "Effect of Cryopreservation on Biological and Immunological Properties of Stem Cells from Apical Papilla." *Journal of Cellular Physiology* 223 (2): 415–22. https://doi.org/10.1002/jcp.22050.

Dominici, M., K. Le Blanc, I. Mueller, I. Slaper-Cortenbach, Fc Marini, Ds Krause, Rj Deans, A. Keating, Dj Prockop, and Em Horwitz. 2006. "Minimal Criteria for Defining Multipotent Mesenchymal Stromal Cells. The International Society for Cellular Therapy Position Statement." *Cytotherapy* 8 (4): 315–17. https://doi.org/10.1080/14653240600855905.

Egusa, Hiroshi, Wataru Sonoyama, Masahiro Nishimura, Ikiru Atsuta, and Kentaro Akiyama. 2012. "Stem Cells in Dentistry – Part I: Stem Cell Sources." *Journal of Prosthodontic Research* 56 (3): 151–65. https://doi.org/10.1016/j.jpor.2012.06.001.

Elsalanty, Mohammed E., and David G. Genecov. 2009. "Bone Grafts in Craniofacial Surgery." *Craniomaxillofacial Trauma & Reconstruction* 2 (3): 125–34. https://doi.org/10.1055/s-0029-1215875.

Ermolaeva, Maria, Francesco Neri, Alessandro Ori, and K. Lenhard Rudolph. 2018. "Cellular and Epigenetic Drivers of Stem Cell Ageing." *Nature Reviews. Molecular Cell Biology* 19 (9): 594–610. https://doi.org/10.1038/s41580-018-0020-3.

Faiella, Whitney, and Rony Atoui. 2016. "Therapeutic Use of Stem Cells for Cardiovascular Disease." *Clinical and Translational Medicine* 5 (1): 34. https://doi.org/10.1186/s40169-016-0116-3.

Feng, Xingmei, Jing Xing, Guijuan Feng, Aimin Sang, Biyu Shen, Yue Xu, Jinxia Jiang, et al. 2013. "Age-Dependent Impaired Neurogenic Differentiation Capacity of Dental Stem Cell Is Associated with Wnt/β-Catenin Signaling." *Cellular and Molecular Neurobiology* 33 (8): 1023–31. https://doi.org/10.1007/s10571-013-9965-0.

Ferretti, Concetta, Guendalina Lucarini, Chiara Andreoni, Eleonora Salvolini, Novella Bianchi, Giovanni Vozzi, Antonio Gigante, and Monica Mattioli-Belmonte. 2015. "Human Periosteal Derived Stem Cell Potential: The Impact of Age." *Stem Cell Reviews* 11 (3): 487–500. https://doi.org/10.1007/s12015-014-9559-3.

Franceschi, Claudio, Paolo Garagnani, Paolo Parini, Cristina Giuliani, and Aurelia Santoro. 2018. "Inflammaging: A New Immune–Metabolic Viewpoint for Age-Related Diseases." *Nature Reviews Endocrinology* 14 (10): 576. https://doi.org/10.1038/s41574-018-0059-4.

Fridlyanskaya, Irina, Larisa Alekseenko, and Nikolay Nikolsky. 2015. "Senescence as a General Cellular Response to Stress: A Mini-Review." *Experimental Gerontology* 72 (December): 124–28. https://doi.org/10.1016/j.exger.2015.09.021.

Friedenstein, A. J., J. F. Gorskaja, and N. N. Kulagina. 1976. "Fibroblast Precursors in Normal and Irradiated Mouse Hematopoietic Organs." *Experimental Hematology* 4 (5): 267–74.

Galler, Kerstin M, Adriana C Cavender, Umut Koeklue, Laura J Suggs, Gottfried Schmalz, and Rena N D'Souza. 2011. "Bioengineering of Dental Stem Cells in a PEGylated Fibrin Gel." *Regenerative Medicine* 6 (2): 191–200. https://doi.org/10.2217/rme.11.3.

Gericke, A., C. Qin, Y. Sun, R. Redfern, D. Redfern, Y. Fujimoto, H. Taleb, W. T. Butler, and A. L. Boskey. 2010. "Different Forms of DMP1 Play Distinct Roles in Mineralization." *Journal of Dental Research* 89 (4): 355–59. https://doi.org/10.1177/0022034510363250.

Gervois, Pascal, Tom Struys, Petra Hilkens, Annelies Bronckaers, Jessica Ratajczak, Constantinus Politis, Bert Brône, Ivo Lambrichts, and Wendy Martens. 2015. "Neurogenic Maturation of Human Dental Pulp Stem Cells Following Neurosphere Generation Induces Morphological and Electrophysiological Characteristics of Functional Neurons." *Stem Cells and Development* 24 (3): 296–311. https://doi.org/10.1089/scd.2014.0117.

Giuliani, Alessandra, Adrian Manescu, Max Langer, Franco Rustichelli, Vincenzo Desiderio, Francesca Paino, Alfredo De Rosa, et al. 2013. "Three Years After Transplants in Human Mandibles, Histological and In-Line Holotomography Revealed That Stem Cells Regenerated a Compact Rather Than a Spongy Bone: Biological and Clinical Implications." *STEM CELLS Translational Medicine* 2 (4): 316–24. https://doi.org/10.5966/sctm.2012-0136.

Gronthos, S., J. Brahim, W. Li, L.W. Fisher, N. Cherman, A. Boyde, P. DenBesten, P. Gehron Robey, and S. Shi. 2002. "Stem Cell Properties of Human Dental Pulp Stem Cells." *Journal of Dental Research* 81 (8): 531–35. https://doi.org/10.1177/154405910208100806.

Gronthos, S., M. Mankani, J. Brahim, P. G. Robey, and S. Shi. 2000. "Postnatal Human Dental Pulp Stem Cells (DPSCs) in Vitro and in Vivo." *Proceedings of the National Academy of Sciences of the United States of America* 97 (25): 13625–30. https://doi.org/10.1073/pnas.240309797.

Gu, Shensheng, Shujun Ran, Bin Liu, and Jingping Liang. 2016. "MiR-152 Induces Human Dental Pulp Stem Cell Senescence by Inhibiting SIRT7 Expression." *FEBS Letters* 590 (8): 1123–31. https://doi.org/10.1002/1873-3468.12138.

Han, Nana, Yong Zheng, Ran Li, Xianyu Li, Mi Zhou, Yun Niu, and Qi Zhang. 2014. "β-Catenin Enhances Odontoblastic Differentiation of Dental Pulp Cells through Activation of Runx2." Edited by Vladimir N. Uversky. *PLoS ONE* 9 (2): e88890. https://doi.org/10.1371/journal.pone.0088890.

Hatakeyama, Akihisa, Soshi Uchida, Hajime Utsunomiya, Manabu Tsukamoto, Hirotaka Nakashima, Eiichiro Nakamura, Cecilia Pascual-Garrido, Ichiro Sekiya, and Akinori Sakai. 2017. "Isolation and Characterization of Synovial Mesenchymal Stem Cell Derived from Hip Joints: A Comparative Analysis with a Matched Control Knee Group." *Stem Cells International* 2017. https://doi.org/10.1155/2017/9312329.

He, Juan, Yan Wang, Shu Sun, Meining Yu, Cuiyu Wang, Xiaohua Pei, Bei Zhu, Jianqing Wu, and Weihong Zhao. 2012. "Bone Marrow Stem Cells-Derived Microvesicles Protect against Renal Injury in the Mouse Remnant Kidney Model." *Nephrology (Carlton, Vic.)* 17 (5): 493–500. https://doi.org/10.1111/j.1440-1797.2012.01589.x.

Hennrich, Marco L., Natalie Romanov, Patrick Horn, Samira Jaeger, Volker Eckstein, Violetta Steeples, Fei Ye, et al. 2018. "Cell-Specific Proteome Analyses of Human Bone

Marrow Reveal Molecular Features of Age-Dependent Functional Decline." *Nature Communications* 9 (1): 4004. https://doi.org/10.1038/s41467-018-06353-4.

Hernandez-Segura, Alejandra, Jamil Nehme, and Marco Demaria. 2018. "Hallmarks of Cellular Senescence." *Trends in Cell Biology* 28 (6): 436–53. https://doi.org/10.1016/j.tcb.2018.02.001.

Hsuan, Yogi Chang-Yo, Cheng-Hsien Lin, Ching-Ping Chang, and Mao-Tsun Lin. 2016. "Mesenchymal Stem Cell-based Treatments for Stroke, Neural Trauma, and Heat Stroke." *Brain and Behavior* 6 (10). https://doi.org/10.1002/brb3.526.

Huang, Chuan-En, Fang-Wei Hu, Chuan-Hang Yu, Lo-Lin Tsai, Tzu-Hsin Lee, Ming-Yung Chou, and Cheng-Chia Yu. 2014. "Concurrent Expression of Oct4 and Nanog Maintains Mesenchymal Stem-like Property of Human Dental Pulp Cells." *International Journal of Molecular Sciences* 15 (10): 18623–39. https://doi.org/10.3390/ijms151018623.

Huang, G. T.-J., S. Gronthos, and S. Shi. 2009a. "Mesenchymal Stem Cells Derived from Dental Tissues vs. Those from Other Sources: Their Biology and Role in Regenerative Medicine." *Journal of Dental Research* 88 (9): 792–806. https://doi.org/10.1177/0022034509340867.

Huang, George T.-J., Wataru Sonoyama, James Chen, and Sang Hyuk Park. 2006. "In Vitro Characterization of Human Dental Pulp Cells: Various Isolation Methods and Culturing Environments." *Cell and Tissue Research* 324 (2): 225–36. https://doi.org/10.1007/s00441-005-0117-9.

Huang, G.T.-J., S. Gronthos, and S. Shi. 2009b. "Mesenchymal Stem Cells Derived from Dental Tissues vs. Those from Other Sources: Their Biology and Role in Regenerative Medicine." *Journal of Dental Research* 88 (9): 792–806. https://doi.org/10.1177/0022034509340867.

Huang, Shishu, Victor Leung, Songlin Peng, Laiching Li, Feng Juan Lu, Ting Wang, William Lu, Kenneth M.C. Cheung, and Guangqian Zhou. 2011. "Developmental Definition of MSCs: New Insights Into Pending Questions." *Cellular Reprogramming* 13 (6): 465–72. https://doi.org/10.1089/cell.2011.0045.

Hui, Hongxiang, Yongming Tang, Min Hu, and Xiaoning Zhao. 2011. "Stem Cells: General Features and Characteristics." *Stem Cells in Clinic and Research*, August. https://doi.org/10.5772/23755.

Iezzi, I., P. Pagella, M. Mattioli-Belmonte, and T. A. Mitsiadis. 2019. "The Effects of Ageing on Dental Pulp Stem Cells, the Tooth Longevity Elixir." *European Cells & Materials* 37: 175–85. https://doi.org/10.22203/eCM.v037a11.

Iohara, Koichiro, Kiyomi Imabayashi, Ryo Ishizaka, Atsushi Watanabe, Junichi Nabekura, Masataka Ito, Kenji Matsushita, Hiroshi Nakamura, and Misako Nakashima. 2011. "Complete Pulp Regeneration After Pulpectomy by Transplantation of CD105+ Stem Cells with Stromal Cell-Derived Factor-1." *Tissue Engineering Part A* 17 (15–16): 1911–20. https://doi.org/10.1089/ten.tea.2010.0615.

Ishkitiev, Nikolay, Ken Yaegaki, Toshio Imai, Tomoko Tanaka, Taka Nakahara, Hiroshi Ishikawa, Vanyo Mitev, and Markus Haapasalo. 2012. "High-Purity Hepatic Lineage Differentiated from Dental Pulp Stem Cells in Serum-Free Medium." *Journal of Endodontics* 38 (4): 475–80. https://doi.org/10.1016/j.joen.2011.12.011.

Isola, Allison L., and Suzie Chen. 2017. “Exosomes: The Messengers of Health and Disease.” *Current Neuropharmacology* 15 (1): 157–65. https://doi.org/10.2174/1570159X14666160825160421.

Jarmalavičiūtė, Akvilė, and Augustas Pivoriūnas. 2016. “Neuroprotective Properties of Extracellular Vesicles Derived from Mesenchymal Stem Cells.” *Neural Regeneration Research* 11 (6): 904–5. https://doi.org/10.4103/1673-5374.184480.

Jeon, Byeong-Gyun, Si-Jeong Jang, Ji-Seong Park, Raghavendra Baregundi Subbarao, Gie-Joon Jeong, Bong-Wook Park, and Gyu-Jin Rho. 2015. “Differentiation Potential of Mesenchymal Stem Cells Isolated from Human Dental Tissues into Non-Mesodermal Lineage.” *Animal Cells and Systems* 19 (5): 321–31. https://doi.org/10.1080/19768354.2015.1087430.

Kanafi, Mohammad Mahboob, Yajaman Bajjappa Rajeshwari, Sarita Gupta, Nidheesh Dadheech, Prabha Damodaran Nair, Pawan Kumar Gupta, and Ramesh Ramchandra Bhonde. 2013. “Transplantation of Islet-like Cell Clusters Derived from Human Dental Pulp Stem Cells Restores Normoglycemia in Diabetic Mice.” *Cytotherapy* 15 (10): 1228–36. https://doi.org/10.1016/j.jcyt.2013.05.008.

Kang, Hara, and Akiko Hata. 2015. “The Role of MicroRNAs in Cell Fate Determination of Mesenchymal Stem Cells : Balancing Adipogenesis and Osteogenesis.” *BMB Reports* 48 (6): 319–23. https://doi.org/10.5483/BMBRep.2015.48.6.206.

Kawasaki, K., J. Ruben, H. Tsuda, M. C. D. N. J. M. Huysmans, and O. Takagi. 2000. “Relationship between Mineral Distributions in Dentine Lesions and Subsequent Remineralization in Vitro.” *Caries Research* 34 (5): 395–403. https://doi.org/10.1159/000016614.

Kim, Yeon Jeong, Soo Hyun Hwang, Hyun Hwa Cho, Keun Koo Shin, Yong Chan Bae, and Jin Sup Jung. 2012. “MicroRNA 21 Regulates the Proliferation of Human Adipose Tissue-Derived Mesenchymal Stem Cells and High-Fat Diet-Induced Obesity Alters MicroRNA 21 Expression in White Adipose Tissues.” *Journal of Cellular Physiology* 227 (1): 183–93. https://doi.org/10.1002/jcp.22716.

Klopp, Ann H., Erika L. Spaeth, Jennifer L. Dembinski, Wendy A. Woodward, Anupama Munshi, Raymond E. Meyn, James D. Cox, Michael Andreeff, and Frank C. Marini. 2007. “Tumor Irradiation Increases the Recruitment of Circulating Mesenchymal Stem Cells into the Tumor Microenvironment.” *Cancer Research* 67 (24): 11687–95. https://doi.org/10.1158/0008-5472.CAN-07-1406.

Kodonas, Kostas, Christos Gogos, Serafim Papadimitriou, Kokona Kouzi-Koliakou, and Dimitrios Tziafas. 2012. “Experimental Formation of Dentin-like Structure in the Root Canal Implant Model Using Cryopreserved Swine Dental Pulp Progenitor Cells.” *Journal of Endodontics* 38 (7): 913–19. https://doi.org/10.1016/j.joen.2012.02.005.

Krifka, Stephanie, Gianrico Spagnuolo, Gottfried Schmalz, and Helmut Schweikl. 2013. “A Review of Adaptive Mechanisms in Cell Responses towards Oxidative Stress Caused by Dental Resin Monomers.” *Biomaterials* 34 (19): 4555–63. https://doi.org/10.1016/j.biomaterials.2013.03.019.

Kulcenty, Katarzyna, Joanna P. Wroblewska, Marcin Rucinski, Emilia Kozlowska, Karol Jopek, and Wiktoria M. Suchorska. 2019. “MicroRNA Profiling During Neural Differentiation of Induced Pluripotent Stem Cells.” *International Journal of Molecular Sciences* 20 (15): 3651. https://doi.org/10.3390/ijms20153651.

Kyrylkova, Kateryna, Sergiy Kyryachenko, Mark Leid, and Chrissa Kioussi. 2012. “Detection of Apoptosis by TUNEL Assay.” *Methods in Molecular Biology (Clifton, N.J.)* 887: 41–47. https://doi.org/10.1007/978-1-61779-860-3_5.

Lai, Ruenn Chai, Fatih Arslan, May May Lee, Newman Siu Kwan Sze, Andre Choo, Tian Sheng Chen, Manuel Salto-Tellez, et al. 2010. “Exosome Secreted by MSC Reduces Myocardial Ischemia/Reperfusion Injury.” *Stem Cell Research* 4 (3): 214–22. https://doi.org/10.1016/j.scr.2009.12.003.

Langer, R, and J. Vacanti. 1993. “Tissue Engineering.” *Science* 260 (5110): 920–26. https://doi.org/10.1126/science.8493529.

Lee, Ryang Hwa, Min Jeong Seo, Roxanne L. Reger, Jeffrey L. Spees, Andrey A. Pulin, Scott D. Olson, and Darwin J. Prockop. 2006. “Multipotent Stromal Cells from Human Marrow Home to and Promote Repair of Pancreatic Islets and Renal Glomeruli in Diabetic NOD/Scid Mice.” *Proceedings of the National Academy of Sciences of the United States of America* 103 (46): 17438–43. https://doi.org/10.1073/pnas.0608249103.

Li, Na, David J. Bates, Jin An, Derek A. Terry, and Eugenia Wang. 2011. “Up-Regulation of Key MicroRNAs, and Inverse down-Regulation of Their Predicted Oxidative Phosphorylation Target Genes, during Aging in Mouse Brain.” *Neurobiology of Aging* 32 (5): 944–55. https://doi.org/10.1016/j.neurobiolaging.2009.04.020.

Lin, Heng, Lili Xu, Huan Liu, Qin Sun, Zhuo Chen, Guohua Yuan, and Zhi Chen. 2011. “KLF4 Promotes the Odontoblastic Differentiation of Human Dental Pulp Cells.” *Journal of Endodontics* 37 (7): 948–54. https://doi.org/10.1016/j.joen.2011.03.030.

Liu, Hongzhi, Hao Su, Xin Wang, and Wei Hao. 2018. “MiR-148a Regulates Bone Marrow Mesenchymal Stem Cells-Mediated Fracture Healing by Targeting Insulin-like Growth Factor 1: LIU et Al.” *Journal of Cellular Biochemistry* 120 (October). https://doi.org/10.1002/jcb.27121.

Liu, Yang, and Sydney C. W. Tang. 2016. “Recent Progress in Stem Cell Therapy for Diabetic Nephropathy.” *Kidney Diseases (Basel, Switzerland)* 2 (1): 20–27. https://doi.org/10.1159/000441913.

Liu, Zhi, Hong Chang, Yihong Hou, Yu Wang, Zhiqiang Zhou, Ming Wang, Zhidong Huang, and Bin Yu. 2018. “Lentivirus-Mediated MicroRNA-26a Overexpression in Bone Mesenchymal Stem Cells Facilitates Bone Regeneration in Bone Defects of Calvaria in Mice.” *Molecular Medicine Reports* 18 (6): 5317–26. https://doi.org/10.3892/mmr.2018.9596.

Livak, K. J., and T. D. Schmittgen. 2001. “Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2(-Delta Delta C(T)) Method.” *Methods (San Diego, Calif.)* 25 (4): 402–8. https://doi.org/10.1006/meth.2001.1262.

López-Otín, Carlos, Maria A. Blasco, Linda Partridge, Manuel Serrano, and Guido Kroemer. 2013. “The Hallmarks of Aging.” *Cell* 153 (6): 1194–1217. https://doi.org/10.1016/j.cell.2013.05.039.

Lyngstadaas, Sp, Jc Wohlfahrt, Sj Brookes, Ml Paine, Ml Snead, and Je Reseland. 2009. “Enamel Matrix Proteins; Old Molecules for New Applications.” *Orthodontics & Craniofacial Research* 12 (3): 243–53. https://doi.org/10.1111/j.1601-6343.2009.01459.x.

Ma, Dandan, Zhaofeng Ma, Xiaojun Zhang, Weihong Wang, Zhenhua Yang, Mi Zhang, Gang Wu, Wei Lu, Zhihong Deng, and Yan Jin. 2009. “Effect of Age and Extrinsic

Microenvironment on the Proliferation and Osteogenic Differentiation of Rat Dental Pulp Stem Cells In Vitro." *Journal of Endodontics* 35 (11): 1546–53. https://doi.org/10.1016/j.joen.2009.07.016.

Maciejewska, Izabela, Cameron Cowan, Kathy Svoboda, William T. Butler, Rena D'Souza, and Chunlin Qin. 2009. "The NH2-Terminal and COOH-Terminal Fragments of Dentin Matrix Protein 1 (DMP1) Localize Differently in the Compartments of Dentin and Growth Plate of Bone." *The Journal of Histochemistry and Cytochemistry: Official Journal of the Histochemistry Society* 57 (2): 155–66. https://doi.org/10.1369/jhc.2008.952630.

Maes, Olivier C., Jin An, Harshini Sarojini, and Eugenia Wang. 2008. "Murine MicroRNAs Implicated in Liver Functions and Aging Process." *Mechanisms of Ageing and Development* 129 (9): 534–41. https://doi.org/10.1016/j.mad.2008.05.004.

Malek, Antoine, and Nick A. Bersinger. 2011. "Human Placental Stem Cells: Biomedical Potential and Clinical Relevance." *Journal of Stem Cells* 6 (2): 75–92.

Martens, W., E. Wolfs, T. Struys, C. Politis, A. Bronckaers, and I. Lambrichts. 2012. "Expression Pattern of Basal Markers in Human Dental Pulp Stem Cells and Tissue." *Cells, Tissues, Organs* 196 (6): 490–500. https://doi.org/10.1159/000338654.

Martinez-Lopez, Nuria, Diana Athonvarangkul, and Rajat Singh. 2015. "Autophagy and Aging." *Advances in Experimental Medicine and Biology* 847: 73–87. https://doi.org/10.1007/978-1-4939-2404-2_3.

Mattar, Philipp, and Karen Bieback. 2015. "Comparing the Immunomodulatory Properties of Bone Marrow, Adipose Tissue, and Birth-Associated Tissue Mesenchymal Stromal Cells." *Frontiers in Immunology* 6. https://doi.org/10.3389/fimmu.2015.00560.

McHugh, Domhnall, and Jesús Gil. 2018. "Senescence and Aging: Causes, Consequences, and Therapeutic Avenues." *The Journal of Cell Biology* 217 (1): 65–77. https://doi.org/10.1083/jcb.201708092.

Mead, Ben, Lisa J. Hill, Richard J. Blanch, Kelly Ward, Ann Logan, Martin Berry, Wendy Leadbeater, and Ben A. Scheven. 2016. "Mesenchymal Stromal Cell–Mediated Neuroprotection and Functional Preservation of Retinal Ganglion Cells in a Rodent Model of Glaucoma." *Cytotherapy* 18 (4): 487–96. https://doi.org/10.1016/j.jcyt.2015.12.002.

Miletich, Isabelle, and Paul T. Sharpe. 2004. "Neural Crest Contribution to Mammalian Tooth Formation." *Birth Defects Research. Part C, Embryo Today: Reviews* 72 (2): 200–212. https://doi.org/10.1002/bdrc.20012.

Minteer, Danielle, Kacey G. Marra, and J. Peter Rubin. 2013. "Adipose-Derived Mesenchymal Stem Cells: Biology and Potential Applications." *Advances in Biochemical Engineering/Biotechnology* 129: 59–71. https://doi.org/10.1007/10_2012_146.

Miron, Richard J., Anton Sculean, David L. Cochran, Stuart Froum, Giovanni Zucchelli, Carlos Nemcovsky, Nikos Donos, et al. 2016. "Twenty Years of Enamel Matrix Derivative: The Past, the Present and the Future." *Journal of Clinical Periodontology* 43 (8): 668–83. https://doi.org/10.1111/jcpe.12546.

Mitsiadis, T. A., A. Feki, G. Papaccio, and J. Catón. 2011. "Dental Pulp Stem Cells, Niches, and Notch Signaling in Tooth Injury." *Advances in Dental Research* 23 (3): 275–79. https://doi.org/10.1177/0022034511405386.

Mitsiadis, T. A., G. Orsini, and L. Jimenez-Rojo. 2015. "Stem Cell-Based Approaches in Dentistry." *European Cells & Materials* 30 (November): 248–57.

Mitsiadis, T.A., and C. Rahiotis. 2004. “Parallels between Tooth Development and Repair: Conserved Molecular Mechanisms Following Carious and Dental Injury.” *Journal of Dental Research* 83 (12): 896–902. https://doi.org/10.1177/154405910408301202.

Mitsiadis, Thimios A., and Daniel Graf. 2009. “Cell Fate Determination during Tooth Development and Regeneration.” *Birth Defects Research. Part C, Embryo Today: Reviews* 87 (3): 199–211. https://doi.org/10.1002/bdrc.20160.

Miura, Masako, Stan Gronthos, Mingrui Zhao, Bai Lu, Larry W. Fisher, Pamela Gehron Robey, and Songtao Shi. 2003. “SHED: Stem Cells from Human Exfoliated Deciduous Teeth.” *Proceedings of the National Academy of Sciences of the United States of America* 100 (10): 5807–12. https://doi.org/10.1073/pnas.0937635100.

Moharamzadeh, K., R. Van Noort, I. M. Brook, and A. M. Scutt. 2007. “HPLC Analysis of Components Released from Dental Composites with Different Resin Compositions Using Different Extraction Media.” *Journal of Materials Science: Materials in Medicine* 18 (1): 133–37. https://doi.org/10.1007/s10856-006-0671-z.

Mokry, Jaroslav, Tomas Soukup, Stanislav Micuda, Jana Karbanova, Benjamin Visek, Eva Brcakova, Jakub Suchanek, Jan Bouchal, Doris Vokurkova, and Romana Ivancakova. 2010. “Telomere Attrition Occurs during Ex Vivo Expansion of Human Dental Pulp Stem Cells.” Research article. BioMed Research International. 2010. https://doi.org/10.1155/2010/673513.

Moore, Kim E., Jeanette F. Mills, and Melissa M. Thornton. 2006. “Alternative Sources of Adult Stem Cells: A Possible Solution to the Embryonic Stem Cell Debate.” *Gender Medicine* 3 (3): 161–68. https://doi.org/10.1016/S1550-8579(06)80204-4.

Morito, Akiyuki, Yujiro Kida, Kazunori Suzuki, Koji Inoue, Noriyuki Kuroda, Kazuhiro Gomi, Takashi Arai, and Tetsuji Sato. 2009. “Effects of Basic Fibroblast Growth Factor on the Development of the Stem Cell Properties of Human Dental Pulp Cells.” *Archives of Histology and Cytology* 72 (1): 51–64.

Morsczeck, C., W. Götz, J. Schierholz, F. Zeilhofer, U. Kühn, C. Möhl, C. Sippel, and K. H. Hoffmann. 2005. “Isolation of Precursor Cells (PCs) from Human Dental Follicle of Wisdom Teeth.” *Matrix Biology: Journal of the International Society for Matrix Biology* 24 (2): 155–65. https://doi.org/10.1016/j.matbio.2004.12.004.

Narayanan, Karthikeyan, Amsaveni Ramachandran, Jianjun Hao, Gen He, Kyle Won Park, Michael Cho, and Anne George. 2003. “Dual Functional Roles of Dentin Matrix Protein 1. Implications in Biomineralization and Gene Transcription by Activation of Intracellular Ca2+ Store.” *The Journal of Biological Chemistry* 278 (19): 17500–508. https://doi.org/10.1074/jbc.M212700200.

Nasef, Aisha, Christelle Mazurier, Sandrine Bouchet, Sabine François, Alain Chapel, Dominique Thierry, Norbert-Claude Gorin, and Loïc Fouillard. 2008. “Leukemia Inhibitory Factor: Role in Human Mesenchymal Stem Cells Mediated Immunosuppression.” *Cellular Immunology* 253 (1–2): 16–22. https://doi.org/10.1016/j.cellimm.2008.06.002.

Oh, Juhyun, Yang David Lee, and Amy J Wagers. 2014. “Stem Cell Aging: Mechanisms, Regulators and Therapeutic Opportunities.” *Nature Medicine* 20 (8): 870–80. https://doi.org/10.1038/nm.3651.

Okada, Motoi, Ha Won Kim, Kaoru Matsu-ura, Yi-Gang Wang, Meifeng Xu, and Muhammad Ashraf. 2016. “Abrogation of Age-Induced MicroRNA-195 Rejuvenates the Senescent

Mesenchymal Stem Cells by Reactivating Telomerase." *Stem Cells (Dayton, Ohio)* 34 (1): 148–59. https://doi.org/10.1002/stem.2211.

Paduano, Francesco, Massimo Marrelli, Lisa J. White, Kevin M. Shakesheff, and Marco Tatullo. 2016. "Odontogenic Differentiation of Human Dental Pulp Stem Cells on Hydrogel Scaffolds Derived from Decellularized Bone Extracellular Matrix and Collagen Type I." *PLOS ONE* 11 (2): e0148225. https://doi.org/10.1371/journal.pone.0148225.

Parner, E.T., J.M. Heidmann, I. Kjær, M. Væth, and S. Poulsen. 2002. "Biological Interpretation of the Correlation of Emergence Times of Permanent Teeth." *Journal of Dental Research* 81 (7): 451–54. https://doi.org/10.1177/154405910208100703.

Patil, Rajreddy, B. Mohana Kumar, Won-Jae Lee, Ryoung-Hoon Jeon, Si-Jung Jang, Yeon-Mi Lee, Bong-Wook Park, et al. 2014. "Multilineage Potential and Proteomic Profiling of Human Dental Stem Cells Derived from a Single Donor." *Experimental Cell Research* 320 (1): 92–107. https://doi.org/10.1016/j.yexcr.2013.10.005.

Pecho, Oscar E., Razvan Ghinea, Erika A. Navarro do Amaral, Juan C. Cardona, Alvaro Della Bona, and María M. Pérez. 2016. "Relevant Optical Properties for Direct Restorative Materials." *Dental Materials* 32 (5): e105–12. https://doi.org/10.1016/j.dental.2016.02.008.

Peng, Li, Ling Ye, and Xue-dong Zhou. 2009. "Mesenchymal Stem Cells and Tooth Engineering." *International Journal of Oral Science* 1 (1): 6–12. https://doi.org/10.4248/ijos.08032.

Piattelli, A., and P. Trisi. 1993. "Pulp Obliteration: A Histological Study." *Journal of Endodontics* 19 (5): 252–54. https://doi.org/10.1016/S0099-2399(06)81303-8.

Pittenger, M. F., A. M. Mackay, S. C. Beck, R. K. Jaiswal, R. Douglas, J. D. Mosca, M. A. Moorman, D. W. Simonetti, S. Craig, and D. R. Marshak. 1999. "Multilineage Potential of Adult Human Mesenchymal Stem Cells." *Science (New York, N.Y.)* 284 (5411): 143–47. https://doi.org/10.1126/science.284.5411.143.

Preshaw, Philip M., Karsten Henne, John J. Taylor, Ruth A. Valentine, and Georg Conrads. 2017. "Age-Related Changes in Immune Function (Immune Senescence) in Caries and Periodontal Diseases: A Systematic Review." *Journal of Clinical Periodontology* 44 (S18): S153–77. https://doi.org/10.1111/jcpe.12675.

Putzeys, Eveline, Siemon De Nys, Stevan M. Cokic, Radu Corneliu Duca, Jeroen Vanoirbeek, Lode Godderis, Bart Van Meerbeek, and Kirsten L. Van Landuyt. 2019. "Long-Term Elution of Monomers from Resin-Based Dental Composites." *Dental Materials* 35 (3): 477–85. https://doi.org/10.1016/j.dental.2019.01.005.

Qin, C., R. D'Souza, and J. Q. Feng. 2007. "Dentin Matrix Protein 1 (DMP1): New and Important Roles for Biomineralization and Phosphate Homeostasis." *Journal of Dental Research* 86 (12): 1134–41. https://doi.org/10.1177/154405910708601202.

Qin, Chunlin, Jan C. Brunn, Richard G. Cook, Ralph S. Orkiszewski, James P. Malone, Arthur Veis, and William T. Butler. 2003. "Evidence for the Proteolytic Processing of Dentin Matrix Protein 1. Identification and Characterization of Processed Fragments and Cleavage Sites." *The Journal of Biological Chemistry* 278 (36): 34700–708. https://doi.org/10.1074/jbc.M305315200.

Qin, Chunlin, J.C. Brunn, Elizabeth Cadena, A Ridall, H Tsujigiwa, H Nagatsuka, N Nagai, and W.T. Butler. 2002. "The Expression of Dentin Sialophosphoprotein Gene in Bone."

Journal of Dental Research 81 (July): 392–94. https://doi.org/10.1177/154405910208100607.

Qu, Tiejun, and Xiaohua Liu. 2013. "Nano-Structured Gelatin/Bioactive Glass Hybrid Scaffolds for the Enhancement of Odontogenic Differentiation of Human Dental Pulp Stem Cells." *Journal of Materials Chemistry B* 1 (37): 4764–72. https://doi.org/10.1039/C3TB21002B.

Ragni, Enrico, Mariele Viganò, Paolo Rebulla, Rosaria Giordano, and Lorenza Lazzari. 2013. "What Is beyond a QRT-PCR Study on Mesenchymal Stem Cell Differentiation Properties: How to Choose the Most Reliable Housekeeping Genes." *Journal of Cellular and Molecular Medicine* 17 (1): 168–80. https://doi.org/10.1111/j.1582-4934.2012.01660.x.

Rani, Sweta, Aideen E Ryan, Matthew D Griffin, and Thomas Ritter. 2015. "Mesenchymal Stem Cell-Derived Extracellular Vesicles: Toward Cell-Free Therapeutic Applications." *Molecular Therapy* 23 (5): 812–23. https://doi.org/10.1038/mt.2015.44.

Ravindran, Sriram, Youbin Zhang, Chun-Chieh Huang, and Anne George. 2013. "Odontogenic Induction of Dental Stem Cells by Extracellular Matrix-Inspired Three-Dimensional Scaffold." *Tissue Engineering Part A* 20 (1–2): 92–102. https://doi.org/10.1089/ten.tea.2013.0192.

Ren, Guangwen, Arthur I. Roberts, and Yufang Shi. 2011. "Adhesion Molecules: Key Players in Mesenchymal Stem Cell-Mediated Immunosuppression." *Cell Adhesion & Migration* 5 (1): 20–22. https://doi.org/10.4161/cam.5.1.13491.

Ren, Guangwen, Liying Zhang, Xin Zhao, Guangwu Xu, Yingyu Zhang, Arthur I. Roberts, Robert Chunhua Zhao, and Yufang Shi. 2008. "Mesenchymal Stem Cell-Mediated Immunosuppression Occurs via Concerted Action of Chemokines and Nitric Oxide." *Cell Stem Cell* 2 (2): 141–50. https://doi.org/10.1016/j.stem.2007.11.014.

Reynolds, Amanda J., and Colin A. B. Jahoda. 2004. "Cultured Human and Rat Tooth Papilla Cells Induce Hair Follicle Regeneration and Fiber Growth." *Differentiation* 72 (9): 566–75. https://doi.org/10.1111/j.1432-0436.2004.07209010.x.

Richard, Suzman, and Beard John. 2011. *Global Health and Aging.* http://www.nia.nih. gov/research/publication/ global-health-and-aging/ preface.

Roberto, Vânia Palma, Paulo Gavaia, Maria João Nunes, Elsa Rodrigues, Maria Leonor Cancela, and Daniel Martins Tiago. 2018. "Evidences for a New Role of MiR-214 in Chondrogenesis." *Scientific Reports* 8 (1): 1–16. https://doi.org/10.1038/s41598-018-21735-w.

Roth V. 2006. "Doubling Time - Online Computing with 2 Points." 2006. http://www.doubling-time.com/compute.php.

Samsonraj, Rebekah M., Michael Raghunath, Victor Nurcombe, James H. Hui, Andre J. van Wijnen, and Simon M. Cool. 2017. "Concise Review: Multifaceted Characterization of Human Mesenchymal Stem Cells for Use in Regenerative Medicine." *STEM CELLS Translational Medicine* 6 (12): 2173–85. https://doi.org/10.1002/sctm.17-0129.

Saxena, Payal, AdityaB Pant, SaurabhK Gupta, and VandanaA Pant. 2012. "Release and Toxicity of Dental Resin Composite." *Toxicology International* 19 (3): 225. https://doi.org/10.4103/0971-6580.103652.

Scheller, E.L., J. Chang, and C.Y. Wang. 2008. "Wnt/β-Catenin Inhibits Dental Pulp Stem Cell Differentiation." *Journal of Dental Research* 87 (2): 126–30. https://doi.org/10.1177/154405910808700206.

Scholzen, Thomas, and Johannes Gerdes. 2000. “The Ki-67 Protein: From the Known and the Unknown.” *Journal of Cellular Physiology* 182 (3): 311–22. https://doi.org/10.1002/(SICI)1097-4652(200003)182:3<311::AID-JCP1>3.0.CO;2-9.

Schubert, Andrea, Christopher Ziegler, Andrea Bernhard, Ralf Bürgers, and Nicolai Miosge. 2019. “Cytotoxic Effects to Mouse and Human Gingival Fibroblasts of a Nanohybrid Ormocer versus Dimethacrylate-Based Composites.” *Clinical Oral Investigations* 23 (1): 133–39. https://doi.org/10.1007/s00784-018-2419-9.

Scribante, Andrea, Marco Bollardi, Marco Chiesa, Claudio Poggio, and Marco Colombo. 2019. “Flexural Properties and Elastic Modulus of Different Esthetic Restorative Materials: Evaluation after Exposure to Acidic Drink.” Research article. BioMed Research International. 2019. https://doi.org/10.1155/2019/5109481.

Selmani, Zohair, Abderrahim Naji, Ines Zidi, Benoit Favier, Emilie Gaiffe, Laurent Obert, Christophe Borg, et al. 2008. “Human Leukocyte Antigen-G5 Secretion by Human Mesenchymal Stem Cells Is Required to Suppress T Lymphocyte and Natural Killer Function and to Induce CD4+CD25highFOXP3+ Regulatory T Cells.” *Stem Cells (Dayton, Ohio)* 26 (1): 212–22. https://doi.org/10.1634/stemcells.2007-0554.

Seo, Byoung-Moo, Masako Miura, Stan Gronthos, Peter Mark Bartold, Sara Batouli, Jaime Brahim, Marian Young, Pamela Gehron Robey, Cun-Yu Wang, and Songtao Shi. 2004. “Investigation of Multipotent Postnatal Stem Cells from Human Periodontal Ligament.” *Lancet (London, England)* 364 (9429): 149–55. https://doi.org/10.1016/S0140-6736(04)16627-0.

Shen, ChongYang, Lin Li, Ting Feng, JinRong Li, MeiXing Yu, Qiao Lu, and Hong Li. 2015. “Dental Pulp Stem Cells Derived Conditioned Medium Promotes Angiogenesis in Hindlimb Ischemia.” *Tissue Engineering and Regenerative Medicine* 12 (1): 59–68. https://doi.org/10.1007/s13770-014-9053-7.

Shi, Yufang, Gangzheng Hu, Juanjuan Su, Wenzhao Li, Qing Chen, Peishun Shou, Chunliang Xu, et al. 2010. “Mesenchymal Stem Cells: A New Strategy for Immunosuppression and Tissue Repair.” *Cell Research* 20 (5): 510–18. https://doi.org/10.1038/cr.2010.44.

Sloan, Alastair J. 2015. “Biology of the Dentin-Pulp Complex.” In *Stem Cell Biology and Tissue Engineering in Dental Sciences*, 371–78. Elsevier. https://doi.org/10.1016/B978-0-12-397157-9.00033-3.

Som, P.M., and I. Miletich. 2018. “Review of the Embryology of the Teeth.” *Neurographics* 8 (5): 369–93. https://doi.org/10.3174/ng.1600049.

Song, Bing, Wenkai Jiang, Amr Alraies, Qian Liu, Vijay Gudla, Julia Oni, Xiaoqing Wei, Alastair Sloan, Longxing Ni, and Meena Agarwal. 2016. “Bladder Smooth Muscle Cells Differentiation from Dental Pulp Stem Cells: Future Potential for Bladder Tissue Engineering.” Research article. Stem Cells International. 2016. https://doi.org/10.1155/2016/6979368.

Sonoyama, Wataru, Yi Liu, Takayoshi Yamaza, Rocky S. Tuan, Songlin Wang, Songtao Shi, and George T.-J. Huang. 2008. “Characterization of the Apical Papilla and Its Residing Stem Cells from Human Immature Permanent Teeth: A Pilot Study.” *Journal of Endodontics* 34 (2): 166–71. https://doi.org/10.1016/j.joen.2007.11.021.

Spath, L., V. Rotilio, M. Alessandrini, G. Gambara, L. De Angelis, M. Mancini, T. A. Mitsiadis, et al. 2010. “Explant-Derived Human Dental Pulp Stem Cells Enhance Differentiation and Proliferation Potentials.” *Journal of Cellular and Molecular Medicine* 14 (6B): 1635–44. https://doi.org/10.1111/j.1582-4934.2009.00848.x.

Squillaro, Tiziana, Gianfranco Peluso, and Umberto Galderisi. 2016. “Clinical Trials With Mesenchymal Stem Cells: An Update.” *Cell Transplantation* 25 (5): 829–48. https://doi.org/10.3727/096368915X689622.

Stolzing, A., E. Jones, D. McGonagle, and A. Scutt. 2008. “Age-Related Changes in Human Bone Marrow-Derived Mesenchymal Stem Cells: Consequences for Cell Therapies.” *Mechanisms of Ageing and Development* 129 (3): 163–73. https://doi.org/10.1016/j.mad.2007.12.002.

Stolzing, Alexandra, Sebastian Sethe, and Andrew M. Scutt. 2006. “Stressed Stem Cells: Temperature Response in Aged Mesenchymal Stem Cells.” *Stem Cells and Development* 15 (4): 478–87. https://doi.org/10.1089/scd.2006.15.478.

Sung, Sumi, Jieun Kim, and Youngmi Jung. 2018. “Liver-Derived Exosomes and Their Implications in Liver Pathobiology.” *International Journal of Molecular Sciences* 19 (12): 3715. https://doi.org/10.3390/ijms19123715.

Sunnegårdh-Grönberg, Karin, Jan W. V. van Dijken, Ulrika Funegård, Anders Lindberg, and Mats Nilsson. 2009. “Selection of Dental Materials and Longevity of Replaced Restorations in Public Dental Health Clinics in Northern Sweden.” *Journal of Dentistry* 37 (9): 673–78. https://doi.org/10.1016/j.jdent.2009.04.010.

Takahashi, Akiko, Naoko Ohtani, Kimi Yamakoshi, Shin-ichi Iida, Hidetoshi Tahara, Keiko Nakayama, Keiichi I. Nakayama, Toshinori Ide, Hideyuki Saya, and Eiji Hara. 2006. “Mitogenic Signalling and the P16 INK4a –Rb Pathway Cooperate to Enforce Irreversible Cellular Senescence.” *Nature Cell Biology* 8 (11): 1291–97. https://doi.org/10.1038/ncb1491.

Takeuchi, S., A. Takahashi, N. Motoi, S. Yoshimoto, T. Tajima, K. Yamakoshi, A. Hirao, et al. 2010. “Intrinsic Cooperation between P16INK4a and P21Waf1/Cip1 in the Onset of Cellular Senescence and Tumor Suppression In Vivo.” *Cancer Research* 70 (22): 9381–90. https://doi.org/10.1158/0008-5472.CAN-10-0801.

Tatullo, Marco, Massimo Marrelli, Kevin M. Shakesheff, and Lisa J. White. 2015. “Dental Pulp Stem Cells: Function, Isolation and Applications in Regenerative Medicine.” *Journal of Tissue Engineering and Regenerative Medicine* 9 (11): 1205–16. https://doi.org/10.1002/term.1899.

Teti, Gabriella, Viviana Salvatore, Stefano Focaroli, Sandra Durante, Antonio Mazzotti, Manuela Dicarlo, Monica Mattioli-Belmonte, and Giovanna Orsini. 2015. “In Vitro Osteogenic and Odontogenic Differentiation of Human Dental Pulp Stem Cells Seeded on Carboxymethyl Cellulose-Hydroxyapatite Hybrid Hydrogel.” *Frontiers in Physiology* 6 (October). https://doi.org/10.3389/fphys.2015.00297.

Thesleff, I. 2014. “Current Understanding of the Process of Tooth Formation: Transfer from the Laboratory to the Clinic.” *Australian Dental Journal* 59 (June): 48–54. https://doi.org/10.1111/adj.12102.

Tian, Ye, Ran Guo, Bin Shi, Longgang Chen, Liqing Yang, and Qin Fu. 2016. “MicroRNA-30a Promotes Chondrogenic Differentiation of Mesenchymal Stem Cells through Inhibiting Delta-like 4 Expression.” *Life Sciences* 148 (March): 220–28. https://doi.org/10.1016/j.lfs.2016.02.031.

Tümpel, Stefan, and K. Lenhard Rudolph. 2019. “Quiescence: Good and Bad of Stem Cell Aging.” *Trends in Cell Biology* 29 (8): 672–85. https://doi.org/10.1016/j.tcb.2019.05.002.

Tziafas, Dimitrios, and Konstantinos Kodonas. 2010. “Differentiation Potential of Dental Papilla, Dental Pulp, and Apical Papilla Progenitor Cells.” *Journal of Endodontics* 36 (5): 781–89. https://doi.org/10.1016/j.joen.2010.02.006.

Uccelli, Antonio, Lorenzo Moretta, and Vito Pistoia. 2008. “Mesenchymal Stem Cells in Health and Disease.” *Nature Reviews. Immunology* 8 (9): 726–36. https://doi.org/10.1038/nri2395.

Wada, Naohisa, Danijela Menicanin, Songtao Shi, P. Mark Bartold, and Stan Gronthos. 2009. “Immunomodulatory Properties of Human Periodontal Ligament Stem Cells.” *Journal of Cellular Physiology* 219 (3): 667–76. https://doi.org/10.1002/jcp.21710.

Wagner, Wolfgang, Frederik Wein, Anja Seckinger, Maria Frankhauser, Ute Wirkner, Ulf Krause, Jonathon Blake, et al. 2005. “Comparative Characteristics of Mesenchymal Stem Cells from Human Bone Marrow, Adipose Tissue, and Umbilical Cord Blood.” *Experimental Hematology* 33 (11): 1402–16. https://doi.org/10.1016/j.exphem.2005.07.003.

Wongsupa, Natkrita, Thongchai Nuntanaranont, Suttatip Kamolmattayakul, and Nuttawut Thuaksuban. 2017. “Assessment of Bone Regeneration of a Tissue-Engineered Bone Complex Using Human Dental Pulp Stem Cells/Poly(ε-Caprolactone)-Biphasic Calcium Phosphate Scaffold Constructs in Rabbit Calvarial Defects.” *Journal of Materials Science: Materials in Medicine* 28 (5): 77. https://doi.org/10.1007/s10856-017-5883-x.

Xu, Dan, and Hidetoshi Tahara. 2013. “The Role of Exosomes and MicroRNAs in Senescence and Aging.” *Advanced Drug Delivery Reviews* 65 (3): 368–75. https://doi.org/10.1016/j.addr.2012.07.010.

Yang, Chi, Xiaohan Liu, Kai Zhao, Youming Zhu, Bin Hu, Yong Zhou, Mohan Wang, et al. 2019. “MiRNA-21 Promotes Osteogenesis via the PTEN/PI3K/Akt/HIF-1α Pathway and Enhances Bone Regeneration in Critical Size Defects.” *Stem Cell Research & Therapy* 10 (1): 65. https://doi.org/10.1186/s13287-019-1168-2.

Yi, Qiao, Ousheng Liu, Fei Yan, Xiao Lin, Shu Diao, Liping Wang, Luyuan Jin, Songlin Wang, Yanqin Lu, and Zhipeng Fan. 2017. “Analysis of Senescence-Related Differentiation Potentials and Gene Expression Profiles in Human Dental Pulp Stem Cells.” *Cells, Tissues, Organs* 203 (1): 1–11. https://doi.org/10.1159/000448026.

Yoder, Mervin C. 2012. “Human Endothelial Progenitor Cells.” *Cold Spring Harbor Perspectives in Medicine* 2 (7). https://doi.org/10.1101/cshperspect.a006692.

Zaragosi, Laure-Emmanuelle, Brigitte Wdziekonski, Kevin Le Brigand, Phi Villageois, Bernard Mari, Rainer Waldmann, Christian Dani, and Pascal Barbry. 2011. “Small RNA Sequencing Reveals MiR-642a-3p as a Novel Adipocyte-Specific MicroRNA and MiR-30 as a Key Regulator of Human Adipogenesis.” *Genome Biology* 12 (7): R64. https://doi.org/10.1186/gb-2011-12-7-r64.

Zhang, Jinlong, Xiaohui Lu, Guijuan Feng, Zhifeng Gu, Yuyu Sun, Guofeng Bao, Guanhua Xu, et al. 2016. “Chitosan Scaffolds Induce Human Dental Pulp Stem Cells to Neural Differentiation: Potential Roles for Spinal Cord Injury Therapy.” *Cell and Tissue Research* 366 (1): 129–42. https://doi.org/10.1007/s00441-016-2402-1.

Zhang, Yanjie, Xiaohan Huang, and Yanhao Yuan. 2017. “MicroRNA-410 Promotes Chondrogenic Differentiation of Human Bone Marrow Mesenchymal Stem Cells through down-Regulating Wnt3a.” *American Journal of Translational Research* 9 (1): 136–45.

Zhang, Zhiwei, Junjie Yang, Weiya Yan, Yangxin Li, Zhenya Shen, and Takayuki Asahara. 2016. “Pretreatment of Cardiac Stem Cells With Exosomes Derived From Mesenchymal Stem Cells Enhances Myocardial Repair.” *Journal of the American Heart Association* 5 (1). https://doi.org/10.1161/JAHA.115.002856.

Zhao, Zhi-Gang, Wei-Ming Li, Zhi-Chao Chen, Yong You, and Ping Zou. 2008. “Immunosuppressive Properties of Mesenchymal Stem Cells Derived from Bone Marrow of Patients with Chronic Myeloid Leukemia.” *Immunological Investigations* 37 (7): 726–39. https://doi.org/10.1080/08820130802349940.

Zhou, Shuanhu, Joel S. Greenberger, Michael W. Epperly, Julie P. Goff, Carolyn Adler, Meryl S. LeBoff, and Julie Glowacki. 2008. “Age-Related Intrinsic Changes in Human Bone-Marrow-Derived Mesenchymal Stem Cells and Their Differentiation to Osteoblasts.” *Aging Cell* 7 (3): 335–43. https://doi.org/10.1111/j.1474-9726.2008.00377.x.