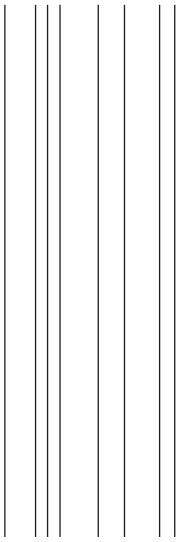

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DOCTORAL DISSERTATION

Dental stem cells and angiogenesis: new strategies for tissue engineering

Doctoral dissertation submitted to obtain the degree of
Doctor of Biomedical Science, to be defended by

Petra Hilkens

Promoter: Prof. Dr Ivo Lambrichts
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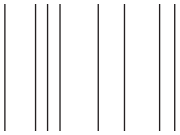

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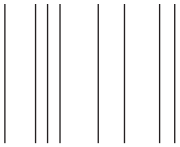

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List of Abbreviations

AAE	American association of endodontists
aMEM	minimal essential medium, alpha modification
ANGPT	angiopoietin
bFGF	basic fibroblast growth factor
BM-MSCs	bone marrow-derived mesenchymal stem cells
CAM	chorioallantoic membrane assay
CFU	colony forming unit
CPC	cetylpyridinium chloride
CSF	colony stimulating factor
DAB	3,3’ diaminobenzidine
DAPI	4’,6-diamidino-2- phenylindole
DMSO	dimethyl sulfoxide
DPPIV	dipeptidyl peptidase IV
DPSCs	dental pulp stem cells
DSCs	dental stem cells
ECM	extracellular matrix
EDN1	endothelin-1
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EZ	enzymatic digestion
FABP-4	fatty acid binding protein
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FSCs	dental follicle precursor cells
G-CSF	granulocyte-colony stimulating factor
HA	hydroxyapatite
hEGF	human epidermal growth factor
HGF	hepatocyte growth factor
HGF-1	human gingival fibroblasts
HIF1α	hypoxia-inducible factor 1 alpha

HLA	human leukocyte antigen
HMEC	human microvascular endothelial cells
HUVEC	human umbilical cord vein endothelial cells
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IL-8	interleukin-8
iPSCs	induced pluripotent stem cells
ITS	insulin transferrin sodium selenite
MCP-1	monocyte chemotactic protein-1
MI	myocardial infarction
MMP	matrix metalloproteinase
MSCs	mesenchymal stem cells
MTA	mineral trioxide aggregate
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NGFR	nerve growth factor receptor
NO	nitric oxide
OG	outgrowth method
PAFSCs	periapical follicular stem cells
PAI-1	plasminogen activator inhibitor-1
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PDLSCs	periodontal ligament stem cells
PE	phycoerythrin
PECAM-1	platelet endothelial cell adhesion molecule-1
PEG	polyethylene glycol
PEGF	pigment epithelium-derived growth factor
PFA	paraformaldehyde
PGA	polyglycolic acid
PLGA	poly(lactic-co-glycolic acid)
P(L)LA	poly-(L-)lactic acid
PRF	platelet-rich fibrin
PRP	platelet-rich plasma
PTX3	pentraxin-3
REP	regenerative endodontic procedure

RER	rough endoplasmic reticulum
RT-PCR	reverse transcriptase polymerase chain reaction
SCAPs	stem cell from the apical papilla
SCID	severe combined immunodeficiency
SEM	scanning electron microscopy
SHEDs	stem cells from human exfoliated deciduous teeth
TCP	tricalcium phosphate
TDM	treated dentin matrix
TEM	transmission electron microscopy
TGF-β	transforming growth factor beta
THBS	thrombospondin
TIMP	tissue inhibitor of matrix metalloproteinase
TNF-α	tumor necrosis factor alpha
uPA	urokinase-type plasminogen activator
VEGF	vascular endothelial growth factor

Chapter 1

Introduction and Aims

Based on:

Hilkens P., Meschi N., Bronckaers A., Lambrechts P. and Lambrichts I. Dental stem cells in pulp regeneration: near future or long road ahead?

Stem Cells and Development (*accepted*)

1.1 Stem cells

Within the human body, stem cells can be found in different developmental stages and tissues. In general, stem cells can be characterized by three well-defined criteria, namely long-term self-renewal, multilineage differentiation potential and the ability of regenerating a certain tissue *in vivo*. Depending on their differentiation capacity, different stem cell populations can be distinguished, such as totipotent, pluripotent, multipotent and unipotent stem cells. **Totipotent stem cells** are derived from a zygote and are capable of forming both embryonic and extra-embryonic tissues, such as the placenta. **Pluripotent stem cells**, on the other hand, such as embryonic stem cells, are derived from the inner cell mass of an early blastocyst. These cells are capable of differentiating into three germ layers, i.e. endoderm, mesoderm and ectoderm, and germ cells. **Multipotent or adult stem cells**, e.g hematopoietic stem cells or mesenchymal stem cells, can only differentiate into one germ layer; they give rise to various organ-specific cell types and are responsible for the natural turn-over of tissues and/or organs. **Unipotent stem cells** have a limited self-renewal capacity and are considered to be progenitor cells of one single cell type [1].

1.1.1 Mesenchymal stem cells

In 1970, Friedenstein et al. were the first to describe the existence of so-called colony-forming unit fibroblasts, a highly clonogenic bone marrow-derived cell population with osteogenic differentiation potential [2]. Further characterization of these mesenchymal stem cells (MSCs), as they were later on called, led to the following minimal criteria according to which these cells can be defined: when maintained under standard culture conditions, MSCs are considered to be plastic-adherent. Furthermore, these cells do not only express certain cell surface markers such as CD73, CD90 and CD105 but they also lack the expression of CD14 or CD11b, CD34, CD45, CD79a or CD19 and HLA-DR surface molecules. Lastly, MSCs are also characterized by a defining trilineage differentiation potential *in vitro*, i.e. differentiation into adipocytes, osteoblasts and chondrocytes [3, 4]. However, multiple studies have indicated that MSCs are able to cross lineage boundaries and to differentiate into non-mesodermal

cell types. In other words, depending on the specific microenvironment, MSCs are able to switch cell fate [1, 5]. For example, when incubated with specific culture media bone marrow-derived MSCs (BM-MSCs) can also give rise to myocardial cells, endothelial cells, hepatocytes and neuronal cells *in vitro* [6-10]. Nevertheless, the potential therapeutic benefits of BM-MSCs in the treatment of several disorders are also offset by their invasive and painful isolation procedure, which all together stresses the need for an alternative source of adult stem cells [11, 12]. MSCs can be found in numerous tissues within the human body, such as adipose tissue, umbilical cord, tendons and also teeth [13-16].

1.1.2 Dental stem cells

Within the human tooth and its environment, several stem cell populations can be distinguished such as dental pulp stem cells (DPSCs), stem cells from the apical papilla (SCAPs), dental follicle precursor cells (FSCs) and periodontal ligament stem cells (PDLSCs) (Figure 1.1) [17-20].

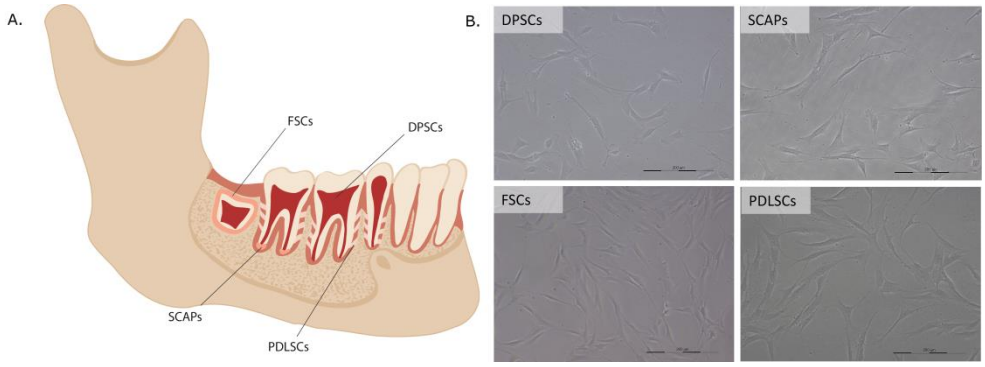

Figure 1.1 The four main dental stem cell populations in the human oral environment. A. The *in vivo* location of dental stem cells. B. Cultured dental stem cells following their isolation. Scale bars = 200 µm. Dental pulp stem cells (DPSCs), stem cells from the apical papilla (SCAPs), dental follicle precursor cells (FSCs), periodontal ligament stem cells (PDLSCs).

Although dental stem cells (DSCs) can be defined as MSCs according to the minimal criteria defined by the International Society for Cellular Therapy, their origin has recently been subject of considerable debate [3, 15, 21-23]. Up until

now, it was assumed that, given the reciprocal interactions between embryonic oral epithelium and neural crest-derived mesenchyme during tooth morphogenesis, DSCs are derived from the neural crest. However, a study by Kaukua *et al.* recently demonstrated the glial origin of a significant subpopulation of DPSCs, suggesting that, depending on the tissue of origin, each DSCs population is defined by its own ability to repair and/or regenerate diseased or damaged (dental) tissues, which will be discussed in the following paragraphs [24].

1.2 Angiogenesis

Within the human body, three mechanisms of blood vessel formation can be distinguished, namely vasculogenesis, arteriogenesis and angiogenesis. During embryonic development, blood vessels are formed by means of **vasculogenesis**, i.e. the assembly of a primitive network through maturation and differentiation of migrated angioblasts [25]. **Arteriogenesis**, on the other hand, can be defined as vessel stabilization or, in pathological conditions, the rapid proliferation of pre-existing collateral arteries in the case of an acute or chronic occlusion of a major artery [25, 26]. The most studied and the most predominant mechanism in adult life, however, is **angiogenesis**. It is defined as the sprouting of new capillaries from pre-existing blood vessels and is considered to be a complex and well-coordinated multi-step process, initiated in response to specific stimuli such as hypoxia or inflammation (Figure 1.2) [25, 27, 28]. The subsequent increase in vascular permeability and vessel destabilization releases sequestered angiogenic proteins and chemokines, which promote endothelial proliferation and migration. Following vascular sprouting and lumen formation, the resulting tubes fuse with the existing capillary after which they are stabilized, i.e. attachment of pericytes and deposition of extracellular matrix [25, 29].

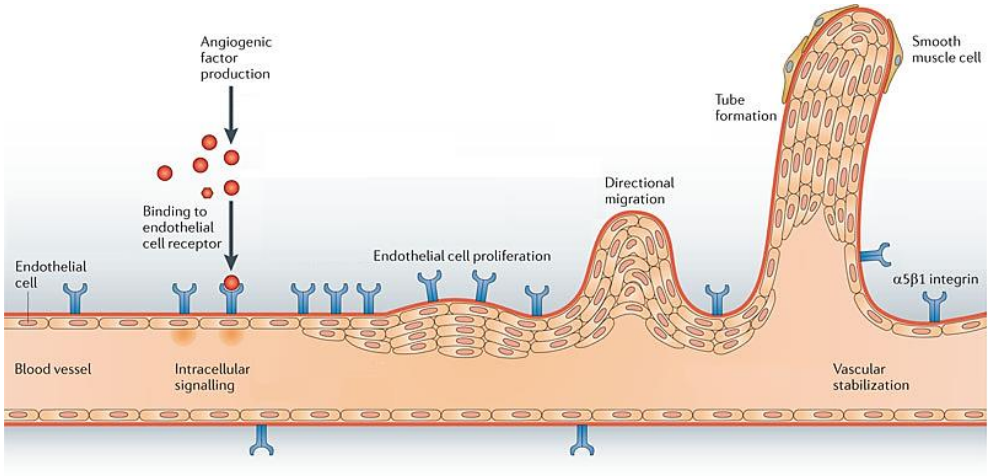

Figure 1.2 Cellular and molecular interactions during angiogenesis. Angiogenesis is a well-orchestrated biological process, which involves vasodilatation, the degradation of extracellular matrix, the activation, proliferation and migration of endothelial cells, tubulogenesis, and stabilization and remodeling of blood vessels. Adapted from Zhang et al., 2012 [30].

The whole process of angiogenesis is carefully regulated by a myriad of regulating proteins, some of which play a dual role depending on the specific microenvironment (Table 1.1a and b) [31-42]. Within the healthy human body, these regulating proteins form a delicate balance between activation and inhibition of blood vessel formation. In general, the effect of the inhibiting factors is dominant and endothelial cells and blood vessels remain in a quiescent state. However, in case of hypoxia, for example, angiogenic growth factors are produced in excess over the angiogenesis inhibitors and an angiogenic switch occurs. In other words, the balance is tipped towards blood vessel growth [29, 32]. Disturbance of the angiogenic balance also takes place in pathological conditions, such as in myocardial ischemia, cerebral ischemic stroke, diabetes mellitus, psoriasis and cancer [31].

Table 1.1a Angiogenic factors and their associated functions

Angiogenesis-stimulating factors		
Angiogenin	Endothelial proliferation and migration. Activation of smooth muscle cells. Basement membrane degradation (indirect).	[32, 43-46]
Angiopoietin-1 (ANGPT1)	Endothelial survival, migration and matrix adhesion. Endothelial sprouting and vessel stabilization.	[32, 47-50]
Angiopoietin-2 (ANGPT2)	Endothelial proliferation, migration and sprouting in the presence of VEGF.	[32, 51, 52]
Basic Fibroblast Growth Factor (bFGF)	Endothelial proliferation, migration and tube formation. Upregulation of uPA, VEGF receptor (KDR) and integrins.	[34, 53-56]
CXC chemokines (ELR^+) e.g. Interleukin-8 (IL-8) and CXCL16	Endothelial survival, proliferation, migration and tube formation. Induction of MMP production.	[34, 37, 57-60]
Colony Stimulating Factors (CSFs)	Endothelial proliferation, migration and differentiation. Induction of proteolytic enzyme release.	[32, 61, 62]
Dipeptidyl peptidase IV (DPPIV)	Vascular remodeling. Endothelial proliferation, migration and tube formation (through cleavage of neuropeptide Y).	[39, 63]
Endothelin 1 (EDN1)	Endothelial proliferation and migration. Stimulation of VEGF-mediated angiogenesis Stimulation of endothelial MMP2 production. Proliferation of vascular smooth muscle cells.	[40, 41, 64, 65]
Epidermal Growth Factor (EGF)	Endothelial proliferation, migration and tube formation.	[32, 34, 66]
Erythropoietin	Endothelial proliferation and tube formation. Stimulation of endothelial MMP2 production.	[67-69]
Hepatocyte Growth Factor (HGF)	Endothelial proliferation, migration and tube formation. Proliferation of vascular smooth muscle cells. Stimulation of VEGF and PIGF production.	[32, 70-72]
Insulin-like Growth Factor-1 (IGF-1)	Endothelial proliferation, migration and tube formation.	[32, 66, 73-75]

	Stimulation of VEGF and plasminogen activator production. Downregulation of endothelial apoptosis.	
Insulin-like Growth Factor Binding Protein 1-3 (IGFBP 1-3)	Endothelial migration and tube formation. Stimulation of IGF-1-mediated angiogenesis. Stimulation of VEGF and MMP2 production.	[33, 76]
Integrins	Endothelial adhesion, proliferation, migration and tube formation. Downregulation of endothelial apoptosis. Stimulation of bFGF-mediated and VEGF-mediated angiogenesis.	[32, 77-82]
Matrix Metalloproteinases (MMPs)	Extracellular matrix degradation and release of sequestered growth factors.	[34, 83, 84]
Monocyte Chemotactic Protein-1 (MCP-1)	Endothelial chemotaxis, tube formation and differentiation. Stimulation of HIF1α and VEGF production.	[36, 85-87]
Nitric Oxide (NO)	Vessel permeability. Stimulation of bFGF and VEGF production. Stimulation of $\alpha_v\beta_3$ integrin expression. Downregulation of endothelial apoptosis. Downregulation of angiostatin production.	[31, 88-91]
Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1)	Endothelial aggregation, migration and tube formation. Vessel stabilization. Essential for bFGF-induced angiogenesis.	[32, 92-94]
Platelet-Derived Growth Factor (PDGF)	Endothelial proliferation, migration and differentiation. Stimulation of VEGF expression. Proliferation of vascular smooth muscle cells and pericytes. Vessel stabilization.	[34, 95-99]
Transforming Growth Factor β (TGF-β)	Endothelial proliferation (dose-dependent). Endothelial differentiation and tube formation. Stimulation of PDGF, VEGF, bFGF, uPA expression. Regulation of MMP2 and MMP9. Vessel stabilization.	[32, 34, 100-103]
Tumor Necrosis Factor α (TNF-α)	Endothelial proliferation (dose-dependent). Endothelial differentiation and tube formation.	[32, 104-107]

	Stimulation of VEGF(R), IL-8 and bFGF expression.	
Urokinase-type Plasminogen Activator (uPA)	Participation in ECM degradation and release of sequestered growth factors. Endothelial migration and invasion. Activation of VEGF and pro-HGF.	[34, 108-110]
Vascular Endothelial Growth Factor (VEGF)	Endothelial proliferation, migration and tube formation. Stimulation of NO synthase and plasminogen activator expression. Downregulation of endothelial apoptosis.	[32, 34, 111-113]

Table 1.1b Angiogenic factors and their associated functions

Angiogenesis-inhibiting factors		
Angiopoietin-2 (ANGPT2)	Natural antagonist of ANGPT1. Upregulation of endothelial apoptosis.	[32, 114]
Angiostatin	Endothelial proliferation, migration and tube formation. Smooth muscle cell proliferation. Upregulation of endothelial apoptosis. Inhibition of HGF-induced angiogenesis.	[32, 34, 115-120]
CXC chemokines (ELR^-)	Inhibition of CXC ELR^+-induced angiogenesis. Endothelial proliferation and migration through inhibition of VEGF and bFGF receptor binding.	[121-123]
DPPIV	Inhibition of endothelial progenitor homing. Inhibition of CXCR3-induced chemotaxis.	[124, 125]
Endostatin	Endothelial proliferation and migration. Upregulation of endothelial apoptosis. Inhibition of MMPs, bFGF-mediated and VEGF-mediated angiogenesis.	[32, 126-129]
IGFBP3	Endothelial migration and tube formation. Inhibition of MMP9 and VEGF production.	[35, 130]
MMPs	Inhibition of FGFR1 and uPAR-mediated signaling. Generation of angiogenic inhibitors by proteolytic cleavage: angiostatin, endostatin, tumstatin, arrestin, canstatin.	[131-136]
Pentraxin-3 (PTX3)	Inhibition of bFGF-mediated angiogenesis.	[42, 137]

Pigment epithelium-derived factor (PEGF)	Endothelial proliferation and migration. Upregulation of endothelial apoptosis. Inhibition of MMPs, bFGF-mediated and VEGF-mediated angiogenesis.	[32, 138-140]
Plasminogen Activator Inhibitor-1 (PAI-1)	Inhibition of uPA.	[34, 108]
Thrombospondin-1 and 2 (THBS-1/2)	Endothelial proliferation, migration and tube formation. Upregulation of endothelial apoptosis.	[31, 141-143]
Tissue Inhibitor of MMPs (TIMP)	Inhibition of MMPs.	[34, 144, 145]
TGF-β	Endothelial proliferation and migration. Downregulation of plasminogen activators. Upregulation of TIMPs Upregulation of endothelial apoptosis.	[32, 102]
TNF-α	Endothelial proliferation (dose-dependent). Inhibition of bFGF-mediated angiogenesis. Upregulation of endothelial apoptosis.	[32, 66]

Besides playing a part in potential life-threatening disorders, angiogenesis also plays a key role in wound healing and tissue engineering; without the development of new blood vessels wound healing would be impaired and newly transplanted tissue would eventually become necrotic. Furthermore, blood supply is also of vital importance in the treatment of ischemic diseases, such as stroke and myocardial infarction [146]. Although early revascularization studies merely focused on the addition and modulation of pro-angiogenic growth factors, the limited success of these studies and the increasing number of 'no-option' patients prompted the need for (stem) cell-based therapies [147, 148].

1.2.1 Angiogenic properties of mesenchymal stem cells

With regard to MSCs, numerous reports indicate their positive impact on angiogenesis either by secreting paracrine factors or by differentiating into endothelial cells themselves. Kagiwada *et al.*, for example, showed that BM-MSCs express pro-angiogenic as well as anti-angiogenic factors such as IL-6, IL-8, TIMP-1, angiogenin, MCP-1 and VEGF [149]. Other angiogenic factors that are secreted by MSCs include: ANGPT1/2, bFGF, HGF, IGF, MMPs, PLGF, TGF-β and uPA, although the angiogenic secretion profile seems to be dependent on the

tissue of origin [150-155]. Due to this paracrine crosstalk, MSCs are able to influence the behavior of endothelial cells *in vitro* as well as *in vivo*. *In vitro*, for example, MSCs have been shown to increase endothelial migration, tube formation and, in certain cases, proliferation [152, 154, 156-159]. In addition, multiple studies indicated positive effects of MSCs in *in vivo* models of wound healing, hind limb ischemia, stroke and myocardial infarction [152, 160-165]. Despite the aforementioned success of MSCs *in vivo*, clinical trials have yielded contrasting results. In particular the exact mechanisms of action often remain subject of controversy [166-168]. Although MSCs have been shown to differentiate into endothelial cells *in vitro*, the rate of MSC engraftment has proven to be extremely low in most *in vivo* settings. Therefore it is currently assumed that the therapeutic benefits of these cells are primarily caused by their paracrine (angiogenic) actions [8, 152, 159, 169-173].

1.2.2 Angiogenic properties of dental stem cells

Despite the elaborate characterization of DSCs over the past decade, little is known about their angiogenic properties. With regard to DPSCs, a number of studies reported their expression of paracrine factors such as VEGF, bFGF and PDGF under basal conditions and after injury or hypoxia [174-177], as well as the potential to ameliorate left ventricular function and to increase angiogenesis in a rat model of myocardial infarction [178]. Furthermore, a couple of studies have indicated the *in vitro* differentiation capacity of DPSCs into endothelial or pericyte-like cells, which could indicate the potential incorporation of these cells into newly formed blood vessels although proof of true functionality is limited at this stage [179-183]. Although SCAPs and FSCs are considered to be more immature dental stem populations residing in strongly vascularized tissues, literature merely indicates their expression of endostatin, bFGF and angiogenesis-regulating transcription factors such as hypoxia-inducible factor 1 alpha (HIF1α) [15, 184]. Despite their expression of angiogenic factors, the effects of both dental stem cell populations on endothelial cell behavior and blood vessel formation have not yet been described. PDLSCs, on the other hand, do not only express VEGF and bFGF, but they are also capable of stimulating endothelial tubulogenesis *in vitro* and *in vivo* [185-187]. In order to potentiate

any therapeutic application of DSCs, either in (dental) tissue engineering or in ischemic diseases, a more elaborate angiogenic profiling is required.

1.3 Dental stem cells in regenerative dentistry

Over the past decade, research has substantially increased in the field of regenerative dentistry, in particular in the field of dental pulp regeneration. Despite being a specialized tissue which serves many physiological functions, the dental pulp is very vulnerable to insults such as caries, infections and trauma, which stresses the need for conservative and effective treatment protocols. Whereas traditional dentistry used to focus on esthetics and merely restoring the function of the compromised tooth, modern dental practice aims for a more regenerative approach [188]. With the emphasis on conservation and viability, present-day regenerative endodontic procedures not only aim to maintain the inherent strength and structure of the tooth but also aim to sustain pulp vitality [188, 189]. However, the technical and biological limitations associated with the current revascularization protocols prompt the need for alternative treatment options. The isolation and extensive characterization of DSCs has raised hope for a new era of dental pulp regeneration, namely an era of stem cell-based dental tissue engineering.

1.3.1 Regenerative endodontics

As any factor which interferes with normal pulp physiology may conflict with the completion of root development, the endodontic treatment of immature teeth in particular holds some major challenges [190, 191]. The viability of Hertwig's epithelial root sheath is a key factor as arrested root development following pulp necrosis may lead to weakening of the root structure, thereby increasing the tooth's susceptibility to fractures and consequently reducing its survival rate [191-194]. In 2008, Huang already mentioned a paradigm shift in the endodontic treatment of necrotic immature permanent teeth; in order to allow continued root development necrotic immature permanent teeth should be treated as minimally invasive as practically possible [191]. The concept of revascularization was first described by Nygaard Östby in 1961, indicating sustained root development following blood clot induction in the root canal of

immature teeth suffering from pulp necrosis [195]. Following numerous adaptations, this regenerative endodontic procedure (REP) was recently described as a standardized procedure by the American Association of Endodontists (AAE) [196]. The treatment protocol encompasses the disinfection of the root canal by means of sodium hypochlorite and triple antibiotic paste, followed by irrigation with ethylenediaminetetraacetic acid (EDTA) and the induction of bleeding with a hand file. Finally, the resulting blood clot is sealed with a resorbable matrix and the access cavity is restored (Figure 1.3) [197]. The induction of a blood clot not only causes the release of growth factors but presumably also serves as a scaffold for stem cells from the apical papilla (SCAPs) which survived the disinfection protocol [190, 198, 199]. Studies also reported the release of pro-angiogenic dentin matrix components following irrigation with EDTA, all together leading to an optimally conductive environment for dental pulp healing and regeneration [200-202].

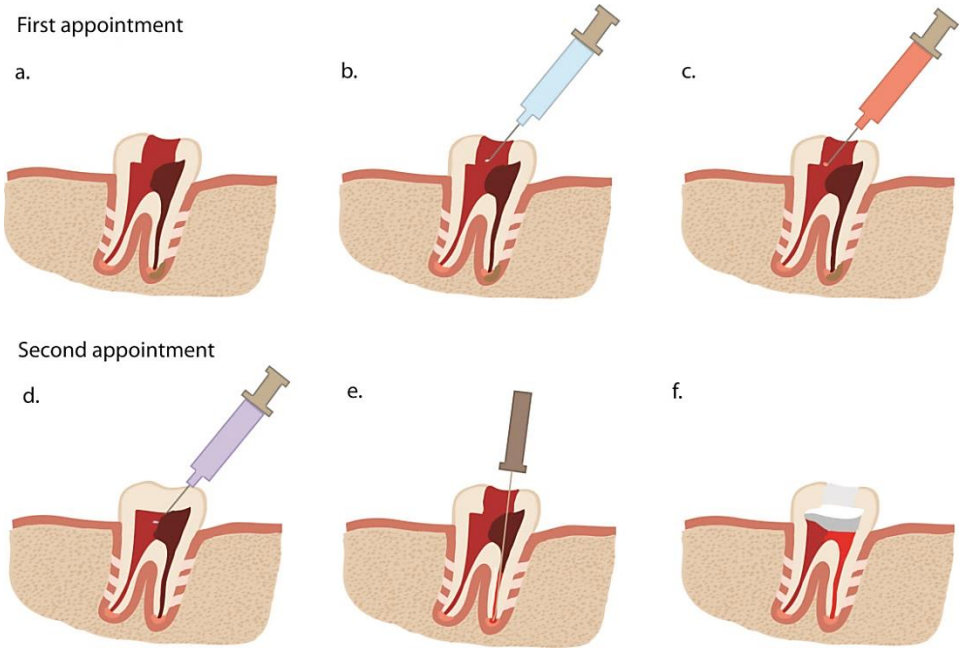

Figure 1.3 Regenerative endodontic procedure. A. Tooth with necrotic pulp and immature apex. B. Irrigation with sodium hypochlorite. C. Disinfection with triple antibiotic paste. D. Irrigation with EDTA. E. Induction of bleeding with a hand file. F. Sealing of blood clot with a resorbable matrix and restoration of access cavity.

Another cell homing-based approach has also been recently described in several case studies, namely the injection of platelet-rich plasma (PRP) or platelet-rich fibrin (PRF) to potentially attract residing stem cell populations and promote tissue restoration [203-209]. However, as already mentioned by Diogenes *et al.* and others, definitive conclusions regarding the reported favorable outcomes and their predictability cannot be drawn at this point due to the different disease etiologies and the lack of standardized treatment protocols in numerous clinical case reports [190, 194, 197]. An additional drawback of the currently used cell homing-based techniques is the fact that the exact nature of the regenerated tissue in the pulp cavity is difficult to determine [210]. Due to its reliance on cell homing, regeneration by triggering the periapical tissue with a hand file and/or application of PRP can cause the invasion of different cell populations into the pulp cavity leading to ingrowth of periodontal tissue and deposition of cementum and bone [208-213]. Another often recurring concern in revascularization/cell homing-based procedures is the size of the apex which is required for adequate pulp revascularization, as the vascular supply within the tooth is restricted by the apical foramen. Several studies suggest a higher rate of dental pulp healing and revascularization with apical sizes ranging from 1.1 to 1.5 mm [210, 214, 215]. In terms of cell-based approaches however, recent studies by Iohara *et al.* indicated regeneration of vascularized pulp tissue in mature dog teeth with an apical opening of 0.7 mm after transplantation of a subpopulation of DPSCs [216, 217]. Furthermore, this research group also reported a more pronounced volume of regenerated dental pulp tissue with a higher capillary density after transplantation of DPSCs in comparison to a cell homing-based approach with granulocyte-colony stimulating factor (G-CSF) and basic fibroblast growth factor (bFGF) [218]. Taken together, these studies suggest the application of DSCs as a potential tool in regenerative endodontic approaches. The following sections offer a comprehensive overview of the current literature on the use of different types of DSCs in dental tissue engineering.

1.3.2 Dental stem cells in dental tissue engineering

Dental pulp stem cells

In 1996, Mooney *et al.* were the first to describe the *in vitro* formation of pulp-like tissue after culturing human pulp-derived fibroblasts onto a polyglycolic acid (PGA) matrix for 60 days [219]. When transplanting these cell-matrix constructs in immunocompromised mice, fibroblasts were able to survive and to produce extracellular matrix [220]. Around the same time, Gronthos *et al.* reported the isolation and characterization of a stem cell population within the dental pulp. Transplantation of these stem cells in combination with hydroxyapatite/tricalcium phosphate (HA/TCP) particles in immunocompromised mice led to the formation of fibrous pulp-resembling tissue containing blood vessels and human odontoblast-like cells which lined a dentin matrix-like structure [17, 221, 222]. In spite of the numerous favorable results indicating the regenerative potential of DPSCs, clinical reality prompted the need for more standardized and representative experimental approaches, such as the tooth slice scaffold model and the ectopic root transplantation model. Next to the investigation of dentinogenesis and the evaluation of pulp vascularization, a human tooth slice can also be adapted as a scaffold to support dental pulp regeneration [223-225]. A couple of studies indicated the formation of vascularized pulp-like tissue after transplantation of tooth slices containing DPSCs or dental pulp stem cells from exfoliated deciduous teeth (SHEDs) in combination with a biodegradable scaffold in immunodeficient mice [226, 227]. As the vascular supply within the tooth is restricted by the diameter of the apical foramen, a more exemplary model is the transplantation of emptied human root canals, i.e. the ectopic root transplantation model. In 2010, Huang *et al.* were one of the first to show that DPSCs, seeded onto a poly(lactic-co-glycolic acid) (PLGA) scaffold, were able to form vascularized pulp / dentin-like tissue in an emptied human root canal which had been subcutaneously transplanted into severe combined immunodeficient (SCID) mice for three months. However, it should be noted that this study applied a rather large apical opening of 2.5 mm, which led the authors to question whether smaller canal openings would affect the efficacy of coronal tissue regeneration [228]. Similar experiments were conducted by Rosa *et al.* using SHEDs in combination with a self-assembling

peptide hydrogel. Although a vascularized pulp / dentin-like tissue was formed after only 35 days, the authors did not seal one of the canal's ends leading to the potential influx of nutrients and cells through both openings of the root canal [229]. In contrast, a recent report on the regenerative potential of different subpopulations of DPSCs indicated the regeneration of vascularized pulp tissue (with no mention of dentin deposition) in human root canals, sealed at one end with mineral trioxide aggregate (MTA), after a mere 21 days of subcutaneous transplantation. In particular a G-CSF mobilized subpopulation of DPSCs showed a significantly larger area of regenerated, vascularized pulp tissue in comparison to $CD105^+$ DPSCs, unfractionated DPSCs and induced pluripotent stem cells (iPSCs), suggesting that the regenerative potential of DPSCs as a whole is determined by the intrinsic properties of certain subsets of cells within the population [230]. Although the ectopic root transplantation model is a feasible, proof-of-principle model for small animals, a more translational approach would require the *in situ* transplantation of DPSCs in (partly) pulpectomized teeth of larger animals. A decade ago, Iohara *et al.* already showed the formation of reparative dentin after autologous transplantation of BMP2-treated DPSC pellets onto the amputated pulp of dog teeth [231, 232]. This group and others also indicated complete pulp regeneration after autologous transplantation of (certain subsets of) DPSCs in pulpectomized dog teeth [216, 233-235]. Recently, concrete steps towards a clinical application of DPSCs have been taken by Iohara and colleagues through the autologous transplantation of clinical-grade G-CSF mobilized DPSCs in permanent dog teeth with an apical opening of 0.6 mm. This study demonstrated the regeneration of vascularized and innervated pulp tissue together with the coronal formation of dentin after assuring the quality of the stem cells by karyotype analysis and ruling out potential tumor formation [189, 217].

Stem cells from the apical papilla

At the apex of developing permanent teeth a loosely attached soft connective tissue can be found, namely the apical papilla. In 2008, Sonoyama et al. were the first to isolate and characterize the papilla's residing stem cell population, i.e. SCAPs. Comparative studies with DPSCs indicated a more pronounced population doubling capacity, a higher proliferation rate and better migratory

properties, indicating a more potent stem cell population. Given their origin from a developing tissue, the regenerative potential of SCAPs has gained increasing interest [236, 237]. In 2006, Sonoyama *et al.* already indicated the formation of dentin-pulp-like complex in immunocompromised mice after transplantation of SCAPs in combination with HA/TCP particles. This study also mentioned the human origin of the dentin-producing cells, suggesting the differentiation of transplanted SCAPs into odontoblasts [236]. The *in vivo* odontogenic potential of SCAPs was also noted by Huang *et al.*, after implantation of SCAPs in combination with a PLGA scaffold in an ectopic root transplantation model. Moreover, the formed dentin-pulp complex showed a more continuous and thicker dentin matrix in comparison with the model utilizing a DPSCs-PLGA construct [228]. More recently, the formation of a vascularized dentin-pulp-like complex with a continuous dentin layer has been observed following *in vivo* transplantation of SCAPs-based cell sheet-derived pellets in dentin matrix fragments [238]. In addition, the vast body of evidence reporting continued root development after conservative treatment of immature teeth suffering from apical periodontitis and/or apical abscesses suggests the survival of SCAPs after infection and their potential contribution to root formation by differentiating into odontoblasts [18, 191, 197, 212, 237, 239-244]. However, to date there has only been one study showing the successful regeneration of a root/periodontal complex in an animal model after autologous transplantation of SCAPs and PDLSCs, indicating that more extensive research is required to unravel the differentiation potential of SCAPs and their potential role in bioroot engineering [18, 236].

Dental follicle precursor cells

Another developing dental tissue which harbors stem cells is the dental follicle [19]. The dental follicle is a loose connective tissue which surrounds the developing tooth and later on in development gives rise to the periodontium. Due to their immature nature FSCs have been widely investigated for their potential role in dental tissue engineering. In 2002, Handa et al. showed that bovine FSCs were able to differentiate into cementoblasts *in vivo* following transplantation in SCID mice [245]. However, initial attempts to reconstruct dental tissues with human FSCs led to disappointing results; despite the

expression of osteoblast-related markers there was no sign of cementum or bone formation eight weeks after transplantation of matrigel clots containing FSCs and HA powder in immunodeficient mice [19]. In contrast, Guo *et al.*, demonstrated the ability of rat FSCs to regenerate dentin by transplanting treated dentin matrix (TDM) - FSCs constructs in the omental pouch of adult rats [246]. The regenerative potential of rat FSCs was later confirmed by the same group in a tooth root reconstruction model. Transplantation of a TDM-FSCs constructs in the alveolar fossa of adult rats led to the generation of dentin-pulp complex, a cementum-like layer and a periodontal ligament (PDL) attached to the host alveolar bone 8 weeks following transplantation [247]. Similar results were obtained after subcutaneous implantation of TDM-FSCs cell sheet constructs, although the addition of calcinated human dentin was required to recreate the appropriate microenvironment for cementum- PDL regeneration [248]. The *in vivo* establishment of PDL was also validated by Yokoi *et al*. by subcutaneously implanting a fibrin clot containing immortalized mouse FSCs and HA powder in immunodeficient mice. Four weeks following transplantation fibrous PDL-like tissue was detected along with scattered bone-like tissue. Cementum on the other hand was not present, according to the authors presumably because of the applied isolation technique for FSCs [249]. More recently, a comparison of human FSCs-sheets and human PDLSCs-sheets by Guo *et al.*, has indicated a stronger periodontal regenerative potential of FSCs-sheets following four weeks of transplantation in combination with TDM in nude mice [250]. Taken together, all the aforementioned data suggest that dental follicles are an easy accessible, favorable source of stem cells for the engineering of different dental tissues. However, more extensive research is required characterizing the proliferation and differentiation properties of FSCs. Furthermore, the impact of different inductive microenvironments on the FSCs' regenerative potential needs to be elucidated in detail. Finally, the species-related differences of FSCs need to be taken into account when contemplating translation into human patients.

Periodontal ligament stem cells

The periodontal ligament is a specialized connective tissue which not only attaches the tooth to the alveolar bone but also has a sensory function [251]. In

2004, Seo *et al.* were able to isolate and characterize the ligament's residing stem cell population, namely PDLSCs [20]. As periodontal diseases can have detrimental effects on the periodontium and eventually lead to premature tooth loss, PDLSCs were quickly assumed to be ideal candidates for cell-based periodontal regeneration. Subcutaneous implantation of PDLSCs in combination with HA/TCP particles in immunocompromised mice led to the formation of a collagen-rich connective tissue containing cementoblast-like cells along a cementum-like structure [20]. Hard tissue formation was also observed when combining PDLSCs-HA/TCP constructs with VEGF or bFGF, although bFGF seemed to have inhibitory effects on mineralization and osteogenic differentiation [252]. In comparison, subcutaneous implantation of DPSCs resulted in the regeneration of a dentin-pulp-like complex, suggesting that next to an inductive microenvironment, the tissue of origin is also a determining factor in the regenerative potential of DSCs [20]. This was highlighted once again in a study by Park *et al.*, indicating PLSCs as the most favorable candidate for periodontal regeneration in comparison to DPSCs and periapical follicular stem cells (PAFSCs), after autologous transplantation in an apical involvement defect in beagles [253]. In contrast, a recent report on PDLSCs-sheets and FSCs-sheets demonstrated a stronger periodontal regenerative potential for FSCs after subcutaneous implantation with TDM [250]. However, as subcutaneous implantation is not sufficient to translate the periodontal regenerative potential of PDLSCs to human periodontitis patients, more clinically relevant animal models as the one applied in the study of Park *et al.* need to be adopted. In rodents, for example, allogeneic PDLSCs seeded onto Gelfoam® scaffolds were able to regenerate bone, periodontal ligament and cementum-like tissue following transplantation in a periodontal fenestration defect [254]. Periodontal repair was also found by Seo *et al.* after transplantation of PDLSCs and HA/TCP particles in immunodeficient rats, although this was not the case in all animal subjects [20]. On the other hand, transplantation of a similar construct in a miniature pig model of periodontitis resulted in the regeneration of bone, cementum and periodontal ligament following 12 weeks of transplantation [255]. Similar outcomes were also demonstrated in an ovine model with periodontal defects after allogeneic or autologous transplantation of PDLSCs in

combination with Gelfoam® scaffolds [256, 257]. Taken together, these data imply that PDLSCs are a favorable cell source for periodontal regeneration.

1.3.3 Scaffolds in dental tissue engineering

Besides responsive (stem) cells and growth factors (which are potentially delivered by cellular vehicles), tissue engineering requires the use of scaffolds in order to support and maintain a complex tissue organization [258, 259]. Within the field of regenerative dentistry, numerous potential tissue substitutes have already been tested, one being more successful than the other. An ideal scaffold should strongly sustain the physiological needs of the cells it supports; i.e. it should closely resemble the natural extracellular matrix. Besides being biocompatible and providing conductive and structural support, scaffolds also require a predictable and controlled biodegradation into non-toxic waste products [259, 260].

Collagen

Collagen, for example, is a naturally occurring macromolecule with excellent biocompatibility which has been widely applied in the regeneration of dental tissues. In 1994, Nakashima already reported the formation of dentin following transplantation of collagen matrix combined with human bone morphogenetic protein (BMP) in the pulp cavity of dogs [261]. To date, a number of studies have described the regeneration of (vascularized) dentin-pulp-like tissue after combining collagen scaffolds with DPSCs in different animal models [227, 229, 262, 263]. However, when contemplating the use of collagen and DSCs, one must take into account the strong matrix contraction caused by DPSCs, potentially limiting cell proliferation and obstructing tissue regeneration [264, 265]. Furthermore, due to its isolation from allogeneic tissue sources, the usage of collagen can hold the risk of potentially transmitting species-related pathologies or of eliciting an immune response [260, 266].

Fibrin and hyaluronic acid

Other naturally occurring biomaterials that are frequently applied in dental tissue engineering are fibrin and hyaluronic acid. Due to its low mechanical

stiffness and rapid shrinkage, fibrin is often used as a composite scaffold in order to improve its mechanical properties [266]. Galler *et al.*, for example, demonstrated the formation of vascularized tissue following transplantation of dentin disks containing polyethylene glycol (PEG)-modified fibrin and DPSCs in immunocompromised mice [267]. Suspension of porcine tooth bud cells in a mix of fibrin glue and platelet-rich fibrin also led to the successful regeneration of dental tissues in a minipig tooth socket [268]. Likewise, the use of fibrin gel on its own resulted in the ingrowth of pulp-like tissue in an ectopic root transplantation model [269]. Regarding the use of hyaluronic acid, Sasaki and Kawamata-Kido indicated the formation of reparative dentin after 60 days of direct pulp capping with this biocompatible glycosaminoglycan [270]. Vascularized pulp tissue formation and periodontal healing was also reported by others when using hyaluronic acid, either as a composite scaffold or on its own [271-273].

Polylactic acid, polyglycolic acid and Poly(lactic-co-glycolic acid)

Besides naturally occurring biomaterials, synthetic polymers are also frequently used in regenerative dentistry. In 1994, Robert and Frank already described the use of poly-(L-) lactic acid (P(L)LA) for guided periodontal tissue regeneration in beagle dogs [274]. Later on, the combination of P(L)LA with DSCs not only led to the successful repair of alveolar bone defects, but the regeneration of dental tissues was also observed, both *in vitro* and *in vivo* [226, 275-280]. With regard to the use of polyglycolic acid (PGA), Mooney *et al.* reported the *in vitro* engineering of dental pulp-like tissue when culturing PGA matrices seeded with dental pulp fibroblasts for a period of 60 days [219]. This outcome was confirmed by Buurma *et al.*, indicating the *in vivo* production of extracellular matrix after three weeks of subcutaneous implantation of dental pulp fibroblasts-PGA constructs in immunocompromised mice [220]. Similar findings were reported by others, showing the *in vivo* regeneration of tooth structures after transplantation of PGA and dental cells for prolonged periods of time in murine models [281-283]. Besides PLLA and PGA, a co-polymer of both materials has also been successfully applied in dental tissue engineering. Young *et al.*, for example, mentioned the formation of recognizable tooth structures following 20 to 30 weeks of transplantation of tooth bud cells seeded on

poly(lactic-co-glycolic acid) (PLGA) scaffolds in the omentum of rats [280]. Similar results were observed by others, indicating the promotion of dentin-pulp-like tissue formation when combining distinct variations of PLGA scaffolds with dental (stem) cells in different animal models [228, 262, 284-286]. In contrast, Zhao *et al.*, reported a lack of periodontal healing when combining mouse dental follicle cells with PLGA sponges in athymic rats, despite the widely accepted regenerative potential of FSCs [245-250, 287]. However, it should be noted that the aforementioned studies applied different scaffold materials together with FSCs derived from different species, yet again underlining the impact of an inductive microenvironment in combination with the inherent species-related differences in DSCs.

Hydroxyapatite, calcium phosphate and dentin matrix fragments

Next to their application in the healing of large bone defects, hydroxyapatite (HA) and tri-calcium phosphate (TCP) are frequently used as scaffold materials for dental tissue engineering purposes. Following the discovery and characterization of DPSCs, Gronthos *et al.* and others elaborately described the regeneration of dentin-pulp-like tissue after subcutaneous implantation of DPSCs and HA/TCP particles [17, 20, 221, 222, 236]. When combining these particles with other DSCs, such as SCAPs or PDLSCs, periodontal repair and regeneration was observed as well as dental pulp regeneration [20, 236, 252, 255]. Hydroxyapatite is a naturally occurring mineral which comprises an extensive part of the composition of dentin. As dentin matrix contains numerous proteins with a substantial influence on biological processes such as cell proliferation, angiogenesis and even dental regeneration, (treated) dentin matrix fragments are often used as a scaffold material [200-202, 227, 288]. In particular the regenerative potential of FSCs in combination with TDM has been investigated extensively [246-248, 250, 289]. More recently, Jiao *et al.* demonstrated the used of cryopreserved TDM as an inductive scaffold for dental follicle cells in the regeneration of dental pulp [290]. SCAPs and PDLSCs also show a distinct regenerative potential when combined with TDM [238, 250]. Transplantation of SCAPs in combination with dentin matrix fragments led to the formation of vascularized dentin-pulp-like complex with a continuous layer of dentin [238].

PDLSCs, on the other hand, were shown to be less potent in comparison to FSCs with regard to periodontal regeneration [250].

Self-assembling peptide hydrogels

Self-assembling peptides are biocompatible peptide chains consisting of naturally occurring amino acids, capable of generating fibrous networks which can be easily modified in order to meet specific requirements such as biodegradability [260]. In 2010, Galler *et al.* addressed the use of a self-assembling peptide hydrogel for regenerative dentistry [291]. Subcutaneous implantation of dentin cylinders filled with growth factor-laden hydrogels led to the ingrowth of fatty tissue and fibrous, connective tissue. However, when encapsulating DPSCs in the same hydrogels, the formation of vascularized pulp-like tissue was observed following a transplantation period of five weeks [292]. With regard to the application of commercially available self-assembling peptide hydrogels, recent work of Cavalcanti *et al.* indicated that DPSCs were not only able to survive and proliferate in Puramatrix ™, but the hydrogel also allowed the cells to differentiate into odontoblasts when applied into a tooth slice model [293]. The same scaffold was used in combination with SHEDs in an emptied root canal, demonstrating the regeneration of a vascularized dentin-pulp complex following transplantation in immunodeficient mice [229].

In summary, this overview suggests a particular role for customized synthetic polymers and peptides in dental tissue engineering. However, more extensive research on the modification of material properties and the *in vivo* behavior of the scaffolds is required before widespread clinical application is possible.

1.4 Aims of the study

As already described in 1.1.2, a number of different stem cell populations can be distinguished within the human tooth and its surrounding tissues. Given their relatively straightforward and minimally invasive isolation from extracted third molars, DSCs have become an attractive source of mesenchymal-like stem cells. DSCs can be isolated according to two different procedures, namely the explant method and enzymatic digestion. Whereas the explant method is based upon the plastic adherence of MSC and subsequent outgrowth of cells out of tissue

fragments, enzymatic digestion involves the application of collagenase I and dispase in order to digest pulp tissue to acquire single cell suspensions [265]. As there is ongoing debate about which isolation procedure is to be favored, **the first aim of this study is to compare the effect of isolation methodology on the stem cell properties of human dental pulp stem cells.**

As mentioned before, DSCs are generally considered to be mesenchymal-like stem cells. However, depending on the tissue of origin, each stem cell population can be characterized by its own intrinsic marker expression and differentiation capacities. In order to ascertain the 'stem cellness' of the cells isolated from different dental tissues, **the second aim of this study is to compare the stem cell properties of DPSCs, SCAPs and FSCs.**

Currently, a vast number of (stem) cell types has emerged as a potential treatment option in vascular medicine, each with their own identity and characteristics [168]. Although bone marrow-derived MSCs have shown great promise in animal models, the increasing controversy regarding their exact mechanism of action in clinical trials in combination with their invasive isolation procedure sparked the search for alternative stem cell sources [12, 166-168]. Therefore, **the paracrine angiogenic potential of postnatal DSCs, in particular DPSCs, SCAPs and FSCs, is compared in chapter 4.**

Within the field of dental tissue engineering, the establishment of adequate tissue vascularization is an important hurdle to overcome, as dental blood supply is restricted to the apical foramen [210, 294]. Besides a functional blood supply and responsive stem cells, the recreation of viable dental tissues also requires the use of scaffolds in order to support and maintain a complex tissue organization. In order to confirm whether DSCs display angiogenic properties in an *in vivo* setting, **the last aim of this study was to implant 3D-printed hydroxyapatite scaffolds containing DPSCs and SCAPs in an *in vivo* model of dental pulp regeneration.**

Chapter 2

Effects of isolation methodology on stem cell properties of dental pulp stem cells

Based on:

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Effect of isolation methodology on stem cell properties and multilineage differentiation potential of human dental pulp stem cells.

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2.1 Abstract

Dental pulp stem cells (DPSCs) are an attractive source of mesenchymal stem cells (MSCs), given their minimally invasive isolation procedure compared to other sources of MSCs. However, the isolation method to be preferred for acquiring DPSCs remains subject of considerable debate. This study compares the stem cell properties and multilineage differentiation capacity of DPSCs obtained by means of the two most widely adapted isolation methods, namely enzymatic digestion (DPSCs-EZ) or the outgrowth method (DPSCs-OG). Assessment of the stem cell properties of DPSCs-EZ and DPSCs-OG showed no significant differences with regard to proliferation rate and colony formation. Phenotypic analysis indicated both groups were positive for CD29, CD44, CD90, CD105, CD117 and CD146 expression without any significant differences. The multilineage differentiation potential of DPSCs was confirmed by using standard immuno(histo/cyto)chemical staining together with an ultrastructural analysis by means of transmission electron microscopy**.** Our results indicate that DPSCs-EZ as well as DPSCs-OG could be successfully differentiated into adipogenic, chondrogenic and osteogenic cell types, although the adipogenic differentiation of both stem cell populations was incomplete. Taken together, these data suggest that both isolation methods can be applied to obtain a stable DPSCs population for the replacement of bone and cartilage tissue.

2.2 Introduction

Since the discovery of bone marrow-derived mesenchymal stem cells (BM-MSCs), additional stem cell niches have not only been identified in umbilical cord, adipose tissue and skeletal muscle but also within the dental pulp [17, 295-298]. Although a number of studies have demonstrated the neural crest origin of human dental pulp stem cells (DPSCs), recent investigations of Feng *et al.* on mouse incisors suggested the contribution of pericyte-derived MSCs to odontoblast differentiation following tissue damage [299-301]. Furthermore, a study of Kaukua *et al.* recently demonstrated the glial origin of a significant subpopulation of DPSCs [24]. Based on their plastic adherence and expression of stem cell markers such as CD29, CD44 and CD90, DPSCs can be characterized as mesenchymal-like stem cells. Moreover, these stem cells have been shown to differentiate into adipocytes, chondroblasts and osteoblasts *in vitro* [17, 221, 302]. Studies have also indicated that DPSCs are not only capable of differentiating into odontoblasts *in vitro* but they are also able to form an organized dentin-pulp-like complex lined with odontoblast-like cells when seeded onto a scaffold and transplanted into immunocompromised mice [17, 228]. In addition to potential applications in tooth regeneration and repair, DPSCs could be clinically applied in other domains, since they are considered to be capable of differentiating into several other lineages *in vitro*, such as muscle cells, endothelial cells and functional neurogenic cells [181, 303, 304]. Next to their higher proliferative and immunomodulatory capacity than BM-MSCs, DPSCs can be isolated relatively easy from extracted third molars without any additional risk to the donor [175, 305]. However, the preferred isolation method, namely enzymatic digestion or the outgrowth method, for obtaining DPSCs remains subject of considerable debate. Whereas the explant method is based upon the plastic adherence of MSCs and subsequent outgrowth of cells out of tissue fragments, enzymatic digestion involves the application of collagenase I and dispase in order to digest pulp tissue to acquire single cell suspensions [265]. Both techniques have been successfully applied for DPSCs, umbilical cord matrix stem cells and adipose tissue-derived MSCs [17, 265, 297, 298, 302, 306-308]. To date, research regarding the preferred isolation method for DPSCs only focused on their differentiation into odontoblasts or osteoblasts, without

considering their stem cell properties or differentiation into other lineages [265, 309]. Therefore, this study aims to compare DPSCs isolated with enzymatic digestion (DPSCs-EZ) and isolated by the outgrowth/explant method (DPSCs-OG) with regard to their stem cell properties and multilineage differentiation potential. Next to the cell morphology, the proliferation rate, colony forming properties and immunophenotype of both DPSCs-EZ and DPSCs-OG are analyzed. Their differentiation potential into osteogenic, chondrogenic and adipogenic lineages is determined by means of immunocytochemical stainings and a detailed ultrastructural analysis.

2.3 Materials and Methods

2.3.1 Cell culture

Dental pulp tissues were acquired with informed consent from patients (15–20 years of age) undergoing extraction of third molars for orthodontic or therapeutic reasons at Ziekenhuis Maas en Kempen, Bree, Belgium. Written informed consent of patients below the age of 18 was obtained through their guardians. This study was approved by the medical ethical committee of Hasselt University. After disinfection of the tooth surface, the teeth were mechanically fractured with surgical chisels and the pulp tissue was obtained with forceps. The tissue was rinsed with Minimal essential medium, alpha modification (αMEM, Sigma-Aldrich, St. Louis, USA) supplemented with 2 mM L-glutamine (Sigma-Aldrich) 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma-Aldrich) and 10% FBS (BiochromAG, Berlin, Germany) (further referred to as standard DPSC culture medium), after which it was minced into fragments of 1–2 mm^3. DPSCs were then isolated according to two different procedures, namely the outgrowth/explant method and enzymatic digestion (Fig. 2.1). With regard to the outgrowth/explant method (DPSCs-OG), a subset of tissue fragments was cultured in 6-well plates in standard DPSC culture medium in order to allow the outgrowth of stem cells (Fig 2.1B). The other subset of pulp tissue fragment (DPSCs-EZ) was enzymatically digested for 60 min at 37°C with a solution containing 3 mg/ml collagenase type I and 4 mg/ml dispase (Sigma-Aldrich). After centrifugation at 300*g*, the resuspended tissue fragments were passed through a 70 µm cell strainer in order to obtain a homogeneous cell suspension (Fig. 2.1A). The cells were cultured in 6-well plates in standard DPSC culture medium. All cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium was changed every 2–3 days and all cultures were regularly monitored with an inverted phase-contrast microscope (Nikon, Eclipse TS100, Nikon Co., Shinjuku, Tokyo, Japan). When reaching 80–90% confluence, cells were harvested using 0.05% Trypsin/EDTA (Sigma-Aldrich) and sub-cultured for further experiments.

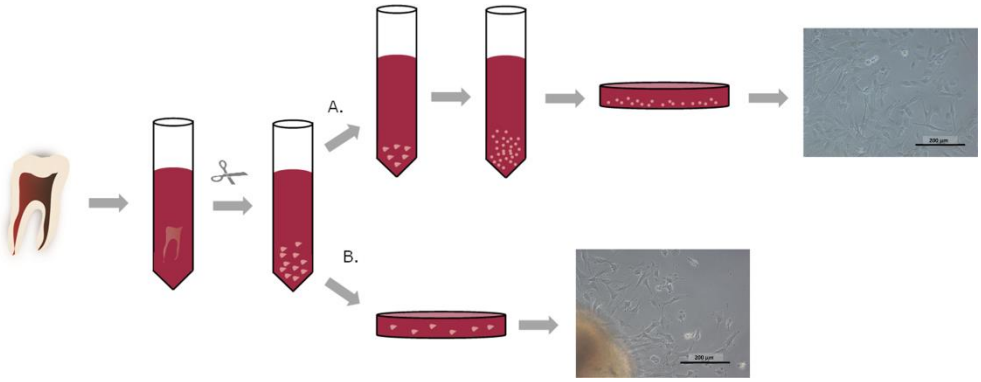


Figure 2.1: Two standard isolation methods for DPSCs. Third molars, extracted for orthodontic reasons, were fractured with surgical chisels after which the dental pulp was removed. Dental pulp tissue was minced into fragments of 1–2 mm³ and DPSCs were then isolated according to two different procedures. A. Enzymatic digestion. Tissue fragments were incubated with a collagenase/dispase solution and passed through a cell strainer in order to obtain a homogeneous cell suspension which was cultured in standard DPSC culture medium. B. Outgrowth method. Tissue fragments were cultured in standard DPSC culture medium in order to allow outgrowth of stem cells. Scale bars = 200 µm.

2.3.2 Colony forming unit assay

A colony forming unit assay was independently conducted on samples of four different donors in order to compare the colony forming properties of both DPSC populations. The cells were seeded in duplicate at 52.6 cells/cm² in a 6-well plate in standard DPSC culture medium. The medium was changed every 2-3 days and after 10 days of culturing the cells were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at room temperature. Following visualization of the colonies with a Toluidine Blue staining (Merck, Darmstadt, Germany), pictures were taken with a high resolution digital camera. Macroscopic colonies (>50 cells) were counted by three independent individuals with Image J cell counter software.

2.3.3 Cell proliferation assay

In order to assess the proliferative capacity of cultured DPSCs, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed on both cell populations. The cells were seeded in triplicate at 6,250

cells/cm² in a 96-well plate in standard DPSC culture medium. Following a culturing period of 24h, 48h, 72h and 96h, the culture medium was replaced by a 500 µg/ml MTT solution (Sigma-Aldrich) in standard DPSC culture medium containing 0.1% FBS. After 4h of incubation the MTT solution was replaced by a mixture of 0.01 M glycine (Sigma-Aldrich) in dimethyl sulfoxide (DMSO) in order to dissolve the formed formazan crystals. The absorbance was measured at a wavelength of 540-550 nm with a Benchmark microplate reader (Bio-Rad Laboratories, Hercules, USA). Data were obtained in parallel for six different donors in three independent assays.

2.3.4 Flow cytometry

The expression of characteristic stem cell markers was evaluated by means of flow cytometry. Following culture dissociation, DSCs were resuspended in PBS containing 2% FBS for 30 min at room temperature in order to allow re-expression of cell surface proteins. For each marker protein, 50,000 cells were incubated with the appropriate phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-conjugated antibodies and their matched isotype controls (Table 2.1) for 45 min at room temperature. Following incubation, 10,000 events per sample were recorded by means of a FACSCalibur flow cytometer. Data were obtained in parallel for three different donors in three independent assays. Analysis was performed using Cell Quest Pro Software (BD Biosciences, Franklin Lakes, USA).

Table 2.1 Conjugated antibodies and matched isotype controls for flow cytometry

Marker protein	Label	Isotype	Dilution	Manufacturer
CD34	PE	Mouse IgG_1, kappa	1:100	Immunotools, Friesoythe, Germany
CD44	PE	Mouse IgG_{2b}	1:100	Immunotools
CD45	PE	Mouse IgG_1, kappa	1:100	eBioscience, Vienna, Austria
CD90	FITC	Mouse IgG_1, kappa	1:100	eBioscience
CD105	PE	Mouse IgG_1, kappa	1:100	eBioscience
p75 NGFR	PE	Mouse IgG_1, kappa	1:100	Biolegend, San Diego, USA
Stro-1	FITC	Mouse IgM	1:20	Santa Cruz Biotechnology, Santa Cruz, USA

2.3.5 Adipogenic differentiation

The multilineage differentiation potential of DPSCs-EZ as well as DPSCs-OG was tested by means of the Human Mesenchymal Stem Cell Functional Identification Kit (R&D Systems, Minneapolis, USA). The adipogenic differentiation protocol was conducted as described by the manufacturer. Briefly, the cells were seeded at 2,100 cells/cm² on glass coverslips (12 mm, Menzel GmbH, Braunschweig, Germany) for light microscopy purposes and plastic coverslips (13 mm, Thermanox, Electron Microscopy Sciences, Hatfield, USA) for electron microscopy in standard DPSC culture medium. When 70-80% confluency was reached, the culture medium was replaced with adipogenic differentiation medium consisting of standard DPSC culture medium and 1% adipogenic differentiation supplement (hydrocortisone, isobutylmethylxanthine and indomethacin in 95% ethanol). The medium was changed every 2-3 days. Following 21 days of culturing the cells were either fixed with 4% PFA at room temperature (for light microscopy) or 2% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.3) at 4°C (for electron microscopy). The adipogenic differentiation potential was eventually evaluated by means of an Oil Red O staining to detect

the accumulation of lipid droplets and immunocytochemistry against Fatty Acid Binding Protein-4 (FABP-4).

2.3.6 Osteogenic differentiation

The osteogenic differentiation of DPSCs was conducted according to the manufacturer's guidelines of the previously mentioned differentiation kit. Briefly, glass or plastic cover slips were coated overnight with 1 µg/ml fibronectin after which the cells were seeded at 4,200 cells/cm² in standard DPSC culture medium. When 50-70% confluency was reached, the culture medium was replaced with osteogenic differentiation medium consisting of standard DPSC culture medium supplemented with 5% osteogenic differentiation supplement (dexamethasone, ascorbate-phosphate and β-glycerolphosphate). The medium was changed every 2-3 days. Following 14-21 days of culturing, the cells were fixed as described earlier in 2.3.5. The osteogenic differentiation potential was evaluated by means of an Alizarin Red S staining to detect the presence of calcium deposits and immunoreactivity against alkaline phosphatase. In addition, a quantitative analysis of the Alizarin Red S uptake was performed with a cetylpyridinium chloride (CPC, Sigma-Aldrich) assay as previously described [310]. Briefly, following staining with 40 mM Alizarin Red S for 10 min at room temperature, the cells were washed thoroughly with distilled water to remove excess staining solution. Subsequently, a 10% CPC solution in 10 mM sodium phosphate was added to the samples for 15 min at room temperature, after which absorption was measured at 550 nm with a Benchmark microplate reader (Bio-Rad Laboratories).

2.3.7 Chondrogenic differentiation

The chondrogenic differentiation potential of both DPSC populations was assessed as described in the manufacturer's guidelines of the previously mentioned differentiation kit. Briefly, a cell pellet containing 250,000 DPSCs was incubated with chondrogenic differentiation medium consisting of DMEM/F12 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% insulin transferrin selenite (ITS) supplement and 1% chondrogenic differentiation supplement (dexamethasone, ascorbate-phosphate,

proline, pyruvate and transforming growth factor-β3 (TGF- β3)). The medium was changed every 2-3 days. After 21 days, the cell pellets were fixed and processed for light microscopy and ultrastructural analysis. A Masson's trichrome stain and immunohistochemistry against aggrecan was performed to assess the presence of cartilaginous tissue.

2.3.8 Immunocytochemistry

Immunofluorescent stainings were performed on non-differentiated DPSCs in order to test the expression of certain stem cell markers. DSCs were seeded at 2,500 cells/cm² on glass cover slips in standard DPSC culture medium. When reaching 70–80% confluency, the cells were fixed in 4% PFA at room temperature. Aspecific binding sites were blocked for 20 min with 10% normal donkey serum (Merck Millipore, Billerica, USA) in PBS at room temperature, after which the cells were incubated overnight with primary antibodies against CD29 and CD117 or CD146 at 4°C. Primary antibodies were omitted in the negative control condition. Afterwards, the cells were washed with PBS and incubated with the appropriate secondary antibodies for 30 min at room temperature. All the applied primary and secondary antibodies are listed in table 2.2. Following counterstaining of cellular nuclei with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen, Carlsbad, USA), the coverslips were mounted with fluorescent mounting medium (Dako, Glostrup, Denmark). Representative pictures were taken with a Nikon Eclipse 80i fluorescent microscope equipped with a Nikon DS-2MBWc digital camera. Non-fluorescent immunostaining was performed on differentiated DPSCs by means of a a DAB EnVision System kit (Dako). After dehydration in graded ethanol of the chondrogenic differentiation samples, the tissues were embedded in paraffin and serial sections of 7 µm were made. Following deparaffinization and rehydration, antigen retrieval was performed by boiling the tissue sections in 1× citrate buffer (Dako). After cooling down, aspecific binding sites were blocked using 10% normal donkey serum for 30 min at room temperature. With regard to the adipogenic and chondrogenic differentiation samples, the cells were pretreated with 0.05 % Triton X-100 (Boehringer, Mannheim, Germany) in PBS for 30 min at 4°C before blocking aspecific binding sites with 10% normal goat serum. Afterwards, the differentiated samples were incubated for 60 min at room temperature with

the primary antibodies listed in table 2.2. The staining was visualized by means of a 3,3' diaminobenzidine (DAB) chromogen solution according to the manufacturer's instructions. Following counterstaining with Mayer's hematoxylin, the coverslips were mounted on glass slides using Aquatex (Merck, Darmstadt, Germany). Representative pictures were taken with a Nikon Eclipse 80i microscope equipped with a DS-5 M digital camera.

Table 2.2 Primary antibodies and matched secondary antibodies for immunostainings

Antibody	**Type**	**Dilution**	**Manufacturer**
Primary antibodies			
CD29	Mouse monoclonal IgG1	1:50	Abcam, Cambridge, UK
CD117	Rabbit Polyclonal IgG	1:100	Santa Cruz Biotechnology
CD146	Rabbit Polyclonal IgG	1:100	Abcam
Aggrecan	Goat Polyclonal IgG	1 µg/100 µl	R&D Systems
FABP-4	Goat Polyclonal IgG	1 µg/100 µl	R&D Systems
Alkaline phosphatase	Mouse monoclonal IgG1	1:50	R&D Systems
Secondary antibodies			
Alexa fluor 555 donkey anti-mouse	Donkey IgG	1:500	Invitrogen
Alexa Fluor 488 donkey anti-rabbit	Donkey IgG	1:500	Invitrogen
Donkey anti-goat	Donkey polyclonal IgG	1:500	Jackson ImmunoResearch Laboratories, Inc., West Grove, USA
Goat anti-mouse	Goat polyclonal IgG	Ready to use	Dako

2.3.9 Transmission electron microscopy

Samples cultured on plastic coverslips were fixed with 2% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.3) at 4 °C. Post-fixation was achieved by treating the samples with 2% osmium tetroxide in 0.05 M sodium cacodylate

buffer (pH 7.3) for 1 h at 4°C after which the samples were stained with 2% uranyl acetate in 10% acetone for 20 min Dehydration was performed by means of ascending concentrations of acetone. The dehydrated samples were then impregnated overnight in a 1:1 mixture of acetone and araldite epoxy resin at room temperature. Following impregnation, the samples were embedded in araldite epoxy resin at 60°C using the pop-off method. The embedded samples were cut into sections of 40–60 nm on a Leica EM UC6 microtome (Leica, Wetzal, Germany) and were then transferred to 0.7% formvar-coated copper grids (Aurion, Wageningen, The Netherlands). The sections were contrasted using a Leica EM AC20 (Leica) with 0.5% uranyl acetate and a stabilized solution of lead citrate. Transmission electron microscopic (TEM) analysis was performed with a Philips EM208 S electron microscope (Philips, Eindhoven, The Netherlands). Digital images were obtained with a Morada Soft Imaging System camera and the corresponding iTEM-FEI software (Olympus SIS, Münster, Germany).

2.3.10 Statistical analysis

Statistical analysis was performed using GraphPad Prism 4 Software (GraphPad Software, La Jolla, CA). Data distribution was assessed by means of a D'Agostino & Pearson normality test, after which the experimental groups were compared using a (non-)parametric t-test. Differences were considered to be statistically significant at P-values ≤ 0.05. All data were expressed as mean ± standard deviation (SD).

2.4 Results

2.4.1 Morphology and immunophenotype of dental pulp stem cells

Following isolation according to the described methods, regular monitoring of the stem cells with a phase-contrast microscope indicated adherent cells in the cultures isolated with enzymatic digestion, while the cells in the explant started to adhere and proliferate after two to four days. After one week, DPSCs-EZ reached 80-90% confluency. In contrast, DPSCs-OG were found to be confluent following 10-14 days of culturing. In terms of cellular morphology, EZ-cultures as well as OG-cultures showed the presence of elongated cells (e, Fig. 2.2A, D). However, given the presence of polygonal cells with multiple processes, enzymatic digestion gave rise to a more heterogeneous cell population in comparison to the outgrowth method (p, Fig. 2.2A). At the ultrastructural level, both DPSCs-EZ and DPSCs-OG were characterized by an elongated cellular appearance with a perinuclear organelle-rich zone (p, Fig. 2.2B, E) containing rough endoplasmic reticulum (r, Fig. 2.1C, F) and mitochondria (m, Fig 2.2C, F), which could clearly be distinguished from an electron-lucent organelle-poor peripheral zone (o, Fig. 2.2B, E). Large euchromatic nuclei with one or more nucleoli (n, Fig. 2.2B, E) were also visible. Although no significant morphological differences between cells from both isolation methods were observed, DPSCs-EZ showed extensive vacuolization throughout the entire cytoplasm (v, Fig. 2.2C) while this was not the case for DPSCs-OG.

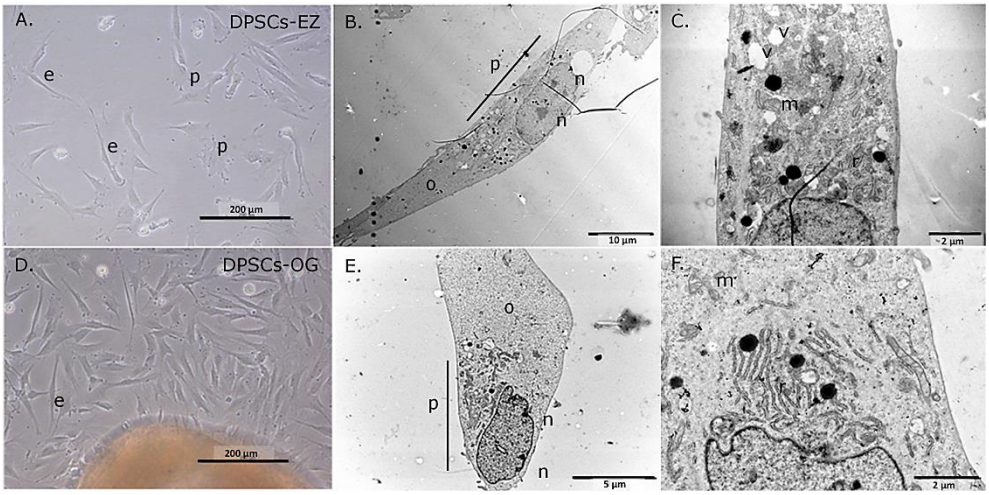
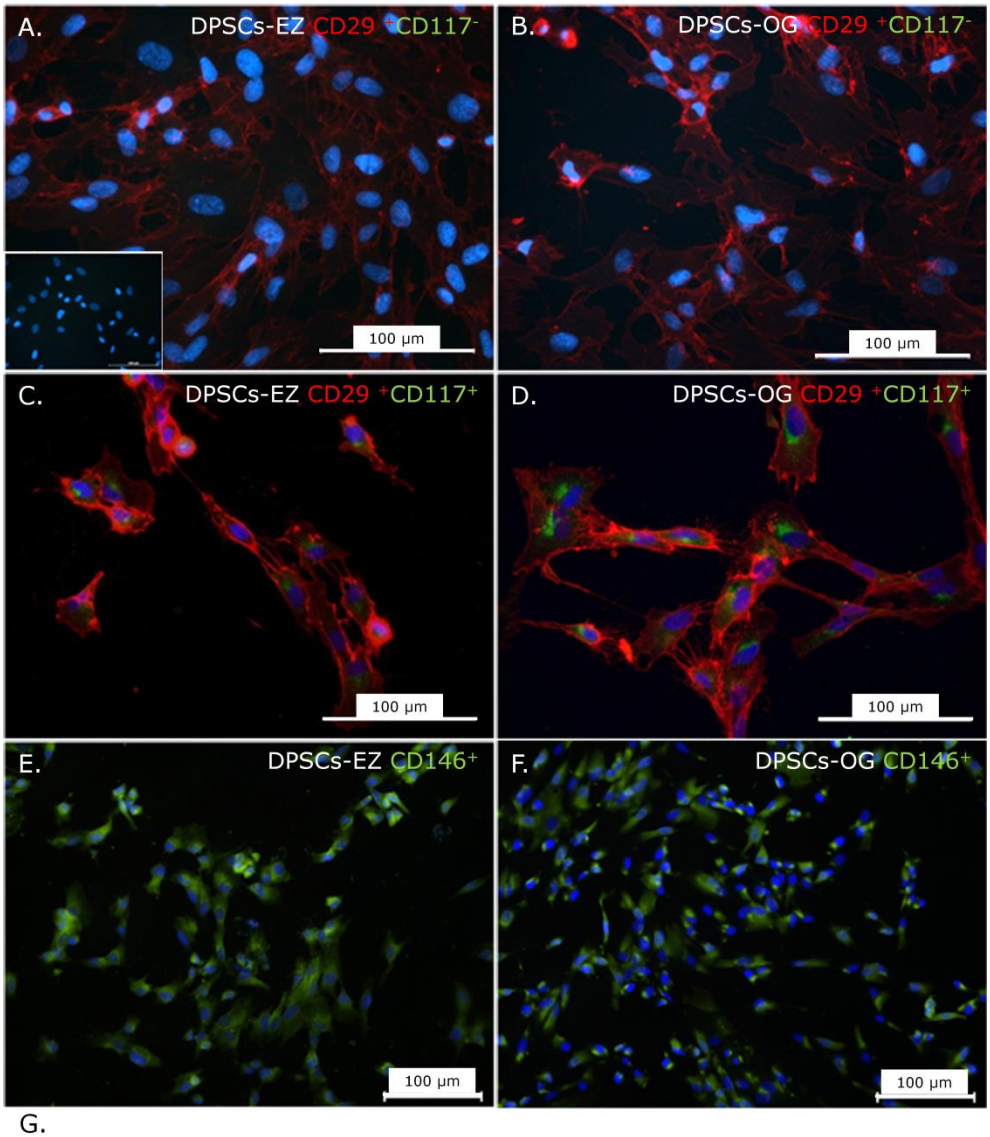


Figure 2.2: Morphology of DPSCs-EZ (A-C) and DPSCs-OG (D-F). A. Heterogeneous DPSCs-EZ culture with elongated (e) and polygonal (p) cells. D. DPSCs-OG culture with elongated (e) fibroblast-like cells. B,E. A perinuclear (p) organelle-rich zone could be distinguished from an electron-lucent organelle-poor (o) region. n = nucleolus. C,F. The organelle-rich zone contained rough endoplasmic reticulum and mitochondria (m). DPSCs-EZ showed extensive vacuolization (v). Scale bars = 200 µm (A, D), 10 µm (B), 5 µm (E), 2 µm (C, F).

The immunophenotype of DPSCs-EZ (Fig. 2.3A, C, E) and DPSCs-OG (Fig. 2.3B, D, F) was evaluated by means of immunocytochemistry and flow cytometry. Both cell cultures showed a uniform expression of stem cell marker CD29 (Fig. 2.3A, B) as well as endothelial marker CD146 (Fig. 2.3E, F). CD117 expression, on the other hand, was characterized by donor variability (Fig. 2.3A-D). With regard to the expression of stem cell markers CD44, CD90 and CD105, flow cytometry pointed out high levels of expression for both DPSCs-EZ and DPSCs-OG (Fig. 2.3G). However, no significant differences could be observed. Analysis also indicated no expression of CD34, CD45 and Stro-1, in addition to a low expression level of NGFR (p75) (Fig. 2.3G).


A.
DPSCs-EZ CD29 +CD117-
100 µm
B.
DPSCs-OG CD29 +CD117-
100 µm
C.
DPSCs-EZ CD29 +CD117+
100 µm
D.
DPSCs-OG CD29 +CD117+
100 µm
E.
DPSCs-EZ CD146+
100 µm
F.
DPSCs-OG CD146+
100 µm

G.

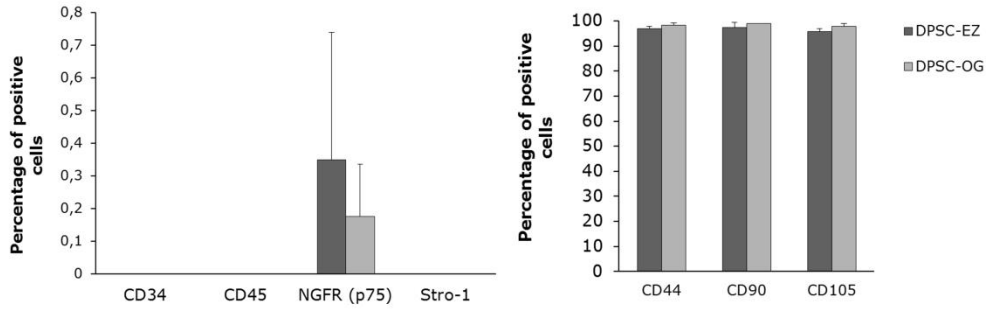

Percentage of positive cells
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
CD34
CD45
NGFR (p75)
Stro-1
Percentage of positive cells
100
90
80
70
60
50
40
30
20
10
0
CD44
CD90
CD105
DPSC-EZ
DPSC-OG

Figure 2.3: Immunophenotype of DPSCs-EZ and DPSCs-OG. A. DPSCs-EZ: $CD29^{+}CD117^{-}$. Inset: Negative control. B. DPSCs-OG: $CD29^{+}$ $CD117^{-}$. C. DPSCs-EZ: $CD29^{+}$ $CD117^{+}$. D. DPSCs-OG: $CD29^{+}$ $CD117^{+}$. E. DPSCs-EZ: $CD146^{+}$. F. DPSCs-OG: $CD146^{+}$. CD29 (red); CD117 (green); CD146 (green); DAPI counterstaining (blue). Scale bars = 100 µm. Pictures are representative for three independent stainings. G. Percentage of positive DPSCs for CD34, CD45, NGFR, Stro-1, CD44, CD90 and CD105. Flow cytometry was conducted in parallel on samples of three different donors in three independent assays. Data are expressed as mean ± SD.

2.4.2 Proliferation rate and colony forming abilities of dental pulp stem cells

In order to assess potential differences in proliferation rate between DPSCs-EZ and DPSC-OGs, an MTT assay was performed on six donor samples. Analysis showed a gradual increase of proliferation during 72h followed by the onset of stabilization for both DPSCs-EZ as well as DPSCs-OG. During this time frame, DPSCs-EZ only showed a significantly higher proliferation rate after 24h (Fig. 2.4A). With regard to their colony forming properties, DPSCs-EZ and DPSCs-OG were both able to form numerous colonies during 10 days of culturing. However, independent quantification of four different donor samples indicated no significant differences between the two isolation methods (Fig. 2.4B).

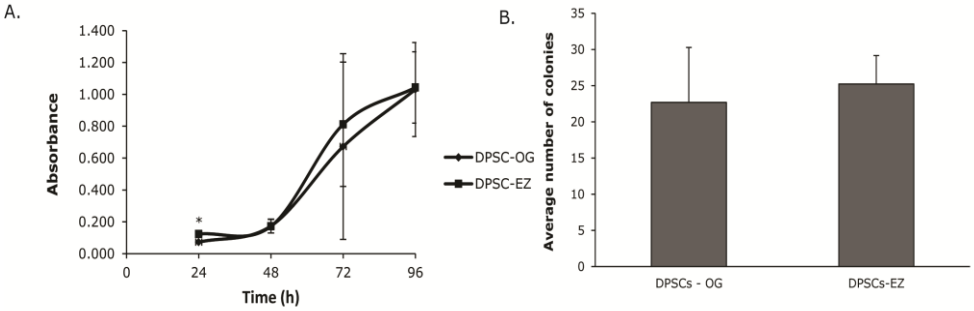


Figure 2.4: Proliferation rate and colony forming properties of DPSCs-EZ and DPSCs-OG. A. MTT analysis of DPSCs-EZ and DPSCs-OG with no significant differences between both groups after 96h. Results were obtained in parallel of samples of six different donors in three independent assays. B. Average number of colonies of DPSCs-EZ and DPSCs-OG after 10 days of culturing. This assay was independently conducted on samples of four different donors. Data are expressed as mean ± SD. * = p-value < 0.05.

2.4.3 Trilineage differentiation potential of dental pulp stem cells

Following 21 days of incubation with **adipogenic differentiation** medium, a subset of DPSCs-EZ and DPSCs-OG showed a strong expression of FABP-4 (Fig. 2.5C, D). Quantification indicated a higher protein level in DPSCs-OG (12.35% vs. 9.5%), although this difference was not found to be statistically significant (Fig. 2.5E). The differentiated cells also displayed large transparent droplets, i.e. lipid-containing vacuoles, in their cytoplasm, which were more pronounced after an Oil Red O staining (Fig. 2.5A, B).

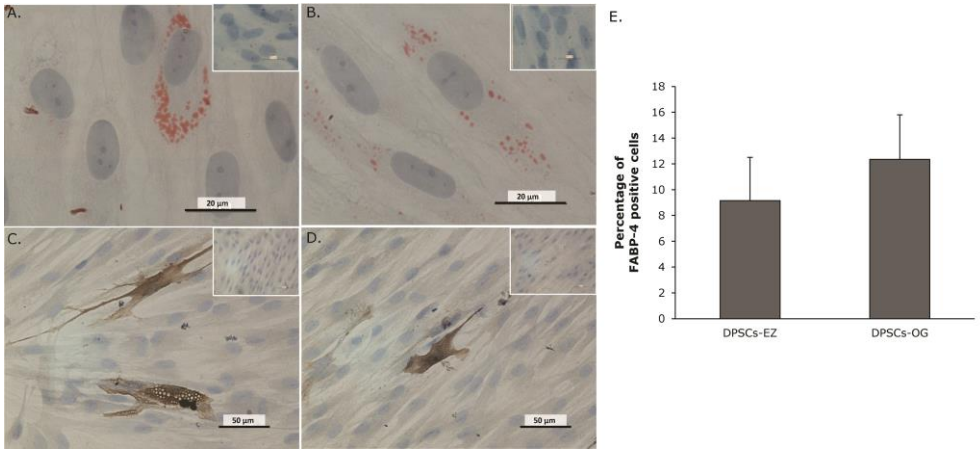


Figure 2.5: Adipogenic differentiation of DPSCs-EZ (A,C) and DPSC-OG (B,D). A, B. Lipid droplet accumulation. Scale bars = 20 µm. C, D. Expression of FABP-4. Scale bars = 50 µm. Insets = undifferentiated DPSCs. E. Quantification of FABP-4 indicated no significant differences between DPSCs-EZ and DPSCs-OG. This assay was conducted on matched samples of four different donors. Data are expressed as mean ± SD.

At the ultrastructural level, differentiated DPSCs-OG and DPSCs-EZ were characterized by a globular appearance (Fig. 2.6A), with cytoplasm containing a dilated rough endoplasmic reticulum (dER, Fig. 2.6B) and branched mitochondria (bMit, Fig. 2.6C). The presence of these organelles was not restricted to the perinuclear area, as was the case in undifferentiated DPSCs. Similar to the Oil Red O staining, a subset of cells also contained small electron-dense lipid droplets (v, Fig. 2.6C, D). No morphological differences were observed between DPSCs-OG and DPSCs-EZ after adipogenic differentiation.

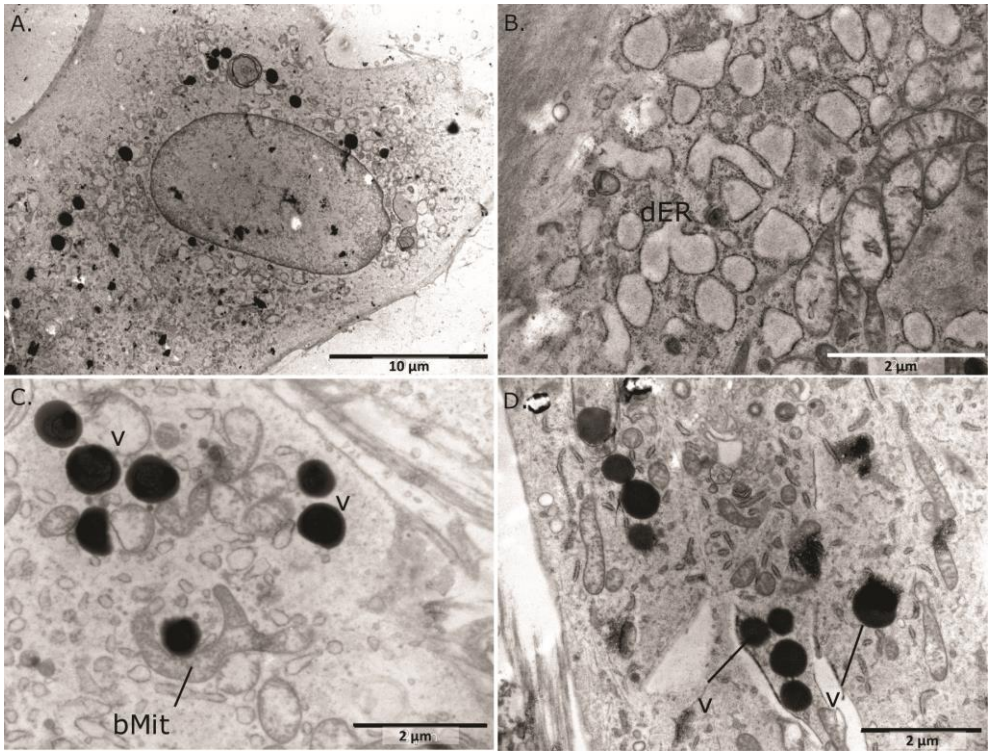


Figure 2.6: Adipogenic differentiation of DPSCs-EZ and DPSCs-OG at the ultrastructural level. A. Globular shape of differentiated DPSCs with organelles spread throughout the cytoplasm. Scale bar = 10 µm. B. Dilated rough endoplasmic reticulum (dRER). Scale bar = 2 µm. C. Electron-dense vesicles (v) and branched mitochondria (bMit). Scale bar = 2 µm. D. Electron-dense vesicles (v). Scale bar = 2 µm. Pictures courtesy of Dr. Annelies Bronckaers and Pascal Gervois.

With regard to **osteogenic differentiation**, alkaline phosphatase synthesis was detected in both groups of DPSCs following 14-21 days of incubation with differentiation medium (Fig. 2.7A, B). Alizarin Red S staining of osteogenic differentiated cells indicated the formation of calcified nodules (Fig. 2.7C, D). These nodules showed donor-related variability in size and number in both experimental groups and were not observed in control samples. Subsequent analysis with CPC demonstrated a significant increase in calcium deposition in both DPSCs-EZ and DPSCs-OG compared to control samples (Fig. 2.7E). However, no significant differences were detected between the two isolation methods.

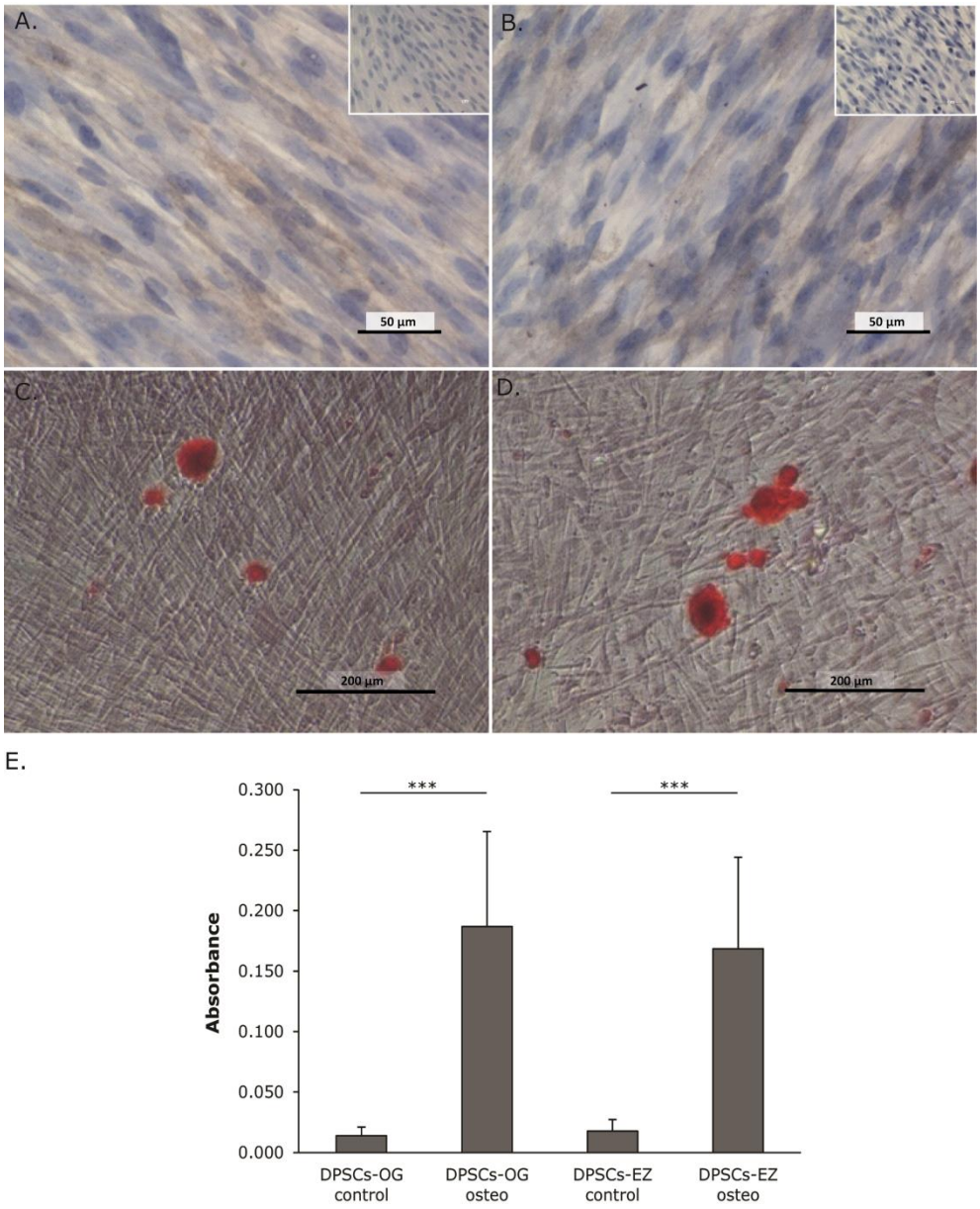


Figure 2.7: Osteogenic differentiation of DPSCs-OG (A,C) and DPSCs-EZ (B,D). A,B. Expression of alkaline phosphatase. Insets = undifferentiated DPSCs. Scale bars = 50 µm. C, D. Detection of calcium deposits with Alizarin Red S. Scale bars = 200 µm. E. Quantification of Alizarin Red S indicated no significant differences between DPSCs-EZ and DPSCs-OG. This assay was conducted on matched samples of eight different donors. Data are expressed as mean ± SD. Data courtesy of Pascal Gervois.

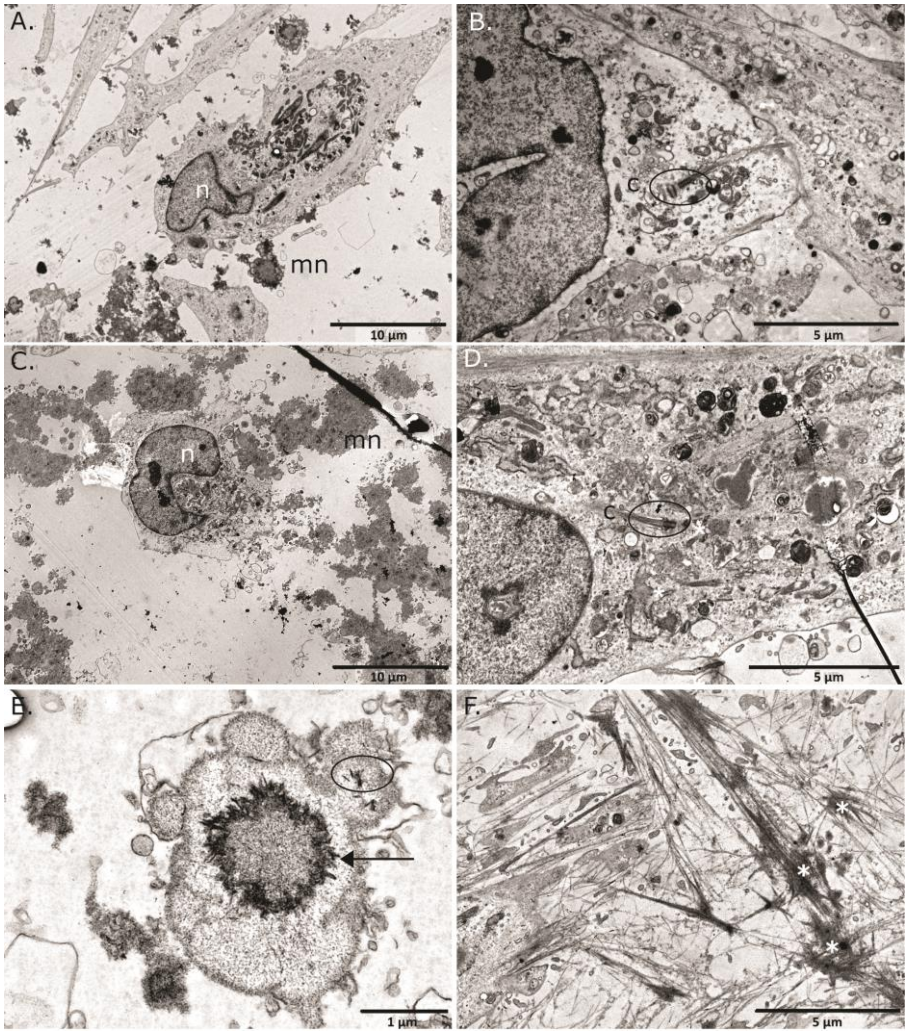


Figure 2.8: Osteogenic differentiation of DPSCs-EZ (A,B) and DPSCs-OG (C-F) at the ultrastructural level. A-D. Differentiated DPSCs-EZ and DPSCs-OG are characterized by an osteoblast-like morphology, displaying an eccentric nucleus (n) and the accumulation of lamellar and matrix-containing vesicles in the cytoplasm. A,C. Mineralized nodules (mn) were also present in the extracellular space. B,D. Primary cilia (c) were observed in both differentiated DPSC subgroups with perpendicular basal bodies present at the base of the cilia (encircled). E. individual hydroxyapatite needles in vesicles (encircled) were appositionally added to the core of the mineralized nodules (arrow). F. Large mineralized structures (asterisks) were found to interact with the surrounding striated collagen fibers. Scale bars = 10 µm (A, C), 5 µm (B, D, F), 1 µm (E). Data courtesy of Pascal Gervois.

Ultrastructurally, osteogenic induced DPSCs-EZ and DPSCs-OG were characterized by an osteoblast-like morphology with an eccentric nucleus (n) and cytoplasm containing multiple lamellar and dark matrix-producing vesicles (Fig. 2.8A-D) The presence of primary cilia was also observed (encircled, Fig. 2.8B, D). In both experimental subgroups, numerous globular mineralized nodules (mn) consisting of hydroxyapatite needles (encircled, Fig. 2.8A, C, E) were detected in the extracellular space in addition to striated collagen fibers, which interacted with large mineralized fragments (asterisks, Fig. 2.8F).

Following three weeks of **chondrogenic differentiation**, DPSCs formed three dimensional cell spheres which tested positive for Aggrecan staining (Fig. 2.9A, B, D, E). Masson's trichrome staining revealed the presence of collagen formation (blue) in the extracellular matrix (ECM) surrounding the differentiated stem cells (Fig. 2.9C, F). Cartilage fragments were also present in a subset of cell spheres (arrow, Fig. 2.9F).

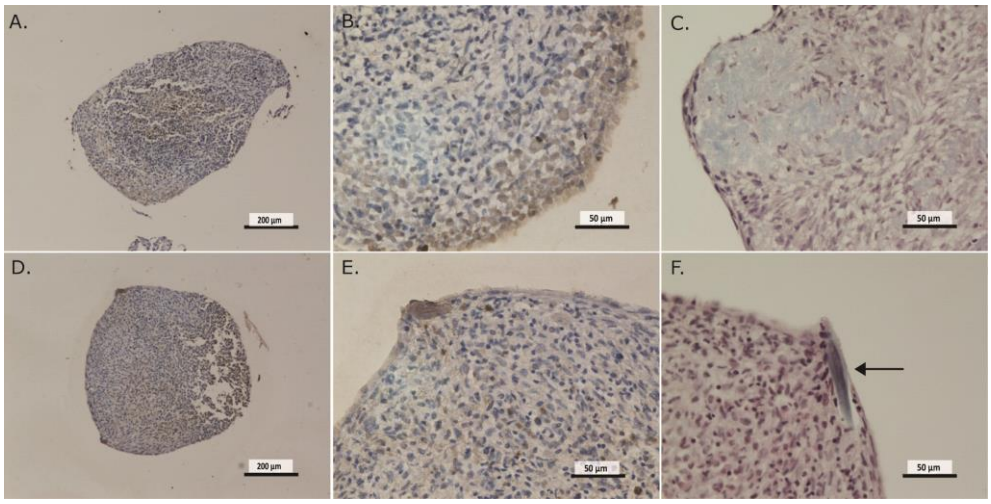


Figure 2.9: Chondrogenic differentiation of DPSCs-EZ (A-C) and DPSCs-OG (D-F). A, B, D, E. Presence of aggrecan in the extracellular matrix of both cell types. C, F. Masson's trichrome staining indicated the presence of collagen after three weeks of differentiation. Cartilage fragments (arrow) were also observed in a subset of cell pellets. Scale bars = 200 µm (A, D), 50 µm (B, C, E, F).

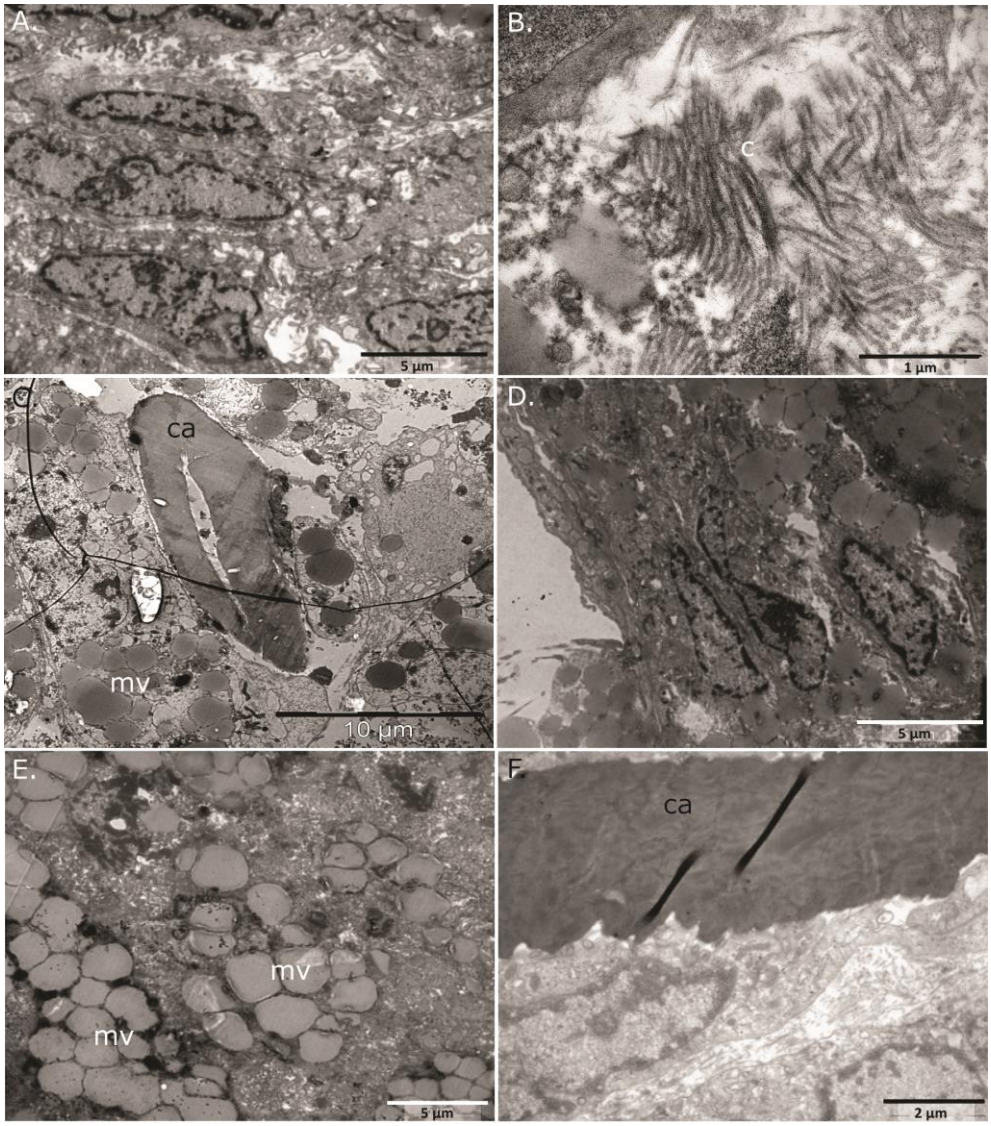


Figure 2.10: Chondrogenic differentiation of DPSCs-EZ (A) and DPSCs-OG (D) at the ultrastructural level. A,D. Elongated cells were observed in both cell cultures. B. Striated collagen fibers (c) in the extracellular matrix. C, F. Extracellular cartilage fragments (ca) closely interacted with the surrounding cells and matrix. Scale bars = 5 µm (A, D, E), 1 µm (B), 10 µm (C), 2 µm (F). Pictures courtesy of Dr. Annelies Bronckaers and Pascal Gervois.

At the ultrastructural level, differentiated DPSCs-EZ (Fig. 2.10A) and DPSCs-OG (Fig. 2.10D) were characterized as elongated cells containing ribosomes and numerous electron-dense intracellular matrix vesicles, probably consisting of glycosaminoglycans (mv, Fig. 2.10C, E). At higher magnifications, striated collagen fibers were observed (c, Fig. 2.10B), as well as cartilage fragments which closely interacted with the surrounding cells and ECM (ca, Fig. 2.10C, F).

2.5 Discussion

In order for dental pulp to be used as a stable source of mesenchymal stem cells in regenerative medicine and tissue engineering, a consensus needs to be established with regard to the applied isolation procedure. Therefore, this study is the first to evaluate the influence of isolation methodology on the stem cell properties and multilineage differentiation potential of DPSC-EZ and DPSC-OG, while keeping all conditions constant throughout the performed experiments. The two most applied isolation methods for DPSCs have been compared, namely (I) the enzymatic digestion method, which subjects the dental pulp to a cocktail of dispase and collagenase in order to obtain a single cell suspension and (II) the outgrowth or explant method, which allows the stem cells to grow out of dental pulp tissue fragments. Gronthos *et al.*, the first to elaborately describe the characteristics of DPSCs, applied enzymatic digestion, whereas many other groups describing the differentiation capacities of DPSCs have applied the outgrowth method [17, 221, 265, 302, 306, 311-314]. Already in 2006, Huang *et al.* compared these two isolation procedures, though the culture conditions differed significantly from each other: DPSCs-EZ were cultured in αMEM containing 20% FBS, whereas DPSC-OG were cultured in DMEM with 10% FBS [265]. This was also the case in a study of Bakopoulou *et al.*, which assesses the impact of isolation methodology on the differentiation potential of DPSCs of deciduous teeth (SHEDs) [309]. In other words, we cannot exclude that the differences observed in these studies might be caused by the applied culture media. In this study, both DPSCs-EZ and DPSCs-OG were derived from the same donors and cultured in the same growth medium, thereby eliminating culture-induced differences in morphology and differentiation potential.

With regard to cellular morphology, DPSCs-EZ as well as DPSCs-OG cultures showed the presence of elongated fibroblast-like cells. However, enzymatic digestion seemed to give rise to a more heterogeneous cell culture, such as cuboidal and polygonal cells. This was also mentioned by Bakopoulou *et al.* regarding SHEDs and supports earlier reports stating that enzymatic digestion not only allows the isolation of fibroblast-like (stem) cells but also the release of endothelial cells and pericytes, whereas the outgrowth method yields a more uniform population of fibroblast-like (stem) cells that migrate out of various

tissue fragments, leaving non-migrating cells to disintegrate within the tissue [17, 221, 265, 309, 315]. Apart from the vacuolization found in DPSCs-EZ, there were no apparent differences between the two cultures at the ultrastructural level.

In spite of the aforementioned culture heterogeneity, there was no general significant difference in the proliferation rate of DPSCs-EZ and DPSCs-OG, except for an increased proliferation of DPSCs-EZ after 24h. Similar results were also found for SHEDs, whereas other studies have suggested an increased proliferation rate in favor of either DPSCs-EZ or DPSCs-OG [265, 309, 316]. However, these discrepancies can be explained by the aforementioned differing culture conditions. CFU analysis also showed no significant differences between DPSCs-EZ and DPSCs-OG: both isolation methods gave rise to pulp cells capable of forming an average amount of 25 versus 22 colonies after 10 days. Although similar culture conditions and seeding density were adopted by Jeon *et al.*, they reported a significantly higher number of colonies for SHEDs isolated by means of enzymatic digestion [317].

In terms of immunophenotype, both isolation methods yielded DPSCs that were strongly positive for CD44, CD90, CD105 and CD146, with no significant differences between both groups. A similar phenotype has recently been observed by Karamzadeh *et al.*, although they mentioned a significantly higher expression of CD105 and CD146 by DPSCs-OG [318]. In contrast to earlier reports but in agreement with Karamzadeh *et al.*, DPSCs were negative for Stro-1 expression [17, 221, 309, 318]. Both groups of DPSCs also showed a low percentage of NGFR (p75) expression (EZ: 0.35%; OG: 0.18%), despite earlier reports from our group and others indicating a p75-positive subset of DPSCs [312, 319]. Although Patel *et al.* have indicated that continued passaging of dental pulp cells leads to the selection of more differentiated cells, as is reflected in a decreased expression of stem cell markers, the absence of Stro-1 and p75 cannot be explained by this phenomenon, since DPSCs of early passage have been used in all experiments [320]. Other potential explanations could be the differences in cell cultivation methods or donor variability attributable to age, dental health and the amount of exerted orthodontic force, factors that all have their impact on cellular homeostasis and marker expression [321-326].

Variability between donors is also observed with regard to the expression of CD117; only subpopulations of certain DPSCs-EZ and DPSCs-OG samples show reactivity for this marker. These findings are supported by earlier reports on various DPSCs subpopulations [327, 328]. Finally, in agreement with previous studies and the general definition of MSCs according to the International Society for Cellular Therapy, both DPSCs-EZ and DPSCs-OG were negative for hematopoietic markers CD34 and CD45 [3, 316, 318, 329]. More recently, other studies have refuted the observation that DPSCs are $CD34^-$ and have even used different cell sorting techniques to identify more potent subtypes of DPSCs with regard to hematopoietic marker expression and targeted differentiation, such as c-kit^+/$CD34^+$/$CD45^-$ DPSCs or $CD31^-$/$CD146^-$ DPSCs [328, 330, 331]. Depending on the clinical situation, a different approach of cell-based therapies can be applied. When using a cell-based therapy in clinically relevant situations, a heterogeneous group of cells might be preferred, as this can react to the various environmental cues within the tissue of interest. However, in other cases, preselected engrafted cells can have a beneficial effect compared with heterogeneous stem cells.

With regard to the trilineage differentiation potential of DPSCs, adipogenic differentiation medium induced the intracellular accumulation of lipid droplets together with the expression of FABP-4, i.e. distinctive characteristics of adipocytes. Although no significant differences were observed between both experimental groups, only a subset of DPSCs seemed to be capable of adipogenic differentiation; indicating that dental pulp might not be an ideal stem cell source for the replacement of fatty tissue. The induction of osteogenic differentiation had a comparable outcome in both groups. Next to the expression of alkaline phosphatase the production of calcified nodules was also observed, although no significant differences were observed. In contrast, Bakopoulou *et al.* reported a higher mineralization rate for SHEDs isolated by means of enzymatic digestion. However, culture conditions were not kept constant throughout the study [309]. Calcified nodules and an osteoid-like extracellular matrix were also observed at the ultrastructural level, which is consistent with the requirement that cells with hard tissue engineering potential must be able to produce both the organic and inorganic bone matrix components [17, 328, 330]. In addition,

primary cilia were detected in both types of osteogenic differentiated DPSCs, suggesting that these cells can translate extracellular chemical and mechanical stimuli into cellular responses [332]. Chondrogenic differentiated DPSCs-EZ and DPSCs-OG demonstrated similar morphological features with no pronounced differences between both groups. The cytoplasm was characterized by the presence of numerous intracellular vesicles and the ECM was comprised of ground substance together with collagen fibers (collagen type II) and, as indisputable evidence for chondrogenic differentiation, cartilage fragments. Taken together, the ultrastructural evaluation of the multilineage differentiated DPSCs-EZ and DPSCs-OG has demonstrated that both cell types show trilineage differentiation potential.

In conclusion, our study reveals no differences in cellular morphology, proliferation rate, stem cell marker expression and mesenchymal differentiation potential between DPSCs isolated by means of enzymatic digestion or the outgrowth method when these cells are kept under the same culture conditions and are derived from the same donors. Both isolation methods yield stem cell populations that are particularly capable of differentiation into osteoblasts and chondroblasts, leading to the conclusion that these stem cells provide a promising strategy for the treatment of bone and cartilage injuries.

Chapter 3

Characterization of dental stem cell populations

3.1 Abstract

Within the human tooth and its surrounding tissues, a number of different stem cell populations can be distinguished, such as dental pulp stem cells (DPSCs), stem cells from the apical papilla (SCAPs) and dental follicle precursor cells (FSCs). Given their relatively straightforward and minimally invasive isolation from extracted third molars, dental stem cells (DSCs) have become an attractive source of mesenchymal-like stem cells. However, in order to ascertain the 'stem cellness' of the cells isolated from different dental tissues and to adequately assess potential tissue-related differences, an thorough empirical comparison with regard to marker expression and multilineage differentiation potential is still required. DPSCs, SCAPs and FSCs expressed CD29, CD44, CD73, CD90, CD105 and CD146, although the percentage of positive DPSCs was slightly lower for certain stem cell markers. In terms of colony forming properties, analysis indicated a significantly lower amount of colonies formed by DPSCs, depending on the seeding density. Only a subset of DSCs expressed pluripotency markers Snail and SOX17, and were capable of differentiating into adipogenic lineages. The induction of osteogenic differentiation significantly differed between the stem cell populations, as FSCs were not able to form fully mineralized nodules. In terms of chondrogenic differentiation, similar morphological features were detected in all DSCs. However, cartilage fragments could only be distinguished in differentiated DPSCs and SCAPs. In conclusion, these data suggest that, although DSCs are all considered to be mesenchymal-like stem cells, there are some differences in between populations that have to be taken into account when considering these cells for therapeutic or tissue engineering applications.

3.2 Introduction

During tooth development, reciprocal spatiotemporal interactions between embryonic oral epithelium and neural crest-derived mesenchyme regulate tooth morphogenesis and differentiation, which eventually results in the formation of an outer layer of enamel and inner layer of primary dentin [333, 334]. Odontoblasts, the cells that are responsible for the formation of primary dentin, were thought to arise from precursor cells residing in the dental pulp, a strongly innervated and vascularized soft connective tissue derived from the dental papilla. In 2000, Gronthos *et al.* were the first to demonstrate the presence of a heterogeneous, clonogenic and highly proliferative cell population within the human dental pulp, namely dental pulp stem cells (DPSCs) [17]. To date, numerous studies have reported on the stem cell characteristics and differentiation potential of these cells. Besides DPSCs, the human tooth and its surrounding tissues comprise a number of other stem cell populations. In 2008, for example, Sonoyama *et al.*, isolated and characterized the so-called SCAPs, i.e. stem cells from the apical papilla, in a loosely attached soft connective tissue at the apex of developing permanent teeth [237]. Around that time, Morsczeck *et al.* mentioned the existence of a stem cell population in another developing dental tissue, namely dental follicle precursor cells (FSCs) [19]. The dental follicle is a loose connective tissue which surrounds the developing tooth and later on in development gives rise to the periodontium. Generally speaking, dental stem cells (DSCs) can be characterized as mesenchymal stem cells (MSCs) according to the minimal criteria defined by the International Society for Cellular Therapy, i.e. they are plastic adherent, they express markers such as CD73, CD90 and CD105, they are negative for markers such as CD14, CD34 and CD45, and they are capable of *in vitro* differentiation into adipogenic, osteogenic and chondrogenic lineages [3, 15, 21-23]. As DSCs can be isolated in a straightforward and minimally invasive way from extracted third molars and demonstrate better proliferative and immunomodulatory properties than bone marrow-derived MSCs, these stem cells have raised hopes for their future clinical applications [175, 305]. Nevertheless, one should always take into account potential origin-related differences. For example, as SCAPs and FSCs are derived from developing dental tissues, they are generally considered to be

more immature and thus, more potent in comparison to DPSCs. Comparative studies between DPSCs and SCAPs already indicated SCAPs to have a more pronounced population doubling capacity, a higher proliferation rate and better migratory properties [237]. Furthermore, the origin of DSCs has recently been subject of considerable debate. Up until now, it was assumed that, given the aforementioned reciprocal interactions between embryonic oral epithelium and neural crest-derived mesenchyme during tooth morphogenesis, DSCs are derived from the neural crest. However, a study of Kaukua *et al.* recently demonstrated the glial origin of a significant subpopulation of DPSCs, suggesting that, depending on the tissue of origin, each DSCs population is defined by its own ability to repair and/or regenerate diseased or damaged (dental) tissues [24]. Therefore, an elaborate comparison of these cells is still required in order to ensure the 'stem cellness' of the isolated cells and to adequately assess potential tissue-related differences. This study aims to characterize and compare DSCs, in particular DPSCs, SCAPs and FSCs, with regard to their stem cell properties. Next to their colony forming ability, the immunophenotype of these stem cells is assessed by means of immunocytochemistry and flow cytometry. Their multilineage differentiation potential, i.e. their ability to differentiate into adipogenic, osteogenic and chondrogenic lineages, is evaluated by means of protein expression and ultrastructural analysis.

3.3 Materials and Methods

3.3.1 Cell Culture

Dental tissues were obtained with informed consent from patients (15–21 years of age) undergoing extraction of third molars for orthodontic or therapeutic reasons at Ziekenhuis Maas en Kempen, Bree, Belgium. Written informed consent of patients below the age of 18 was obtained via their guardians. This study was approved by the medical ethical committee of Hasselt University. Immediately after extraction, dental follicles and apical papillae were gently removed from the teeth with forceps. The pulp tissue was obtained by means of forceps after mechanically fracturing the teeth with surgical chisels. Dental tissues were rinsed with Minimal essential medium, alpha modification (αMEM, Sigma-Aldrich, St. Louis, USA) supplemented with 100 U/ml Penicillin and 100 µg/ml Streptomycin (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich) (further referred to as standard DSC culture medium) containing 10% FBS (BiochromAG, Berlin, Germany), after which DSCs were isolated according to the explant method as described in chapter 2 (2.3.1). NTERA cells (CRL-1973, ATCC, Molsheim Cedex, France and kindly provided by Raf Donders, Biomedical Research Institute, Diepenbeek, Belgium) were cultured in Dulbecco's Modified Eagle Medium (DMEM) high glucose (Sigma-Aldrich) supplemented with 100 U/ml Penicillin and 100 µg/ml Streptomycin, 2 mM L-glutamine, sodium pyruvate and 10% FBS. All cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium was changed every 2–3 days and all cultures were regularly monitored with an inverted phase-contrast microscope (Nikon, Eclipse TS100, Nikon Co., Shinjuku, Tokyo, Japan). When reaching 80–90% confluence, cells were harvested using 0.05% Trypsin/EDTA (Sigma-Aldrich) and sub-cultured for further experiments.

3.3.2 Colony forming Unit assay

The colony forming efficacy of all three dental stem cell populations was evaluated by means of a colony forming unit (CFU) assay as described in chapter 2 (2.3.2). Results were independently obtained for samples of four (DPSCs) or five (SCAPs, FSCs) different donors.

3.3.3 Flow cytometry

In order to assess and compare the expression of characteristic stem cell markers, flow cytometry was performed on six samples of each dental stem cell population. Following culture dissociation, DSCs were resuspended in PBS containing 2% FBS for 30 min at room temperature in order to allow re-expression of cell surface proteins. For each marker protein, 50,000 cells were incubated with a 1:100 dilution of the appropriate phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-conjugated antibodies and their matched isotype controls (Table 3.1) for 45 min at room temperature. Following incubation, 10,000 events per sample were recorded by means of a FACSCalibur flow cytometer. Data analysis was performed using Cell Quest Pro Software (BD Biosciences, Franklin Lakes, USA).

Table 3.1 Conjugated antibodies and matched isotype controls for flow cytometry

Marker protein	Label	Isotype	Manufacturer
CD29	FITC	Mouse IgG_1, kappa	eBioscience, Vienna, Austria
CD31	PE	Mouse IgG_1, kappa	Immunotools, Friesoythe, Germany
CD34	PE	Mouse IgG_1, kappa	Immunotools
CD44	PE	Mouse IgG_{2b}	Immunotools
CD45	PE	Mouse IgG_1, kappa	eBioscience
CD73	FITC	Mouse IgG_1, kappa	eBioscience
CD90	FITC	Mouse IgG_1, kappa	eBioscience
CD105	PE	Mouse IgG_1, kappa	eBioscience
CD117	PE	Mouse IgG_1, kappa	eBioscience

3.3.4 Pluripotency array

In order to identify which pluripotency-related proteins are expressed by DSCs, a general screening was performed by means of a human pluripotent stem cell array (Proteome profiler™, R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. Briefly, while array membranes were blocking, cell lysates are prepared for incubation by adding the appropriate buffers. Cell lysate from NTERA cells, a pluripotent cell line, was included as a positive control.

Following 1h of incubation, samples are added to the array membranes for an overnight incubation period at 4°C. After rinsing the membranes with the complementary wash buffer, the reconstituted detection antibody cocktail was added for 2h at room temperature. Following another wash, Streptavidin-HRP was added to the array membranes for 30 min at room temperature. In order to allow visualization of the results, chemiluminescent reagents (ECL plus, GE Healthcare, Little Chalfont, UK) were added to the array membranes, after which the membranes were exposed to X-ray film during 5 min (empirically determined optimal exposure time). This screening was performed two independent times on matched samples of two different donors. NTERA cells were included as a positive control. The results were validated by immunofluorescent staining against selected markers.

3.3.5 Adipogenic differentiation

The adipogenic differentiation potential of DPSCs, SCAPs and FSCs was assessed as described earlier in chapter 2 (2.3.5). This protocol was conducted on at least two donor samples of each stem cell population.

3.3.6 Osteogenic differentiation

The osteogenic differentiation capacity of at least two donors samples of all three dental stem cell populations was evaluated as described in chapter 2 (2.3.6).

3.3.7 Chondrogenic differentiation

The capability of DSCs to differentiate into chondrogenic lineages was determined as described in chapter 2 (2.3.7). This protocol was performed on at least two donor samples of each stem cell population.

3.3.8 Immunocytochemistry

Immunofluorescent stainings were performed on DPSCs, SCAPs and FSCs in order to evaluate the expression of certain pluripotency markers. Fluorescent and non-fluorescent stainings were conducted to assess the trilineage differentiation potential of DSCs. All stainings were accomplished as described in

chapter 2 (2.3.8). Primary antibodies and matched secondary antibodies are listed in table 3.2.

Table 3.2: Primary antibodies and matched secondary antibodies for immunostainings

Antibody	Type	Dilution	Manufacturer
Primary antibodies			
CD146	Rabbit Polyclonal IgG	1:100	Abcam, Cambridge ,UK
Snail	Mouse monoclonal IgG1	1:50	eBioscience
SOX17	Mouse monoclonal IgG1	1:50	Abcam
Aggrecan	Goat Polyclonal IgG	1 µg/100 µl	R&D Systems
FABP-4	Goat Polyclonal IgG	1 µg/100 µl	R&D Systems
Alkaline phosphatase	Mouse monoclonal IgG1	1:50	R&D Systems
Secondary antibodies			
Alexa Fluor 555 donkey anti-mouse	Donkey IgG	1:500	Invitrogen, Carlsbad, USA
Alexa Fluor 488 donkey anti-rabbit	Donkey IgG	1:500	Invitrogen
Alexa Fluor 488 donkey anti-goat	Donkey IgG	1:500	Invitrogen
Donkey ant-goat	Donkey polyclonal IgG	1:500	Jackson ImmunoResearch Laboratories, Inc., West Grove, USA

3.3.9 Transmission electron microscopy

The morphological features and trilineage differentiation potential of DSCs at the ultrastructural level were assessed as described in chapter 2 (2.3.9).

3.3.10 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 Software (GraphPad Software, La Jolla, CA, USA). Data distribution was assessed by means of a D'Agostino & Pearson normality test. When n < 8, data distribution was considered to be non-parametric. Data were compared with a Kruskall-Wallis test combined with a Dunns post-hoc test. Differences were considered to be statistically significant at P-values ≤0.05. All data were expressed as mean ± standard deviation (SD) * = P-value < 0.05; ** = P-value < 0.01; *** = P-value < 0.001.

3.4 Results

3.4.1 Morphology and colony forming properties of human dental stem cells

Following isolation of the different stem cell populations, regular monitoring of the cell cultures with a phase-contrast microscope indicated cell adherence and proliferation after four to five days. While DPSCs were found to be confluent following 10-14 days of culturing, SCAPs and FSCs usually reached 80-90% confluency after 7-10 days. With regard to cellular morphology, all cell cultures showed the presence of elongated, fibroblast-like cells (Fig. 3.1A-C). At the ultrastructural level, DPSCs (Fig 3.1D), SCAPs (Fig. 3.1E) and FSCs (Fig. 3.1F) were characterized as elongated cells comprising a perinuclear organelle-rich zone (p, Fig. 3.1D-F), consisting of rough endoplasmic reticulum (r, Fig. 3.1G-I) and mitochondria (m, Fig. 3.1G-I), and a peripheral electron-lucent organelle-poor zone (o, Fig. 3.1D-F). Large euchromatic nuclei with one or more clear nucleoli were also detected (n, 3.1D-F). No significant morphological differences were observed between the three dental stem cell populations.

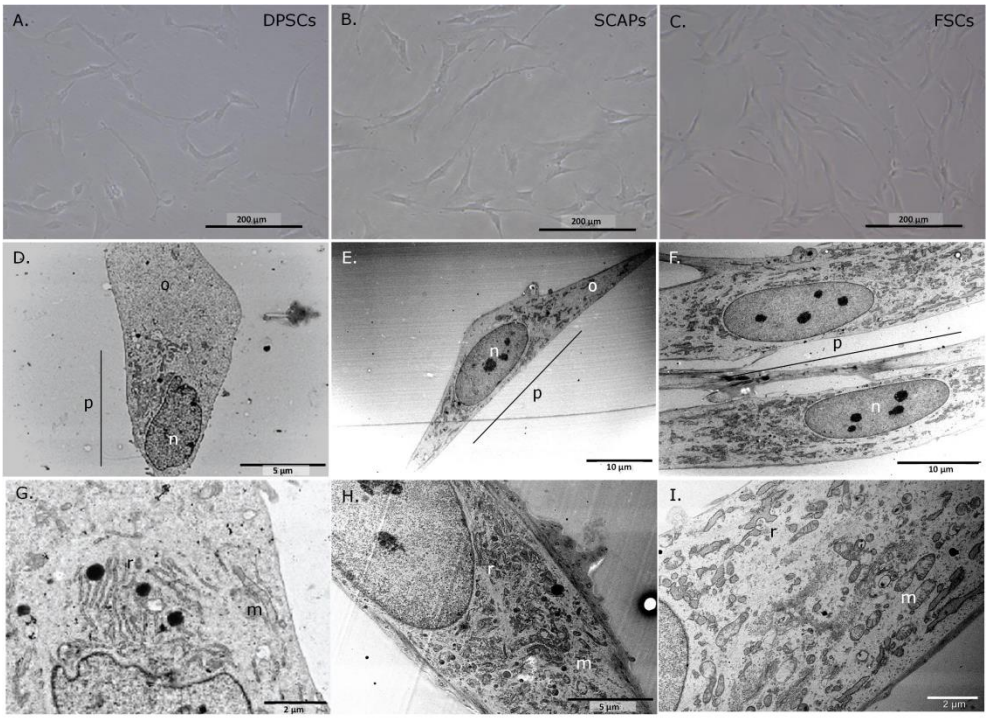


Figure 3.1: Morphology of DPSCs (A, D, G), SCAPs (B, E, H) and FSCs (C, F, I) A-C. Dental stem cell cultures with elongated, fibroblast-like cells. D-F. A perinuclear (p) organelle-rich zone could be distinguished from an electron-lucent organelle-poor (o) region. n = nucleolus. G-I. The organelle-rich zone contained rough endoplasmic reticulum and mitochondria (m). Scale bars = 200 µm (A-C), 10 µm (E, F), 5 µm (D, H), 2 µm (G, I).

With regard to the colony forming abilities of DSCs, analysis indicated a significantly lower amount of colonies formed by DPSCs in comparison to SCAPs (Fig. 3.2A). However, when applying a higher seeding density, this significant difference disappeared and DPSCs even showed a trend towards a higher amount of formed colonies compared to the similar colony forming potential of SCAPs and FSCs (Fig. 3.2B).

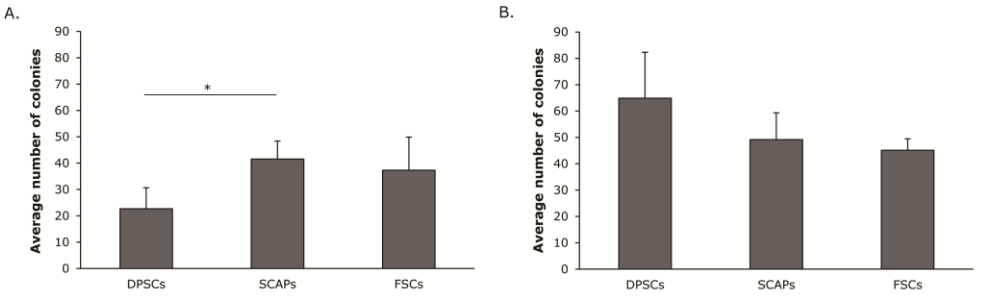


Figure 3.2: Colony forming properties of DPSCs, SCAPs and FSCs. A. Average number of colonies of DSCs after 10 days of culturing with a seeding density of 52.6 cells/cm². B. Average number of colonies of DSCs after 10 days of culturing with a seeding density of 210.5 cells/cm². This assay was independently conducted on samples of four (DPSCs) or five (SCAPs, FSCs) different donors. All data are expressed as mean ± SD. * = P-value < 0.05

3.4.2 Immunophenotype of human dental stem cells

The immunophenotype of DPSCs, SCAPs and FSCs was assessed by means of immunofluorescent stainings and flow cytometry (Fig. 3.3). All three cell cultures showed a uniform expression of endothelial marker CD146 (Fig. 3.3A). With regard to the expression of stem cell markers CD29, CD44, CD73, CD90 and CD105, flow cytometry indicated high percentages of positive cells in all dental stem cell populations (Fig. 3.3B). However, DPSCs displayed a significantly lower expression of CD29, CD105 and CD90 in comparison to FSCs and SCAPs respectively. Analysis also indicated the absence of CD31, CD34 and CD45 expression and negligible percentage (<1%) of CD117 positive dental stem cells.

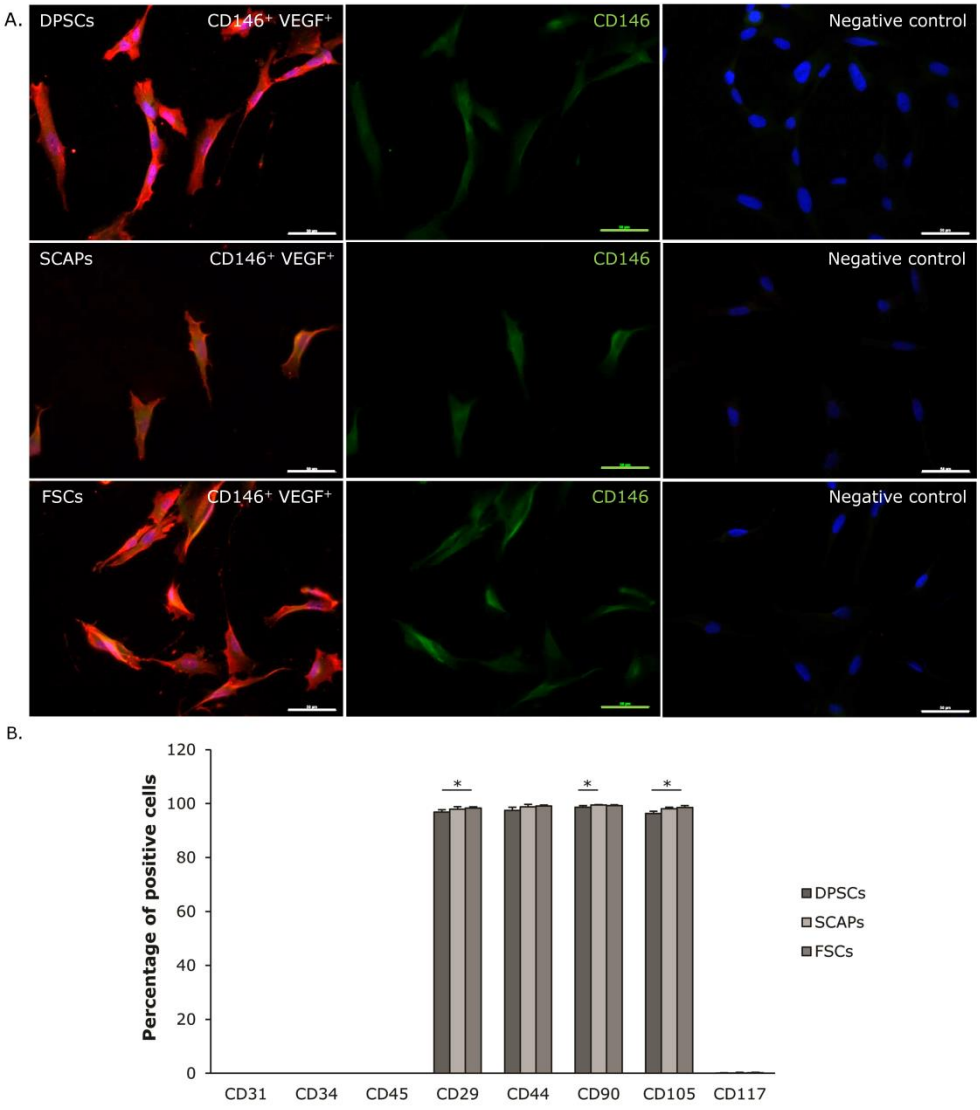


Figure 3.3: Immunophenotype of DPSCs, SCAPs and FSCs. A. Dental stem cells show a uniform expression of CD146. Scale bars = 50 µm. Pictures are representative of three independent stainings. B. Percentage of positive DSCs for CD31, CD34, CD45, CD29, CD44, CD90, CD105 and CD117. Flow cytometry was conducted in parallel on samples of six different donors in three independent assays. All data are represented as mean ± SD. * = P-value < 0.05.

3.4.3 Expression of pluripotency markers by human dental stem cells

In order to evaluate the expression of pluripotency markers by DPSCs, SCAPs and FSCs, a general screening was performed on samples of two different donors using an antibody array. While the NTERA cells (included as a positive control condition) expressed a wide variety of pluripotency-related proteins, such as Oct 3/4, Nanog, SOX2, E-cadherin, SOX17 and Snail, dot blot analysis of DPSCs, SCAPs and FSCs cell lysates in particular indicated a differential expression of Snail (red frame, Fig. 3.4A, B) and SOX17 (blue frame, Fig. 3.4A, B). Validation of these results by means of immunocytochemistry confirmed the expression of Snail and SOX17 in a subset of DPSCs, SCAPs and FSCs (Fig. 3.4C).

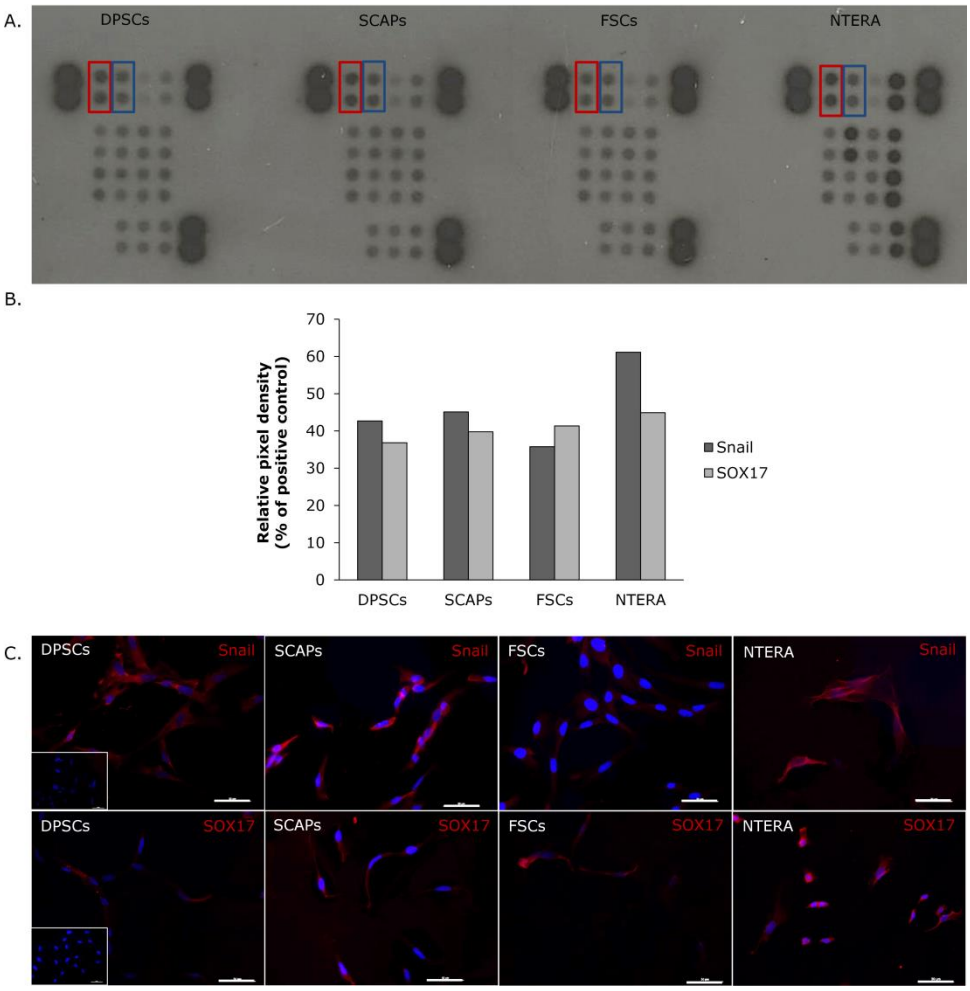


Figure 3.4: Expression of pluripotency markers by dental stem cells. A. Dot blot analysis indicated the differential expression of Snail (red frame) and SOX17 (blue frame) by DPSCs, SCAPs, FSCs and NTERA cells. This assay was independently performed on samples of two different donors. B. Quantification of Snail and SOX17 expression. Expression levels were normalized against positive reference spots. C. Subsets of DPSCs, SCAPs and FSCs expressed Snail and SOX17. Pictures are representative of four independent stainings. Insets: negative control. Scale bars = 50 µm.

3.4.5 Trilineage differentiation potential of dental stem cells

Following 21 days of incubation with adipogenic differentiation medium, a subset of DPSCs, SCAPs and FSCs showed a strong expression of FABP-4 (Fig. 3.5A-C). The differentiated cells also displayed large transparent droplets, i.e. lipid-containing vacuoles, in their cytoplasm, which were more apparent after Oil Red O staining (Fig. 3.5D-F).

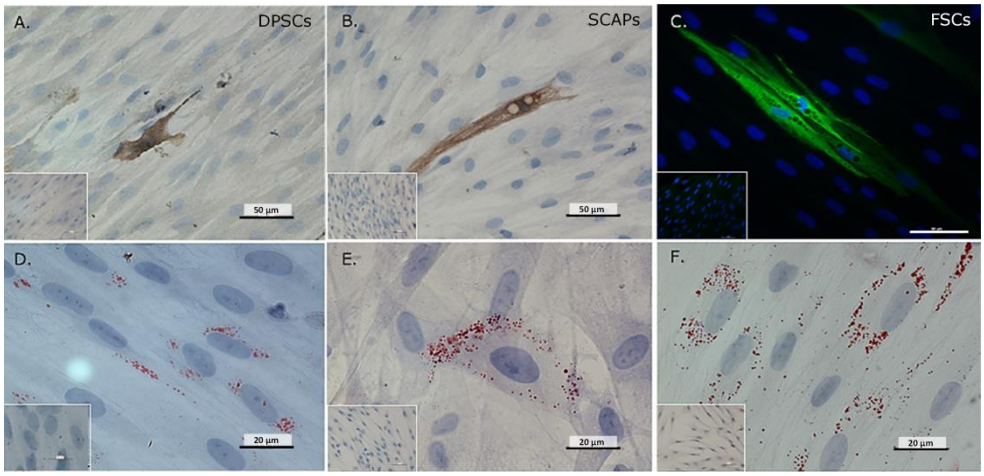


Figure 3.5: Adipogenic differentiation potential of dental stem cells. A-C. Expression of FABP-4 in subsets of differentiated DPSCs (A), SCAPs (B) and FSCs (C). Insets = undifferentiated cells. Scale bars = 50 µm. D-F. Oil Red O staining indicated the accumulation of lipid droplets in subsets of DPSCs (D), SCAPs (E) and FSCs (F). Insets = undifferentiated cells. Scale bars = 20 µm. SCAPs data courtesy of Dr. Wendy Martens.

At the ultrastructural level, differentiated cells displayed a globular appearance, with no distinctive organelle-poor zone, i.e organelles were spread throughout the cytoplasm (Fig. 3.6A-C). Next to dilated rough endoplasmic reticulum (dER, Fig. 3.6D-F) and branched mitochondria (bMit, Fig. 3.6D-F), differentiated cells also contained small electron-dense lipid droplets (asterisks, Fig. 3.6G-I). No morphological differences were observed between the three stem cell populations after adipogenic differentiation.

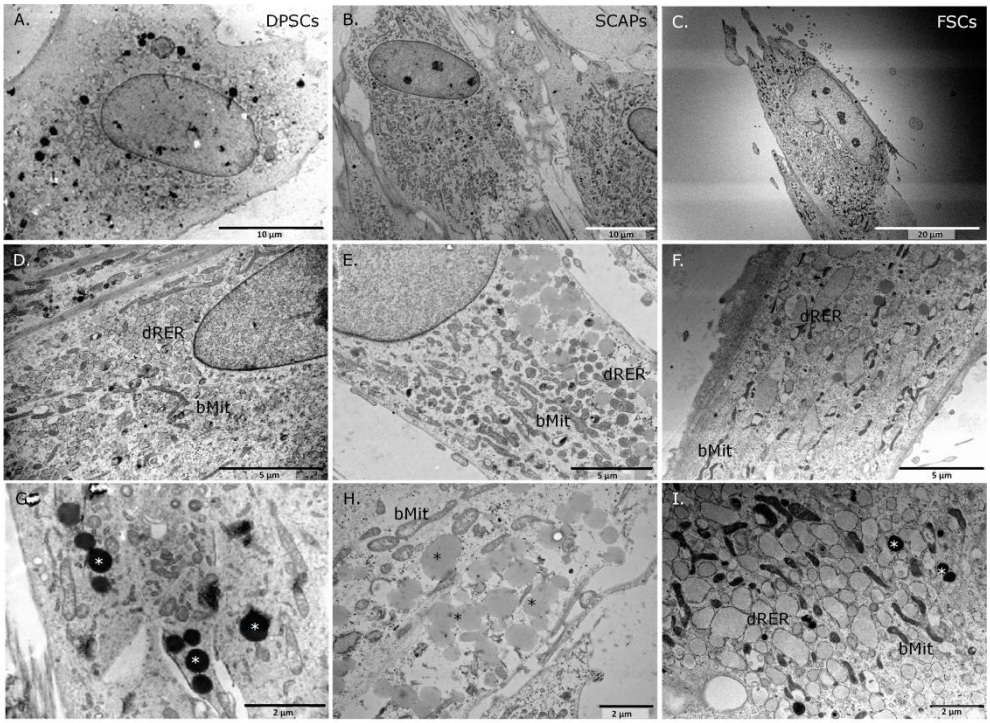


Figure 3.6: Adipogenic differentiation potential of dental stem cells at the ultrastructural level. A-C. Differentiated DPSCs (A), SCAPs (B) and FSCs (C) were characterized by a globular appearance. Scale bars = 10 µm (A, B), 20 µm (C). D-F. Differentiated cells contained branched mitochondria (bMit) and dilated rough endoplasmic reticulum (dRER). Scale bars = 5µm. G-I. Electron-dense lipid droplets (asterisks) were also observed in differentiated DPSCs (G), SCAPs (H) and FSCs (I). Scale bars = 2µm. DPSCs pictures courtesy of Pascal Gervois. SCAPs data courtesy of Dr. Wendy Martens.

In terms of osteogenic differentiation, alkaline phosphatase (ALP) synthesis was observed in DPSCs, SCAPs and FSCs following 14-21 days of culturing in differentiation medium (Fig. 3.7A-C). Alizarin Red S staining of osteogenic differentiated cells demonstrated the presence of calcified nodules in all three stem cell populations, although the number and size of the nodules varied significantly (Fig. 3.7D-F).

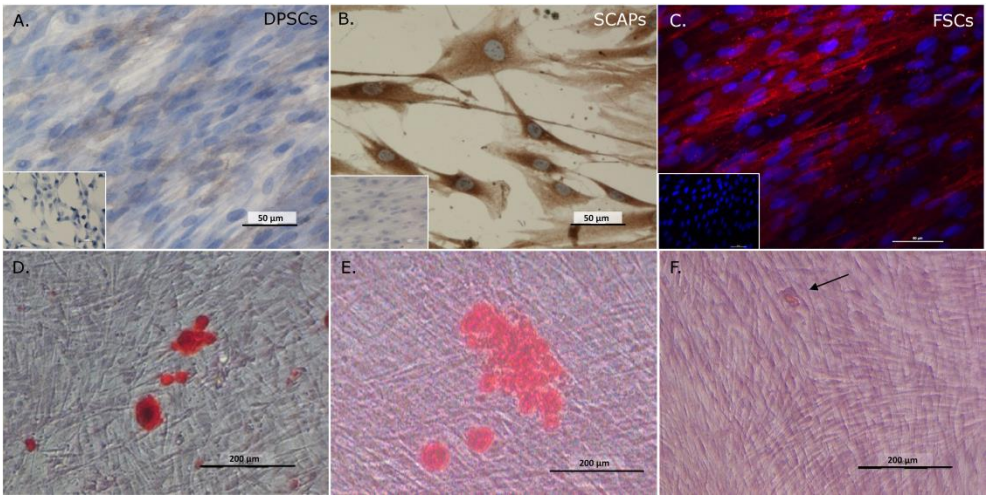


Figure 3.7: Osteogenic differentiation potential of dental stem cells. A-C. Expression of alkaline phosphatase in differentiated DPSCs (A), SCAPs (B) and FSCs (C). Insets = undifferentiated cells. Scale bars = 50 µm. D-F. Alizarin Red S staining indicated the presence of calcified nodules in of DPSCs (D), SCAPs (E) and FSCs (arrow, F). Scale bars = 200 µm. DPSCs data courtesy of Pascal Gervois. SCAPs data courtesy of Dr. Wendy Martens.

Ultrastructurally, some pronounced differences were detected between the different stem cell populations. While osteogenic differentiated DPSCs and SCAPs were characterized by a clear osteoblast-like morphology with an eccentric nucleus (Fig. 3.8A, B), this was not the case for FSCs. Most of the FSCs still showed an elongated fibroblast-like appearance, though cell organelles were not restricted to a perinuclear zone (Fig. 3.8C). Mineralized nodules were observed in DPSCs and SCAPs, while in FSCs only hydroxyapatite needles could be distinguished (encircled, Fig. 3.8D-F). Striated collagen was present in the extracellular matrix of all differentiated DSCs (Fig. 3.8G-I).

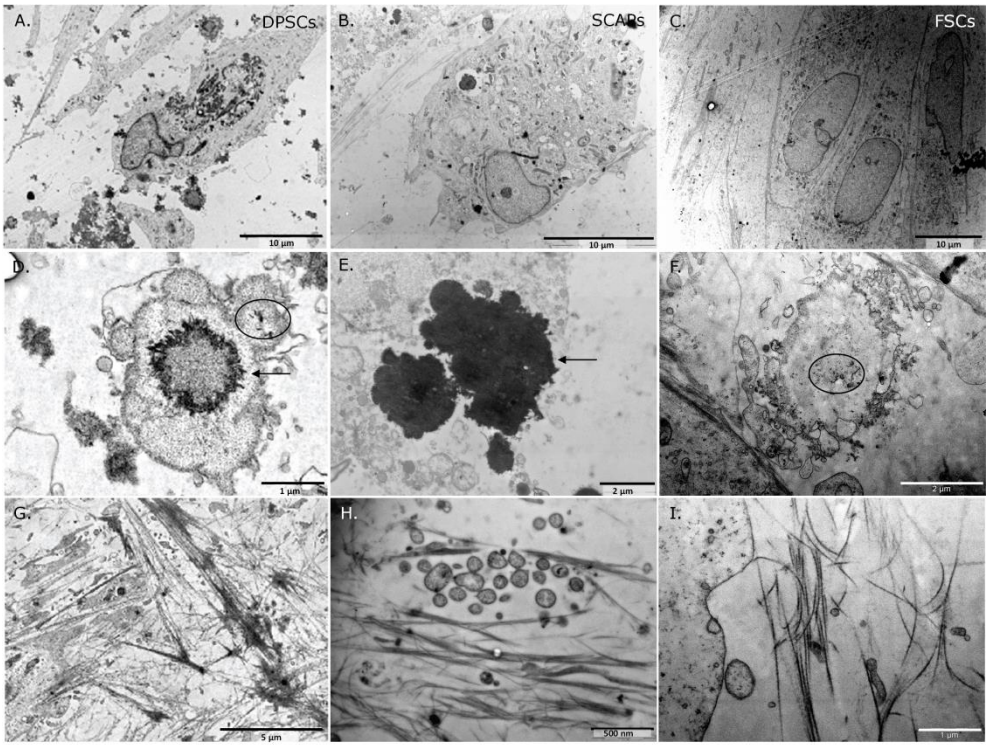


Figure 3.8: Osteogenic differentiation potential of dental stem cells at the ultrastructural level. A-B. Differentiated DPSCs (A) and SCAPs (B) were characterized by a globular appearance. Scale bars = 10 µm. C. Differentiated FSCs displayed an elongated cellular morphology. Scale bar = 10 µm. D-E. Presence of mineralized nodules in DPSCs (arrow, D) and SCAPs (arrow, E) with distinguishable hydroxyapatite needles (encircled). Scale bars = 1 µm (D), 2 µm (E). F. Mineralized nodules were absent in differentiated FSCs. Deposition of hydroxyapatite needles was observed (encircled). Scale bar = 2 µm. G-I. Abundant striated collagen fibers were present in the extracellular matrix of all differentiated DSCs. Scale bars = 5µm (G), 500 nm (H), 1 µm (I). DPSCs data courtesy of Pascal Gervois. SCAPs data courtesy of Dr. Wendy Martens.

Following three weeks of chondrogenic differentiation, DPSCs, SCAPs and FSCs formed three dimensional cell spheres which were positive for Aggrecan expression (Fig. 3.9A-C). Masson's trichrome and Alcian Blue staining also indicated the presence of collagen and glycosaminoglycans in the extracellular matrix of differentiated DSCs (Fig. 3.9D-F).

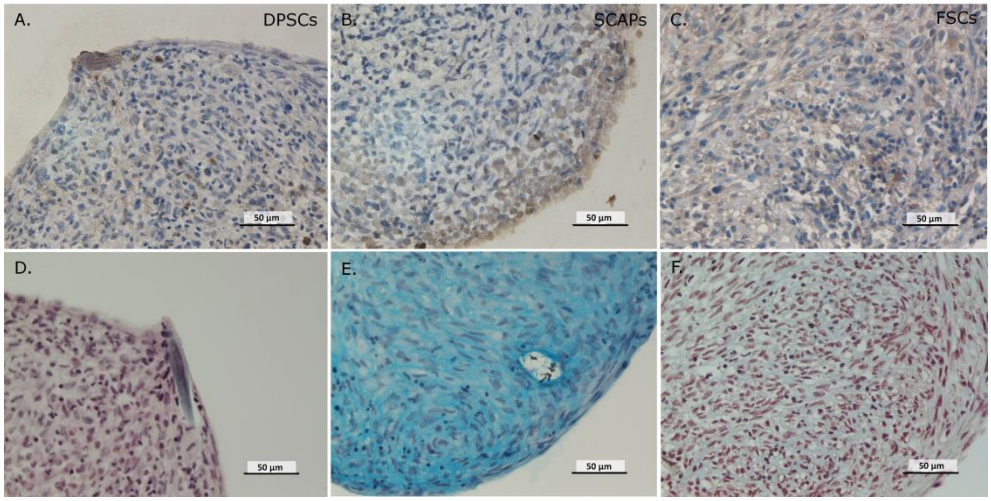


Figure 3.9: Chondrogenic differentiation potential of dental stem cells. A-C. Pellets derived from differentiated DPSCs (A), SCAPs (B) and FSCs (C) were positive for aggrecan expression. D-F. Masson's trichrome (D, F) and Alcian Blue staining (E) indicated the presence of collagen and glycosaminoglycans, respectively. Scale bars = 50 µm. DPSCs pictures courtesy of Annelies Bronckaers. SCAPs data courtesy of Dr. Wendy Martens and Dr. Annelies Bronckaers.

At the ultrastructural level, chondrogenic differentiated DSCs could be characterized as elongated cells (Fig. 3.10A-C) containing matrix vesicles (Fig. 3.10D-F). Although these vesicles showed a clear interaction with striated collagen in the extracellular space of all differentiated stem cell populations (Fig. 3.10G-I, L), cartilage fragments could only be distinguished in cell pellets derived from DPSCs and SCAPs (ca, Fig. 3.10 J, K).

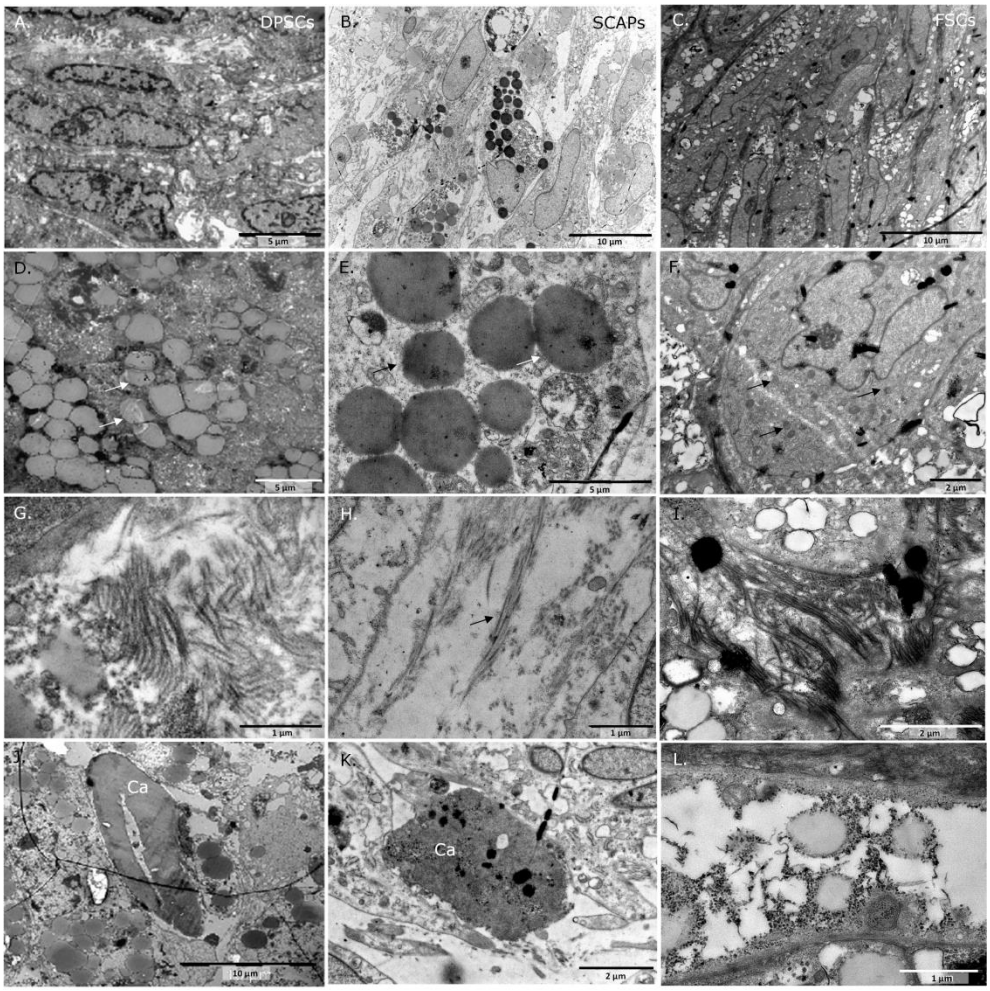


Figure 3.10: Chondrogenic differentiation potential of dental stem cells at the ultrastructural level. A-C. Differentiated DPSCs (A), SCAPs (B) and FSCs (C) showed an elongated cellular morphology. Scale bars = 5 µm (A), 10 µm (B, C). D-F. Differentiated DSCs contained matrix vesicles (arrows). Scale bars = 5 µm (D, E), 2 µm (F). G-I. Striated collagen was present in the extracellular space of all differentiated DSCs. Scale bars = 1 µm (G, H), 2 µm (I). J-L. Cartilage fragments were only found to be present in cell pellets derived from DPSCs (J) and SCAPs (K). In the extracellular space of differentiated FSCs (L) there was a clear interaction between secreted matrix vesicles and collagen fibers. Scale bars = 10µm (J), 2 µm (K), 1 µm (L). DPSCs pictures courtesy of Pascal Gervois. SCAPs data courtesy of Dr. Wendy Martens and Dr. Annelies Bronckaers.

3.5 Discussion

Over the past decade, a number of different stem cell populations have been isolated from the human tooth and its environment, such as DPSCs, SCAPs and FSCs. Their straightforward and minimally invasive isolation from extracted third molars, together with their proliferative and immunomodulatory advantages over bone marrow-derived MSCs, has indicated they are an attractive source of mesenchymal-like stem cells for clinical applications. Nevertheless, one should take into account potential differences related to their developmental origin, as it defines the inherent capability of each DSC population to repair and/or regenerate diseased and damaged tissues. Therefore, an elaborate empirical comparison of these cells with regard to colony formation, marker expression and multilineage differentiation potential is required to adequately assess these differences.

In terms of colony forming properties, CFU analysis indicated a significantly lower amount of colonies formed by DPSCs (22.69 ± 7.98) in comparison to SCAPs (41.55 ± 6.82). Similar differences were found by Tamaki *et al.*, reporting a significantly higher number of colonies formed by SCAPs compared to DPSCs [335]. Although this group also reported enhanced colony forming properties for FSCs, this was not the case in our study (FSCs: 37.33 ± 12.53). Furthermore, the average number of formed colonies reported by Tamaki *et al.* was notably higher, this can be explained by the higher seeding density of 127.3 cells/cm². However, when we seeded the cells at a higher density of 210.5/cm², DPSCs (64.90 ± 17.42) showed a propensity to form a higher amount of colonies in comparison to SCAPs (49.17 ± 10.19) and FSCs (45.10 ± 4.39), yet no statistically significant differences were detected.

With regard to the immunophenotype, DPSCs, SCAPs and FSCs were all positive for CD29, CD44, CD73, CD90 and CD105 and CD146. Nevertheless, DPSCs showed slightly, but significantly lower levels of CD29, CD90 and CD105, in comparison to SCAPs and FSCs. Similar findings were also reported by Huang *et al.* and others, although no significant differences were observed [15, 335]. There were also no notable differences for CD31, CD34 and CD45 negativity, although there have been reports indicating certain subpopulations of DPSCs

and SCAPs expressing CD31 or CD34 [180, 330, 331, 336]. Analysis also indicated a small percentage (<1%) of CD117 positive dental stem cells. However, the expression of CD117 appears quite susceptible to donor variability, as already indicated by our group and others reporting subsets of $CD117^+$ DPSCs [330, 337].

Along with the standard stem cell markers, the expression of pluripotency markers by DSCs was also assessed. Although the general screening indicated the expression of SOX17 and Snail by all three stem cell populations, not all cells of each stem cells population were positive for these markers, as was pointed out by immunocytochemical stainings performed on samples of four different donors. Given their neural crest origin, the expression of Snail, a neural crest marker which plays an important role in the regulation of the epithelial-mesenchymal transition and the maintenance of stem cell function, by DSCs was not surprising [338, 339]. Comparable observations were made by Vanacker *et al.* and others, in particular in DPSCs and SCAPs at the transcriptional level [340-342]. The expression of SOX17, on the other hand, an endodermal marker which plays an important role in cardiovascular development, has only been reported in early passage DPSCs at the gene expression level [343, 344].

With regard to the trilineage differentiation potential of DPSCs, adipogenic differentiation medium induced the intracellular accumulation of lipid droplets together with the expression of FABP-4, i.e. distinctive characteristics of adipocytes. Although no significant morphological differences between the stem cells populations were found, only a subset of cells was clearly capable of adipogenic differentiation. These results support earlier reports on DPSCs, SCAPs and FSCs indicating either incomplete adipogenic differentiation or adipogenic differentiation in a subpopulation of cells [221, 237, 335, 345, 346]. Following the induction of osteogenic differentiation, the expression of alkaline phosphatase and the production of calcified nodules was observed. However, while the osteogenic differentiation of DPSCs and SCAPs had similar outcomes, differentiated FSCs clearly contained less calcified nodules. This difference was also apparent at the ultrastructural level, as the FSCs still displayed an elongated fibroblast-like morphology and contained, besides some distinguishable hydroxyapatite needles, no mineralized nodules. Striated

collagen fibers, on the other hand, were present in DPSCs, SCAPs and FSCs. These results are in contrast with earlier studies reporting on the successful osteogenic differentiation of FSCs [19, 347, 348]. However, the aforementioned studies did not include an ultrastructural analysis, which, in this case is required for an in-depth assessment of the cellular characteristics as we also observed alkaline phosphatase expression and (small) calcified nodules following Alizarin Red S staining. Following incubation with chondrogenic induction medium, aggrecan expression, together with the presence of collagen and glycosaminoglycans, was detected in all cell pellets derived from differentiated DSCs. Collagen and glycosaminoglycan-containing matrix vesicles were also detected at the ultrastructural level. However, cartilage fragments were only observed in the extracellular space of differentiated DPSCs and SCAPs. While the successful chondrogenic differentiation of DPSCs has been reported previously, limited data is available with regard to the chondrogenic differentiation potential of SCAPs and FSCs [15, 329, 341, 349, 350].

In conclusion, this study compared three DSC stem cell populations with regard to their colony forming properties, immunophenotype and trilineage differentiation potential. DPSCs displayed a lower colony forming potential in comparison to SCAPs and FSCs. All three stem cell populations expressed the classical panel of stem cell markers, with some minor differences between populations. Our results also suggest a strong chondrogenic/osteogenic potential of DPSCs and SCAPs, in comparison to FSCs. Furthermore, the incomplete adipogenic differentiation of all three stem cell populations indicates that DSCs in general might not be the ideal stem cells for the replacement of fatty tissue. Taken together, this indicates that, although DSCs are all considered to be mesenchymal-like stem cells, the aforementioned differences surely have to be kept in mind when considering these cells for therapeutic or tissue engineering applications.

Chapter 4

Angiogenic properties of dental stem cells

Based on:

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Pro-angiogenic impact of dental stem cells in vitro and in vivo.

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4.1 Abstract

Vascular supply is a key process in tissue engineering and ischemic diseases such as myocardial infarction and stroke. However, both the traditional revascularization methods and the current stem cell therapies no longer fulfil the expectations for an increasing number of patients. Therefore, the present study aimed to elucidate the paracrine angiogenic properties of postnatal dental stem cells (DSCs), in particular dental pulp stem cells (DPSCs), stem cells from the apical papilla (SCAPs) and dental follicle precursor cells (FSCs). An antibody array, together with RT-PCR and ELISA, pointed out the differential expression of pro-angiogenic as well as anti-angiogenic factors by cultured DSCs and human gingival fibroblasts (HGF-1). Despite the secretion of proliferation-promoting factors, DSCs caused no notable increase in the proliferation of human microvascular endothelial cells (HMEC-1). With regard to other aspects of the angiogenic cascade, DPSCs, SCAPs and HGF-1 significantly promoted endothelial transmigration in a transwell migration assay, though a notable effect on directional migration was absent. DPSCs also had a pronounced effect on endothelial tubulogenesis, as was shown by an *in vitro* Matrigel™ assay. In the final part of this study, a chorioallantoic membrane assay demonstrated a sustained pro-angiogenic impact of DPSCs and SCAPs in an *in vivo* setting. Collectively, these data indicate a predominant pro-angiogenic influence of DPSCs and SCAPS *in vitro* and *in vivo* in comparison to FSCs, encouraging further investigation of both these stem cell populations as therapeutic tools in vascular medicine as well as (dental) tissue engineering.

4.2 Introduction

Adequate vascularization is not only a limiting factor for the survival of transplanted cells and tissues, but it also plays a key role in tooth development, wound healing and ischemic disorders such as stroke and myocardial infarction (MI). As angiogenesis is a multi-step process which is regulated by a delicate balance of stimulating and inhibiting factors, early revascularization studies merely focused on the modulation of pro-angiogenic growth factors [147]. However, the limited success of these studies and the increasing number of 'no-option' patients prompted the need for (stem) cell-based therapies [148]. Bone marrow-derived mesenchymal stem cells (BM-MSCs), in particular, showed great promise in the enhancement of tissue perfusion in animal models of MI and hind limb ischemia [351-353]. Although early clinical studies regarding the use of BM-MSCs in cardiovascular diseases showed improved tissue function after therapy, the exact contribution of these cells is not clear as most of these studies involved the use of a heterogeneous bone marrow-derived cell population. Furthermore, the mechanisms underlying the improved tissue function and increased neovascularization remain subject of controversy [166, 167]. As the rate of engraftment of stromal cells derived from different tissue sources has proven to be extremely low in most *in vivo* settings, it is currently assumed that the therapeutic benefits of these cells are primarily caused by their paracrine (angiogenic) actions [152, 169-172]. The potential therapeutic benefits of BM-MSCs in the treatment of several disorders are also offset by their invasive and painful isolation procedure, which all together stresses the need for an alternative source of adult stem cells [12]. Over a decade ago, Gronthos *et al.* were the first to report the presence of a stem cell population within the dental pulp [17]. Besides dental pulp stem cells (DPSCs), dental tissues also harbor other stromal cells such as stem cells from the apical papilla (SCAPs) and dental follicle precursor cells (FSCs) [19, 237]. Although dental stem cells (DSCs) display cell surface characteristics similar to BM-MSCs, their proliferative and immunomodulatory properties have proven to be more pronounced [15, 312, 354]. Furthermore, these cells can relatively easily be isolated from human third molars, a procedure which involves little discomfort for the patient compared to bone marrow aspiration [12, 15, 355]. Numerous studies also

suggest the potential role of DSCs in the repair of diseased and damaged tissues, as they are not only capable of forming dentin-pulp complexes and periodontal tissues *in vivo*, but also display an *in vitro* potential towards myogenic and neurogenic cells [15, 17, 18, 237, 303]. However, little is known about the angiogenic properties of DSCs. With regard to DPSCs, a number of studies mentioned the expression of paracrine angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) under basal conditions, after injury or hypoxia [174-177], as well as the potential to ameliorate left ventricular function and to increase angiogenesis in a rat model of MI [178]. Furthermore, a couple of studies have indicated the *in vitro* differentiation capacity of DPSCs into endothelial or pericyte-like cells, which could indicate the potential incorporation of these cells into newly formed blood vessels although proof of true functionality is limited at this stage [180-182]. Although SCAPs and FSCs are considered to be more immature dental stem cell populations residing in strongly vascularized tissues, literature merely indicates their expression of endostatin, bFGF and angiogenesis-regulating transcription factors such as hypoxia-inducible factor 1 alpha (HIF1A) [15, 184]. Despite their expression of angiogenic factors, the effects of both dental stem cell populations on endothelial cell behavior and blood vessel formation have not yet been described. Within the oral environment an additional cell population with potential angiogenic properties can be found, namely gingival fibroblasts. In certain inflammatory and pathological conditions human gingival fibroblasts (HGF) have been known to secrete angiogenic factors such as urokinase (uPA), monocyte chemotactic protein-1 (MCP-1), interleukin 8 (IL-8) and VEGF [356-358]. Furthermore, these cells are capable of promoting the proliferation of human umbilical cord vein endothelial cells (HUVEC) [357]. In order to potentiate any therapeutic application of DSCs, either in (dental) tissue engineering or in ischemic diseases, a more elaborate angiogenic profiling is required. In the present study, the paracrine angiogenic properties of DSCs and HGF are further elucidated by determining the angiogenic expression profile at mRNA and protein level, after which the impact of these cells on endothelial proliferation, migration and tube formation is assessed *in vitro*. Finally, a

chorioallantoic membrane (CAM) assay is performed to evaluate the potential of DSCs and HGF to induce blood vessel formation *in vivo*.

4.3 Materials and Methods

4.3.1 Cell culture

Dental tissues were acquired with informed consent from patients (15–21 years of age) undergoing extraction of third molars for orthodontic or therapeutic reasons at Ziekenhuis Maas en Kempen, Bree, Belgium. Written informed consent of patients below the age of 18 was obtained through their guardians. This study was approved by the medical ethical committee of Hasselt University. Dental stem cells (DSCs) were isolated according to the explant method described in chapter 2 (2.3.1) and 3 (3.3.1). A human microvascular endothelial cell line (HMEC-1) was purchased from the Center of Disease Control and Prevention (Atlanta, USA). The cells were cultured in MCDB 131 medium (Invitrogen, Carlsbad, USA) supplemented with 100 U/ml Penicillin and 100 µg/ml Streptomycin (Sigma-Aldrich, St.-Louis, USA), 10 mM L-glutamine (Sigma-Aldrich), 10% FBS (Biochrom AG, Berlin, Germany), 10 ng/ml human epidermal growth factor (hEGF, Gibco, Paisley, UK) and 1 µg/ml hydrocortisone (Sigma-Aldrich) (further referred to as standard endothelial culture medium). Human gingival fibroblasts (HGF-1, CRL-2014, ATCC, Molsheim Cedex, France) were cultured in αMEM supplemented with 100 U/ml Penicillin and 100 µg/ml Streptomycin, 2 mM L-glutamine and 20% FBS (further referred to as standard HGF-1 culture medium). All cell cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO2. The culture medium was changed every 2–3 days and all cultures were regularly monitored with an inverted phase-contrast microscope (Nikon, Eclipse TS100, Nikon Co., Shinjuku, Tokyo, Japan). When reaching 80–90% confluence, cells were harvested using 0.05% Trypsin/EDTA (Sigma-Aldrich) and sub-cultured for further experiments.

4.3.2 Conditioned medium

DSCs as well as HGF-1 (passage 2-4) were seeded at 20,000 cells/cm² in standard culture medium. After 24h of culturing, the cells were rinsed twice with phosphate buffered saline (PBS) after which the cells were incubated with standard DSC culture medium containing 0.1% FBS. After 48h, the conditioned medium was harvested and stored at -80°C for further experiments.

4.3.3 Antibody array

In order to identify which angiogenesis-related proteins are secreted by DSCs and HGF-1, a general screening was performed by means of a human angiogenesis antibody array (Proteome profiler™, R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. Briefly, while array membranes were blocking, conditioned medium of both DSCs and HGF-1 was incubated with a detection antibody cocktail. Standard DSC culture medium supplemented with 0.1% FBS was incorporated as a negative control. Following 1h of incubation, the sample/antibody mixture was added to the array membranes for an overnight incubation period. After rinsing the membranes with the complementary wash buffer, Streptavidin-HRP was added for 30 min at room temperature. In order to allow visualization of the results, chemiluminescent reagents (ECL plus, GE Healthcare, Little Chalfont, UK) were added to the array membranes, after which the membranes were exposed to X-ray film during 2 min (empirically determined optimal exposure time). The visualized dots were quantified by means of Image J software complemented with a dot blot analyzer plug-in. Expression levels were normalized against positive and negative reference dots. This screening was performed three independent times on stem cell samples of three different donors. The results were validated by reverse transcriptase polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA).

4.3.4 Reverse transcriptase polymerase chain reaction

DSCs and HGF-1 (passages 2–3) were seeded at 4,000 cells/cm² and 2,000 cells/cm², respectively, in standard culture medium. When reaching 80–90% confluence, the cells were harvested using 0.05% Trypsin/EDTA and cell pellets were made. Total RNA was extracted from the cell pellets as described by the RNeasy Plus Mini Kit (Qiagen, Venlo, the Netherlands). After determining the concentration and the purity of the isolated RNA by means of a spectrophotometer (NanoDrop 2000, Thermo Scientific, Pittsburgh, USA), 700 ng of RNA was reverse-transcribed into cDNA according to the Reverse Transcription System (Promega, Leiden, The Netherlands). cDNA was amplified through RT-PCR according to the protocol described in table 4.1. All primers

(Table 4.2, Eurogentec S.A., Seraing, Belgium) were generated by means of Primer-BLAST, except for angiogenin and tissue inhibitor of matrix metalloproteinase-4, which were derived from literature [359-361]. Primer efficiency was tested by means of a gradient PCR. The resulting samples were separated on a 1.2% agarose gel and visualized by means of ethidium bromide. This reaction was performed on six different patient samples of each cell population, of which three representative samples are shown.

Table 4.1 PCR protocol

PCR mix per sample (µl)		**PCR cycles**		
10x PCR buffer	2.5	1x	5 min	94°C
Forward primer (25 µM)	1	35x	1 min	95°C
Reverse primer (25 µM)	1		1 min	T_m
dNTPs (10 mM)	0.5		45 sec.	72°C
Taq polymerase	0.75	1x	10 min	95°C
MilliQ	18.25	1x	∞	4°C

4.3.5 Enzyme-linked immunosorbent assay

In order to obtain a clear view of the concentration range of secreted angiogenic factors, the following ELISAs were performed on conditioned medium of DSCs and HGF-1 (passages 2–4) according to the manufacturer's instructions: angiopoietin-1 (ANGPT1) (Raybiotech, Norcross, USA), insulin-like growth factor binding protein 3 (IGFBP3) (Raybiotech) and VEGF (Raybiotech). All ELISAs were conducted on at least six different patient samples of each cell population.

Table 4.2 Primer designation for angiogenic factors and housekeeping genes

Gene	Accession number	Primer	Sequence	Tm (°C)	Product size (bp)
Angiogenic factors					
Angiogenin (ANG)	NM_001145.4	Forward Reverse	CCT-GGG-CGT-TTT-GTT-GTT-GG TGT-GGC-TCG-GTA-CTG-GCA-TG	62.2	352
Dipeptidyl peptidase IV (DPPIV)	NM_001935.3	Forward Reverse	GGC-ACC-TGG-GAA-GTC-ATC-GGG-A GGG-CAG-ACC-AGG-ACC-GGA-AC	65.6	237
Endothelin 1 (EDN1)	NM_001955.4	Forward Reverse	TTG-CCA-AGG-AGC-TCC-AGA-AAC-AGC ACG-GAA-CAA-CGT-GCT-CGG-GA	64	206
Pentraxin 3 (PTX3)	NM_002852.3	Forward Reverse	TCC-CCA-TTC-AGG-CTT-TCC-TCA-GCA ACG-GCG-TGG-GGT-CCT-CAG-TG	65.2	277
Pigment epithelial-derived factor (PEDF)	NM_002615.5	Forward Reverse	ATC-CAC-AGG-CCC-CAG-GAT-GCA-G GCT-CGT-GCT-GGA-TCG-CAC-CC	65.4	235
Plasminogen activator inhibitor 1 (PAI-1)	NM_003256.3	Forward Reverse	ATA-CTG-AGT-TCA-CCA-CGC-CC GTG-GAG-AGG-CTC-TTG-GTC-TG	62.1	320
Tissue inhibitor of matrix metalloproteinase 1 (TIMP-1)	NM_003254.2	Forward Reverse	GCT-TCT-GGC-ATC-CTG-TTG-TT TTT-GCA-GGG-GAT-GGA-TAA-AC	60	462
Tissue inhibitor of matrix metalloproteinase 4 (TIMP-4)	NM_003256.3	Forward Reverse	CAA-GAG-GTC-AGG-TGG-TAA ACA-GCC-AGA-AGC-AGT-ATC	54	446
Thrombospondin 1 (THBS1)	NM_003246.2	Forward Reverse	CAG-GGC-TCC-TGT-CGC-TCT-CCA ACA-TTC-TGC-AGC-ACC-CCC-TGG-AA	56.6	793
Urokinase (uPA)	NM_002658.3	Forward Reverse	GCC-ATC-CCG-GAC-TAT-ACA-GA AGG-CCA-TTC-TCT-TCC-TGG-GT	59.7	417
Reference genes					
B2-microglobulin	NM_004048.2	Forward Reverse	CTC-ACG-TCA-TCC-AGC-AGA-GA CGG-CAG-GCA-TAC-TCA-TCT-TT	60	213
B-actin	NM_001101.3	Forward Reverse	AAA-TCT-GGC-ACC-ACA-CCT-TC AGA-GGC-GTA-CAG-GGA-TAG-CA	60	185

4.3.6 Immunofluorescent stainings

In order to evaluate whether the expression of angiogenic factors was affected by culture conditions, immunofluorescent stainings were performed on three different series of dental tissues. All stainings were performed as described earlier in chapter 2 (2.3.8). The applied primary and secondary antibodies are listed in table 4.3. Before mounting the tissue sections, all stained tissues were incubated with 0.1% Sudan Black (Sigma-Aldrich) in 70% ethanol for 10 min in the dark in order to minimize autofluorescence. Pictures were taken with a Nikon Eclipse 80i fluorescent microscope equipped with a Nikon DS-2MBWc digital camera.

Table 4.3 Primary antibodies and matched secondary antibodies for immunofluorescent stainings

Antibody	Type	Dilution	Manufacturer
Primary antibodies			
CD29	Mouse monoclonal IgG1	1:50	Abcam, Cambridge, UK
CD146	Rabbit Polyclonal IgG	1:50	Abcam
VEGF	Mouse monoclonal IgG_{2B}	1:50	R&D Systems Minneapolis, USA
ANGPT1	Rabbit Polycloncal IgG	1:100	R&D Systems
IGFBP3	Mouse monoclonal IgG_{2B}	1:50	R&D Systems
Secondary antibodies			
Alexa fluor 555 donkey anti-mouse	Donkey IgG	1:500	Invitrogen
Alexa Fluor 488 donkey anti-rabbit	Donkey IgG	1:500	Invitrogen

4.3.7 Cell proliferation assay

The ability of DSCs and HGF-1 to promote endothelial proliferation was evaluated by means of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HMEC-1 were seeded at 15,625 cells/cm² in a 96-well plate in standard endothelial culture medium. After 24h, the cells were rinsed with PBS and incubated with conditioned medium of DSCs and HGF-1, standard

culture medium supplemented with 0.1% FBS (negative control condition) or standard DSC culture medium supplemented with 10% FBS (positive control condition). Experimental conditions were all performed in triplicate. Following an incubation period of 72h, a MTT assay was performed as described in chapter 2 (2.3.3). This assay was repeated four independent times on a total of at least nine patient samples of each stem cell population.

4.3.8 Transwell migration assay

In order to assess the potential influence of DSCs and HGF-1 on endothelial migration, a transwell migration assay was performed. Both DSCs and HGF-1 (passages 2-4) were seeded at 50,000 cells/cm² in a 24-well plate in standard culture medium. After 24h, the cells were rinsed with PBS and incubated with standard DSC culture medium supplemented with 0.1% FBS in order to allow the secretion of angiogenic factors. Following another culture period of 24h, tissue culture inserts (ThinCert™, 8 µm pore size, Greiner Bio-One, Frickenhausen, Germany) were seeded with HMEC-1 at 150,000 cells/cm² in standard DSC culture medium containing 0.1% FBS and placed above the cells. Standard DSC culture medium containing 10% FBS and 0.1% FBS was used as a positive and negative control in the wells beneath. Following 24h of incubation, migrated HMEC-1 were fixed with 4% paraformaldehyde (PFA) at room temperature and stained with 0.1% Crystal Violet (Sigma-Aldrich) in 10% ethanol. Per insert, two representative pictures were taken with an inverted phase-contrast microscope (Nikon Eclipse TS100) equipped with a ProgRes® C3 digital microscope camera (Jenoptik AG, Jena, Germany). The amount of migration (expressed as mean area percentage) was quantified using AxioVision software 4.6.3 (Carl Zeiss Vision, Aalen, Germany). This assay was independently performed on at least six different patient samples of each stem cell population.

4.3.9 Wound healing assay

The potential impact of DSCs and HGF-1 on the directional migration of HMEC-1 was evaluated by means of a wound healing assay. HMEC-1 were seeded at 11,600 cells/compartment in a culture insert (Ibidi GmbH, Planegg/Martinsried, Germany). Following 24h of culturing, the insert was removed, leaving a linear

opening between two monolayers of endothelial cells. The endothelial cells were incubated with DSCs and HGF-1 culture medium for 24h. Standard culture medium containing 0.1% FBS and 10% FBS was used as a negative and positive control, respectively. To eliminate potential bias from cellular proliferation, 4µg/ml Mitomycin C (Sigma-Aldrich) was added to each condition. After 24h, the cells were fixed with 4% PFA at room temperature and stained for 10 min with 0.1% Crystal Violet (Sigma-Aldrich) in 10% ethanol. Of each experimental condition, two representative pictures were taken with an inverted phase-contrast microscope (Nikon Eclipse TS100) equipped with a ProgRes® C3 digital microscope camera (Jenoptik AG). The number of migrated cells within the linear opening was quantified using Image J cell counter software. This assay was performed eight independent times on different donor samples of each stem cell population.

4.3.10 Tube formation assay

In order to evaluate the effect of DSCs and HGF-1 on endothelial tubulogenesis, a tube formation assay was performed. The inner wells of an Angiogenesis µ-slide (Ibidi) were coated with growth factor-reduced BD Matrigel™ Basement Membrane Matrix (BD Biosciences, Franklin Lakes, USA). After the matrix had set, the outer wells of the µ-slide were seeded with a cell suspension containing 5,000 HMEC-1 and conditioned medium of DSCs or HGF-1. Standard DSC culture medium containing 10% FBS and 0.1% FBS was used as a positive and negative control, respectively. The cultures were maintained for 24h at 37°C in a humidified atmosphere containing 5% CO2. The next day, two representative pictures per well were taken with an inverted phase-contrast microscope (Nikon Eclipse TS100) equipped with a ProgRes® C3 digital microscope camera (Jenoptik AG). Per picture, five blood vessels were measured starting from the middle of each branching point using Image J Software. This assay was performed five independent times on samples of five different donors.

4.3.11 Chorioallantoic membrane assay

A chorioallantoic membrane (CAM) assay was performed in order to examine the angiogenic behavior of DSCs and HGF-1 in an *in vivo* setting. Fertilized white leghorn chicken eggs (*Gallus gallus*) were incubated for three days at 37 °C in a humidified atmosphere. After three days (E3), 3–4 ml albumen was removed from the eggs in order to detach the developing CAM from the egg shell. A small opening was made in the shell, which was covered afterwards with cellophane tape before the eggs were returned to the incubator. Six days later (E9), the CAM was incubated with Matrigel™ droplets (BD Biosciences) containing 50,000 DSCs (passages 3–4) or HGF-1. Pure Matrigel™ droplets were applied as a negative control. Following three days of incubation (E12), the eggs were opened and the CAM was carefully dissected out of the eggs to assess angiogenesis. Pictures were taken with a stereomicroscope (Wild M3Z Stereomicroscope, Heerbrugg, Switzerland) equipped with a Nikon digital net camera DN100. In order to quantify angiogenesis, two concentric circles (radii 1.5 mm and 2 mm) were drawn and intersecting blood vessels were counted independently two times in double-blind fashion. This assay was performed four independent times on samples of four different donors, leading to a total of at least 26 eggs per experimental condition.

4.3.12 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 Software (GraphPad Software, La Jolla, CA, USA). Data distribution was assessed by means of a D'Agostino & Pearson normality test. In case of a normal distribution, experimental groups were compared using a one way ANOVA with a Dunnett's multiple comparison post-hoc test, a Tukey-Kramer multiple comparison post-hoc test or a Newman-Keuls post-hoc test. Non-parametric data were compared with a Kruskall-Wallis test combined with a Dunns post-hoc test. Differences were considered to be statistically significant at P-values ≤ 0.05. All data were expressed as mean ± standard deviation (SD). * = P-value < 0.05; ** = P-value < 0.01; *** = P-value < 0.001.

4.4 Results

4.4.1 The angiogenic expression profile of DSCs and HGF-1

The first part of this study focused on the identification of the angiogenic factors expressed by DSCs and HGF-1. In a first stage, a general screening of conditioned medium was performed using an antibody array, which indicated the expression of a wide variety of pro-angiogenic (green) as well as anti-angiogenic (red) proteins by both DSCs and HGF-1 (Fig. 4.1A). Following normalization against positive and negative reference spots, dot blot analysis pointed out the relative expression of angiogenic factors by all (stem) cell populations (Fig. 4.1B). Proteins which displayed a relatively low expression pattern or which showed no pronounced differences in expression levels between the different cell populations were validated at mRNA level by means of RT-PCR. Analysis indicated variable expression levels of urokinase (uPA), endothelin-1 (EDN1), dipeptidyl peptidase IV (DPPIV), angiogenin (ANG), plasminogen activator inhibitor 1 (PAI-1), thrombospondin-1 (THBS1), tissue inhibitor of matrix metalloproteinase 1 and 4 (TIMP1/4), pentraxin-3 (PTX3) and pigment epithelium-derived factor (PEGF), not only between the different (stem) cell populations but also between donor samples of the same stem cell population (Fig. 4.2).

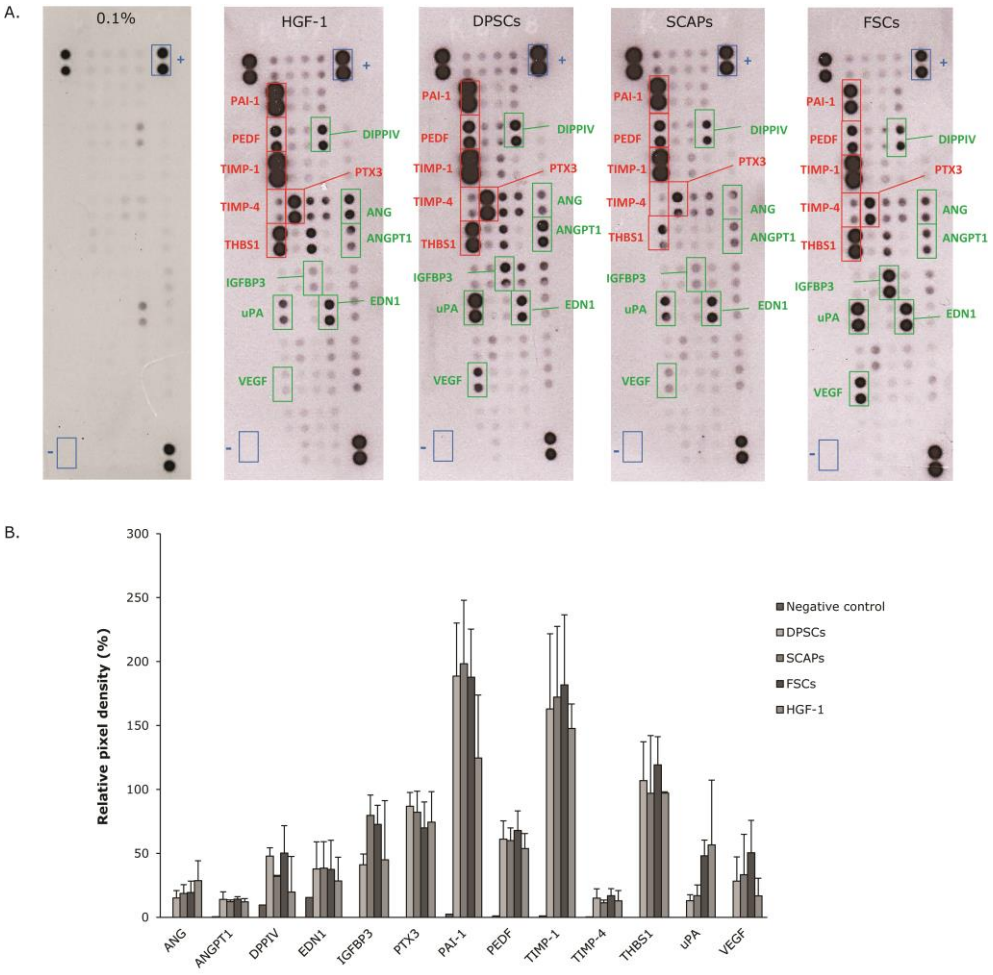


Figure 4.1: Angiogenic secretion profile of dental stem cells and human gingival fibroblasts. A. Detection of protein spots on the array membrane. Pro-angiogenic factors (green) as well as anti-angiogenic factors (red) were differentially secreted in the conditioned medium of dental pulp stem cells (DPSCs), stem cells from the apical papilla (SCAPS), dental follicle precursor cells (FSCs) and human gingival fibroblasts (HGF-1). Culture medium containing 0.1% FBS was used as a negative control condition. Expression levels were normalized against positive and negative reference spots (blue). B. Relative levels of protein secretion by the different cell populations. This assay was performed three times on matched samples of three different donors. Data are expressed as mean ± SD.

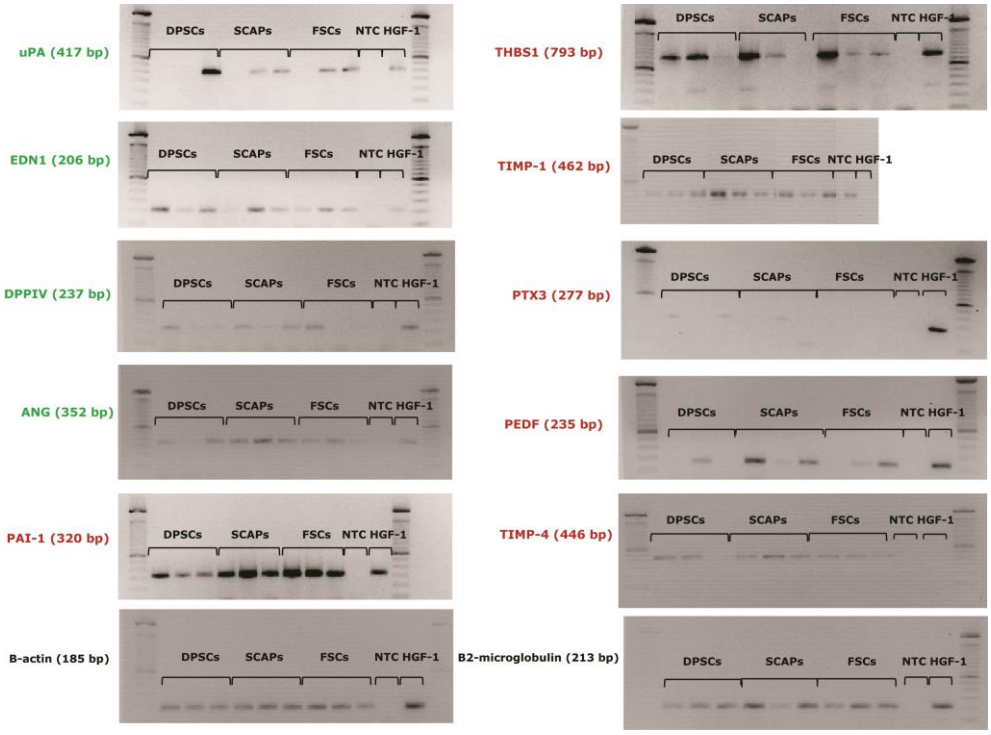


Figure 4.2: Expression of angiogenic factors at mRNA level. RT-PCR indicated variable levels of gene expression of pro-angiogenic (green) and anti-angiogenic (red) factors between the studied cell populations as well as between different patient samples. Pro-angiogenic factors: urokinase (uPA), endothelin-1 (EDN1), dipeptidyl peptidase IV (DPPIV), angiogenin (ANG). Anti-angiogenic factors: plasminogen activator inhibitor 1 (PAI-1), thrombospondin-1 (THBS1), tissue inhibitor of matrix metalloproteinase 1 and 4 (TIMP1/4), pentraxin-3 (PTX3), pigment epithelium-derived factor (PEGF). Reference genes (black) were expressed by DSCs and HGF-1. This assay was performed on at least six different patient samples of each stem cell population. Three representative samples are shown.

Angiogenic factors of which the antibody array indicated a trend towards differential expression (Fig. 4.1A), namely ANGPT1, IGFBP3 and VEGF, were validated through ELISA in order to obtain a clear view of the range of the secreted concentrations (Fig. 4.3). Analysis revealed no significant differences between the different cell populations in terms of ANGPT1 expression levels. With regard to the expression of IGFBP3, FSCs secreted a significantly higher concentration compared to DPSCs and SCAPs. DPSCs, on the other hand,

demonstrated notably higher levels of VEGF secretion in comparison to the other cell populations (Fig. 4.3).

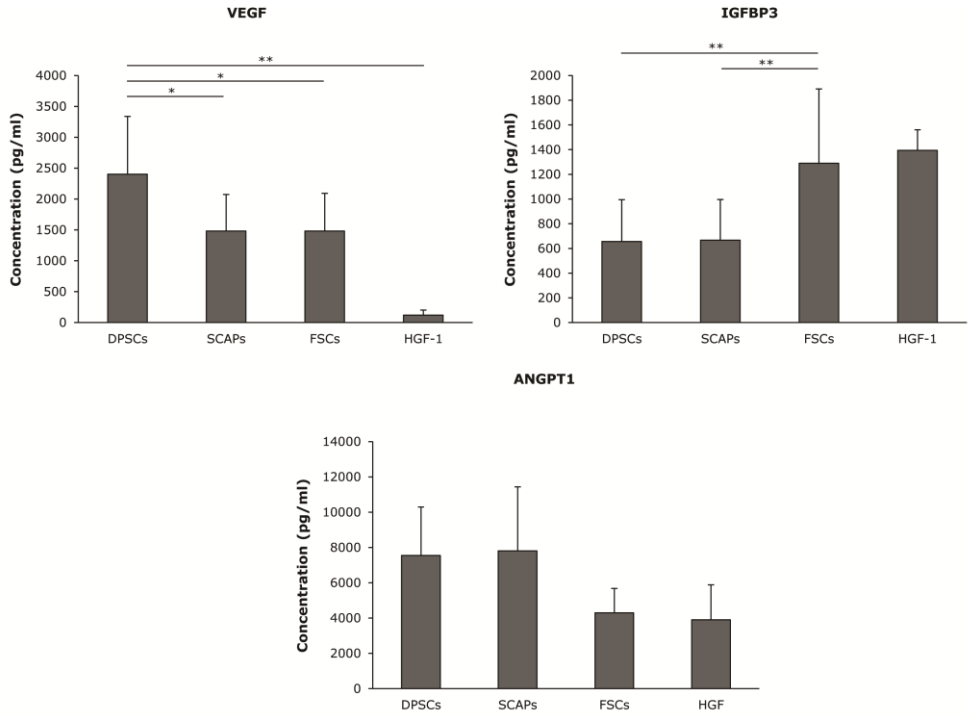
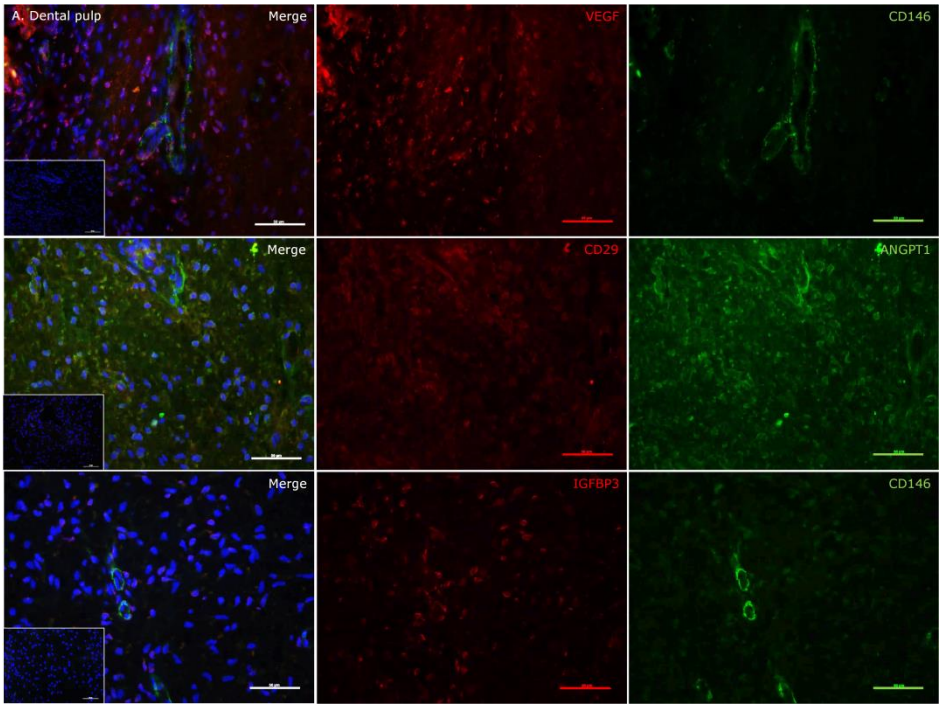
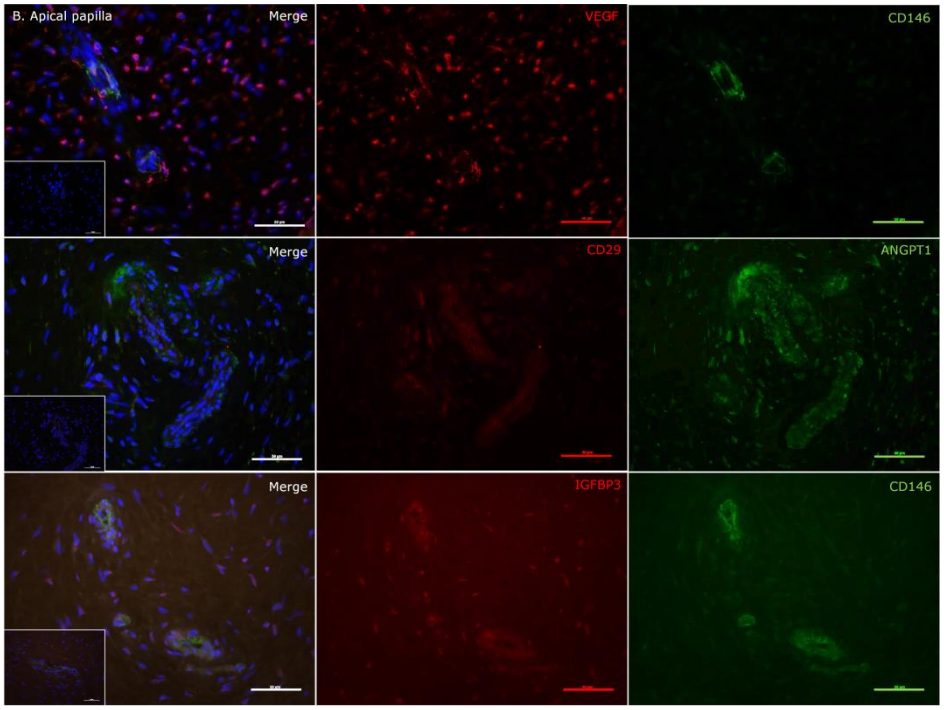


Figure 4.3: Expression of angiogenic factors at protein level. ELISA indicated the differential expression of vascular endothelial growth factor (VEGF), insuline-like growth factor binding protein 3 (IGFB3) and angiopoietin 1 (ANGPT1) by the studied cell populations. DPSCs showed a significantly higher expression of VEGF, while FSCs expressed a notably higher level of IGFBP3. There were no significant differences with regard to ANGPT1 expression. ELISAs were performed on conditioned medium of at least six different patient samples of all three stem cell populations. Data are expressed as mean ± SD. * = p-value < 0.05; ** = p-value < 0.01.

As the expression of angiogenic factors such as VEGF can be induced by culture conditions [158], an immunofluorescent staining was carried out in order to evaluate the *in situ* expression of this protein as well as ANGPT1 and IGFBP3 in dental tissues. Dental pulp (Panel A, Fig. 4.4), as well as apical papilla (Panel B, Fig. 4.4) and dental follicle (Panel C, Fig. 4.4) showed positive expression of VEGF throughout the whole tissue. Co-expression with CD146 was mainly apparent in perivascular regions. Similarly, IGBP3 positive cells were present throughout all dental tissues while co-expression with CD146 was only observed around blood vessels. All dental tissues showed an overall expression of ANGPT1, while the expression of CD29 was restricted to certain cells.




B. Apical papilla
Merge
VEGF
CD146
Merge
CD29
ANGPT1
Merge
IGFBP3
CD146

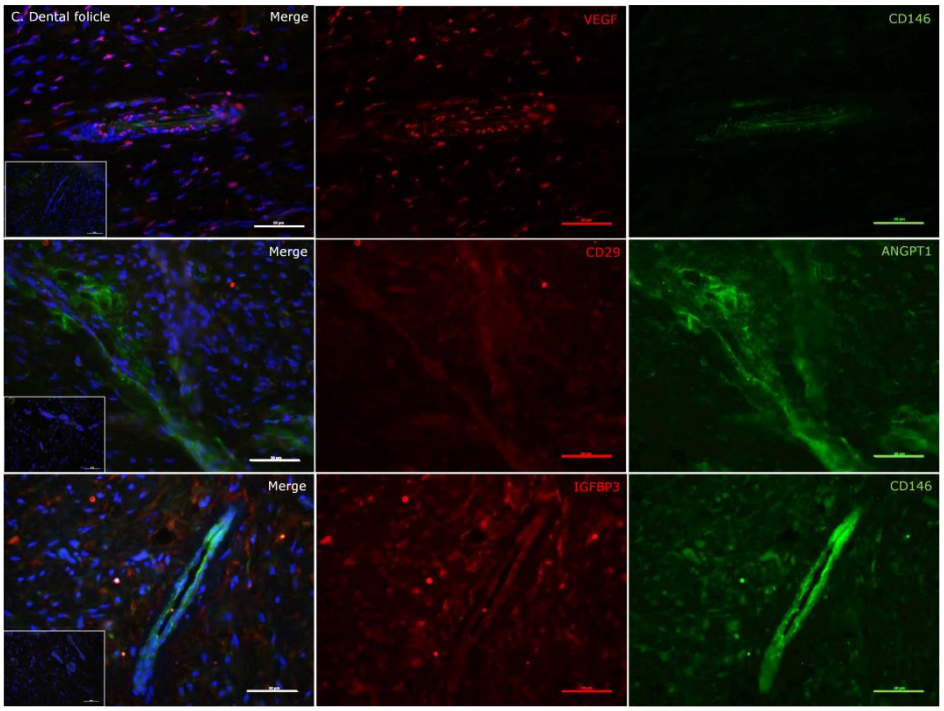

C. Dental folicle
Merge
VEGF
CD146
Merge
CD29
ANGPT1
Merge
IGFBP3
CD146

Figure 4.4: in situ expression of angiogenic proteins in dental tissues. A. Dental pulp: $VEGF^+CD146^+$; $CD29^+ANGPT1^+$; $IGFBP3^+CD146^+$. B. Apical papilla: $VEGF^+CD146^+$; $CD29^+ANGPT1^+$; $IGFBP3^+CD146^+$. C. Dental follicle: $VEGF^+CD146^+$; $CD29^+ANGPT1^+$; $IGFBP3^+CD146^+$. Insets: negative control. Scale bars: 50 µm.

4.4.2 Functional analysis of the angiogenic potential of DSCs and HGF-1 *in vitro*

As angiogenesis is a well-orchestrated biological reaction, involving a myriad of regulating factors, it is not only important to determine which of these factors are secreted by DSCs but also to assess the potential influence of DSCs on the behavior of endothelial cells. Therefore, multiple *in vitro* assays were performed with HMEC-1, mimicking the different steps of the angiogenic process. One of the first events within the angiogenic cascade comprises the proliferation of endothelial cells. Hence, in a first stage the proliferation-stimulating capacity of DSCs was tested by means of a MTT assay. After incubating HMEC-1 with conditioned medium for 72h, no apparent difference in proliferation was detected compared to the control conditions (Fig. 4.5).

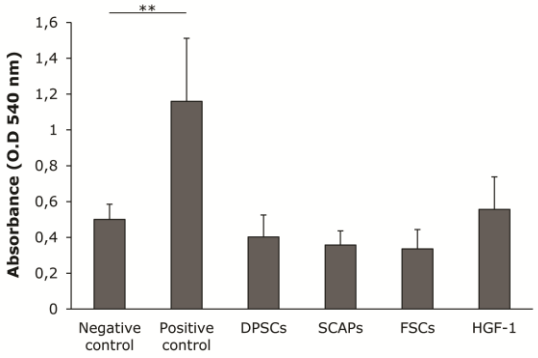


Figure 4.5: Influence of dental stem cells on endothelial proliferation. Endothelial proliferation following 72 h of incubation with conditioned medium from dental pulp stem cells (DPSCs), stem cells of the apical papilla (SCAPs), dental follicle precursor cells (FSCs) and human gingival fibroblasts (HGF-1). Negative control: culture medium containing 0.1% FBS. Positive control: culture medium containing 10% FBS. The conditioned medium of DSCs as well as HGF-1 was not able to increase endothelial proliferation, as compared to the positive control medium. This assay was repeated four independent times on at least nine different patient samples. Data are expressed as mean ± SD. ** = p-value < 0.01.

During angiogenesis, endothelial cells also respond to chemotactic stimuli in order to migrate towards the site which requires vascular supply [25]. Accordingly, the chemotactic potential of DSCs was evaluated using a transwell migration system, which involved 24h incubation of HMEC-1 (seeded in a culture insert) with DSCs and HGF-1 (seeded in the wells beneath) in order to induce transmigration (Fig. 4.6A). In particular DPSCs, SCAPs and HGF-1 caused a significant increase in endothelial cell migration (Fig. 4.6B).

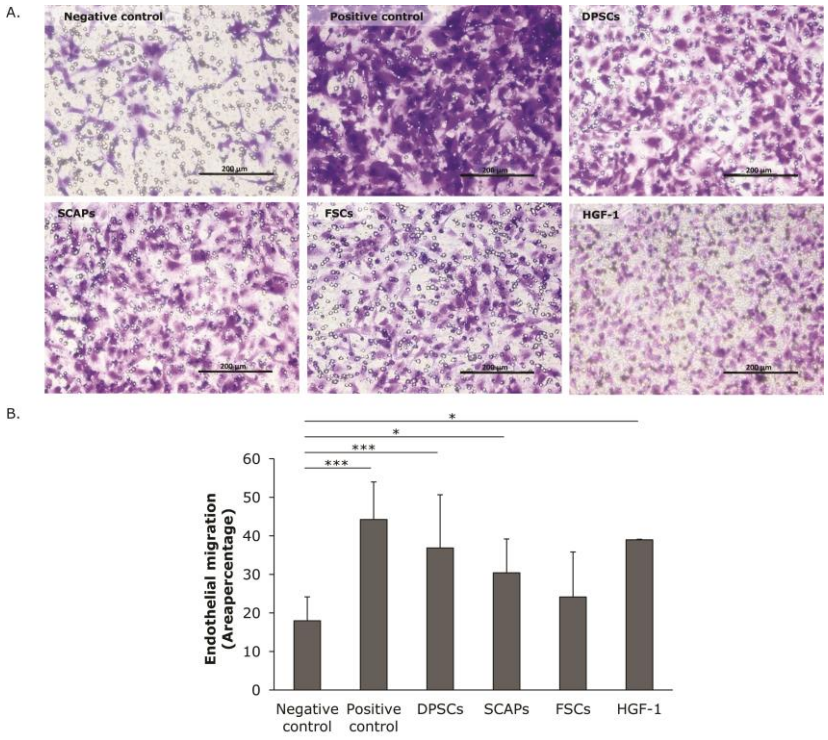


Figure 4.6: The impact of dental stem cells on endothelial migration. A. Representative endothelial transmigration following 24 h of incubation with dental pulp stem cells (DPSCs), stem cells of the apical papilla (SCAPs), dental follicle precursor cells (FSCs) and human gingival fibroblasts (HGF-1). Negative control: culture medium containing 0.1% FBS. Positive control: culture medium containing 10% FBS. Scale bars = 200 µm. B. The graph shows the mean area percentage of endothelial migration. DPSCs, SCAPs and HGFs significantly increased endothelial migration. This assay was independently performed on at least six different patient samples of each stem cell population. Data are expressed as mean ± SD.* = p-value < 0.05; *** = p-value < 0.0001.

Another way of assessing endothelial migration is directional migration, which plays an important role in wound healing in particular [362]. Therefore, a wound healing assay was performed, allowing the migration of endothelial cells within a linear opening between two monolayers of cells (Fig. 4.7A). Following 24h incubation of HMEC-1 with conditioned medium of DSCs and HGF-1, statistical analysis showed no significant change in the number of migrated endothelial cells (Fig. 4.7B).

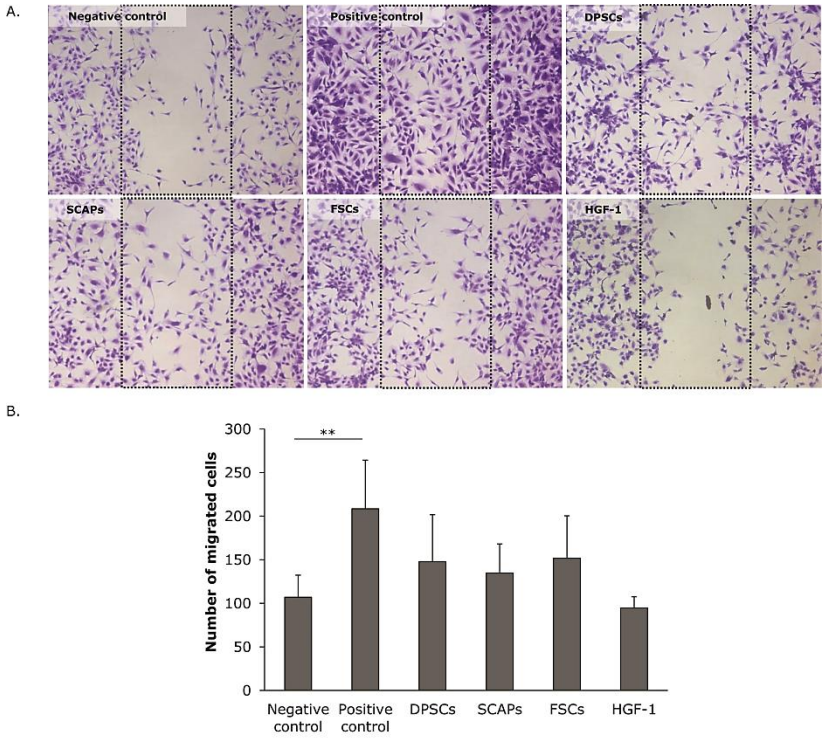


Figure 4.7: The influence of dental stem cells on directional migration of endothelial cells. A. Representative endothelial migration following 24h of incubation with conditioned medium of dental pulp stem cells (DPSCs), stem cells of the apical papilla (SCAPs), dental follicle precursor cells (FSCs) and human gingival fibroblasts (HGF-1). Negative control: culture medium containing 0.1% FBS. Positive control: culture medium containing 10% FBS. Delineated area = migration area. B. The graph shows the average number of migrated cells within the delineated area. This assay was performed eight independent times on different donor samples of each stem cell population. Data are express as mean ± SD. ** = p-value < 0.01.

With regard to tubulogenesis, a Matrigel™ assay was applied to examine the effect of DSCs on endothelial tube formation. Following 24h of incubation, the conditioned medium of DPSCs in particular induced a pronounced increase in endothelial tube length compared to the negative control situation (Fig. 4.8A). Furthermore, analysis indicated a significantly higher impact of DPSC conditioned medium compared to SCAPs and HGF-1, respectively (Fig. 4.8B).

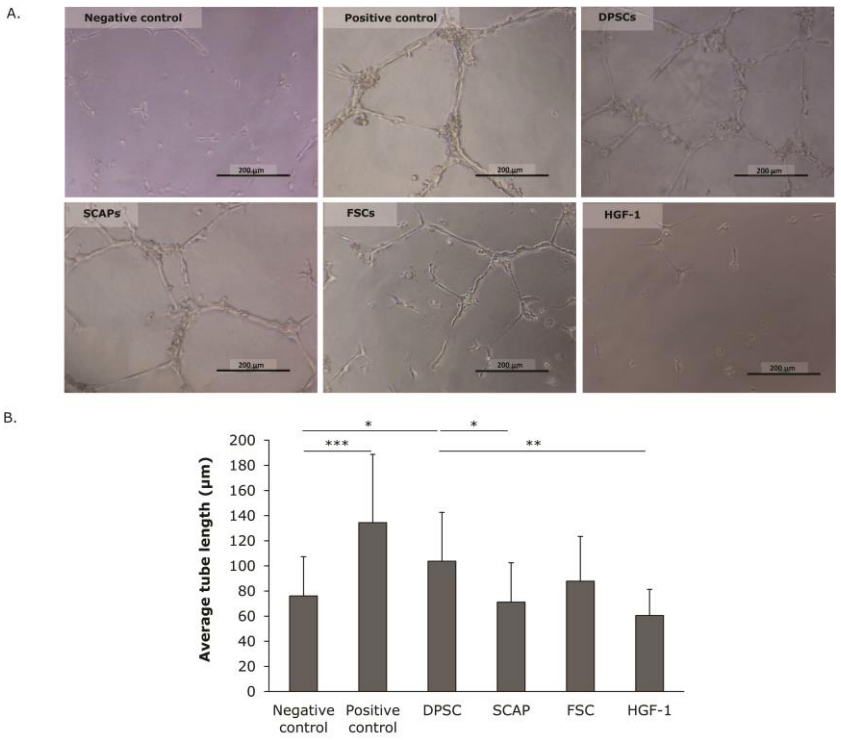
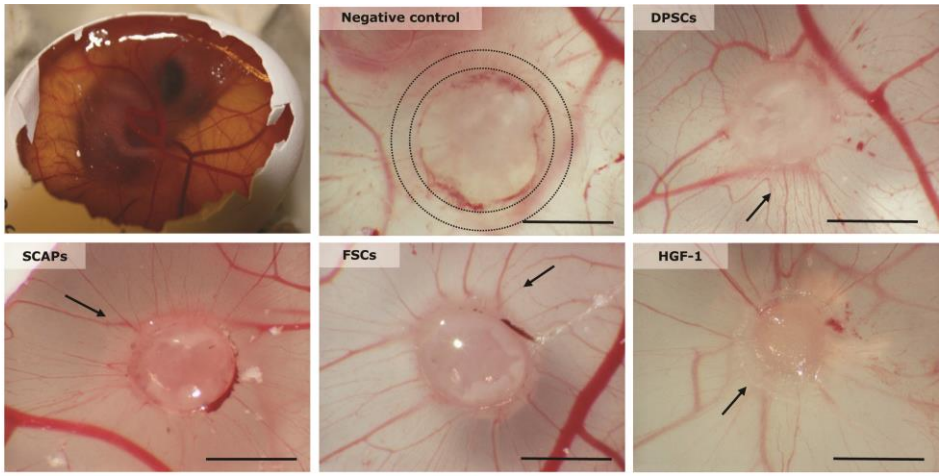


Figure 4.8: Impact of dental stem cells on endothelial tubulogenesis. A. Representative endothelial tube formation following 24h of incubation with conditioned medium of dental pulp stem cells (DPSCs), stem cells from the apical papilla (SCAPs), dental follicle precursor cells (FSCs) and human gingival fibroblasts (HGF-1). Negative control: culture medium containing 0.1% FBS. Positive control: culture medium containing 10% FBS. Scale bars = 200 µm. B. The graph shows the average endothelial tube length (µm). DPSC conditioned medium significantly increased endothelial tube formation. DPSCs significantly differed from SCAPs and HGF-1 regarding their impact on tubulogenesis. This assay was performed five independent times on matched samples of 5 different donors. Data are expressed as mean ± SD.* = p-value < 0.05; ** = p-value < 0.01; *** = p-value < 0.0001.

4.2.3 Angiogenesis *in vivo*

To confirm whether DSCs could sustain their paracrine effects in an *in vivo* setting, a CAM assay was performed. After 3 days of incubation, a characteristic spoke wheel pattern was observed in all test samples, as the capillaries grew radially towards the different cell populations (Fig. 4.9A). Representative pictures were taken and two concentric circles were drawn to allow quantification of intersecting blood vessels in a double-blind fashion. Compared to the control condition, DPSCs and SCAPs caused a significant increase in blood vessel count, while this was not the case for FSCs and HGF-1 (Fig. 4.9B).

A.



B.

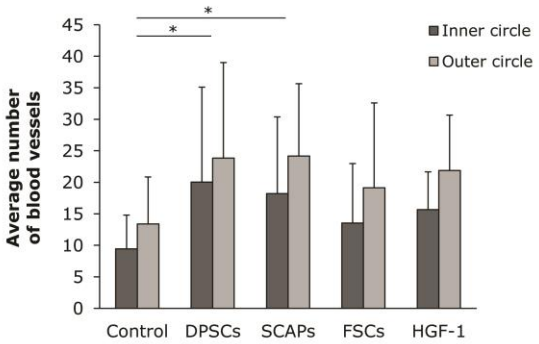


Figure 4.9: Angiogenic properties of dental stem cells in a chorioallantoic membrane assay. A. Representative vascularization of the chorioallantoic membrane (CAM) following 3 days of incubation with growth factor-reduced Matrigel™ containing dental pulp stem cells (DPSCs), stem cells of the apical papilla (SCAPs), dental follicle precursor cells (FSCs) and human gingival fibroblasts (HGF-1). Matrigel™ alone was used as a negative control condition. A characteristic spoke wheel pattern was observed in all test conditions (arrows). Scale bars = 2 mm. B. Average number of blood vessels. DPSCs and SCAPs significantly increased the number of capillaries intersecting both circles. This assay was performed four independent times on matched samples of four different donors, leading to a total of at least 26 eggs per experimental condition. Data are expressed as mean ± SD. * = p-value < 0.05.

4.5 Discussion

Within the human body, three mechanisms of blood vessel formation can be distinguished, namely vasculogenesis, arteriogenesis and angiogenesis. In particular angiogenesis, i.e. the sprouting of new capillaries from pre-existing blood vessels, is the most predominant mechanism in the adult body [25]. However, over a decade ago several studies suggested the existence of circulating progenitor cells that could support endothelial regeneration and blood vessel formation [363, 364]. Currently, a vast number of (stem) cell types has emerged as a potential treatment option in vascular medicine, with each their own identity and characteristics [168]. Although transplanted cells, such as BM-MSCs, are supposed to contribute to therapeutic angiogenesis either through secretion of pro-angiogenic factors or by differentiating into endothelial cells, the exact mechanisms of action often remain subject of controversy [166-168]. As the rate of stromal cell engraftment has proven to be extremely low in most *in vivo* settings, it is currently assumed that the therapeutic benefits of these cells are primarily caused by their paracrine (angiogenic) actions [152, 169-172]. Therefore, the main focus of this study was to compare the paracrine angiogenic potential of postnatal DSCs, in particular DPSCs, SCAPs and FSCs, together with a HGF-1 cell line as an additional population from the oral environment with potential angiogenic properties.

In a first part of this study, the presence of angiogenic proteins in the conditioned medium of DSCs and HGF-1 was determined by means of an antibody array. This general screening demonstrated the differential secretion of multiple angiogenesis-related factors by the studied cell populations. In-depth expression analysis at mRNA level indicated the expression of angiogenesis-stimulating (uPA, EDN1, DPPIV and ANG) as well as angiogenesis-inhibiting (PAI-1, THBS1, TIMP1/4, PTX3 and PEGF) factors by DSCs and HGF-1. A subset of these factors, namely uPA, EDN1 and THBS1, were previously reported in primary rat and human cultures of gingival fibroblasts [356, 365, 366]. A number of studies also indicated the (inflammation-induced) expression of other angiogenic proteins by primary HGF-1, such as MCP-1, bFGF, ANGPT2, CXCL12 and CXCR4, some of which were also minimally detected in our antibody array [357, 366, 367]. At the protein level, ELISA showed the secretion of ANGPT1,

IGFB3 and VEGF by DSCs and HGF-1. ANGPT1 primarily plays a role in tubulogenesis and vessel stabilization, while IGFB3 is thought to have a dual function; i.e. stimulation of angiogenesis on the one hand by promoting endothelial motility and inhibition of angiogenesis on the other hand by downregulating pro-angiogenic factors such as VEGF and bFGF [33, 35, 368]. VEGF is a well-known protein which serves many functions within the angiogenic cascade, such as the regulation of vessel permeability, the stimulation of endothelial proliferation and migration, and the downregulation of endothelial apoptosis [32]. No significant differences were demonstrated between the different cell populations in terms of ANGPT1 expression. However, VEGF was shown to be secreted at substantially high levels by DPSCs, as previously reported by others [176, 177]. Earlier studies also mentioned the inflammation-induced expression of VEGF by primary cultures of HGF-1 [358, 367]. In accordance with a previous report of Götz *et al.* which highlighted the expression of the IGF system in human permanent teeth, IGBP3 was secreted by all DSCs populations, though at significantly higher concentrations by FSCs [369].

With regard to the functional analysis of DSCs and HGF-1, several *in vitro* tests were performed with endothelial cells (HMEC-1) to assess their influence on the different steps of the angiogenic process. Despite the expression of multiple mitogens such as VEGF, EDN1, DPPIV and ANG, DSCs were not able to increase the proliferation of HMEC-1. A potential explanation for this discrepancy could be the concentration of the secreted proliferation-stimulating factors, which was presumably too low to cause a sustainable effect. Another explanation may lie in the expression of proliferation-inhibiting factors, such as PTX3 and THBS1; the actions of these proteins could be predominant in comparison to the angiogenesis-promoting factors and tip the angiogenic balance towards an inhibitory state. In contrast, Iohara *et al.*, demonstrated a significant increase of human umbilical vein endothelial cell (HUVECs) proliferation following incubation with conditioned medium of porcine pulp-derived $CD31^-$ $CD146^-$ stem cells [331]. However, besides considering the potential species-related differences between porcine and human DPSCs, it also has to be kept in mind that the $CD31^-$ $CD146^-$ subset of DPSCs could potentially display more pronounced angiogenic properties than DPSCs in general [175]. In comparison, studies

regarding the proliferation-stimulating capacity of BM-MSCs yielded similar conflicting results. While Potapova *et al*. and others mentioned a significant increase in HUVECs proliferation caused by BM-MSCs, Gruber *et al.*, demonstrated no proliferation-promoting effect of BM-MSC conditioned medium [157, 158, 162, 370]. However, the constitution of the applied conditioned medium appears to play an important role, as FBS itself contains several growth factors and it has the potential to increase the expression of proliferation-stimulating factors such as VEGF, which can bias the outcome of the experiment [158]. For that reason, the conditioned medium in this study only contained 0.1% FBS in order to avoid artificial upregulation of VEGF or other mitogens.

During angiogenesis, endothelial cells migrate along a gradient of chemotactic proteins [25]. Since the expression analysis demonstrated that DSCs and HGF-1 express multiple factors which are known to affect migration, such as ANGPT1, EDN1, IGFBP3, uPA and VEGF, a transwell migration assay was carried out to assess their chemotactic potential. Following 24h of incubation, DPSCs, SCAPs and HGF-1 significantly increased endothelial transmigration, while FSCs had no substantial impact. Given the high secretion of IGFBP3 by FSCs, the lack of a pronounced migration-stimulating effect was rather unexpected. However, the aforementioned dual role of IGFBP3 taken together with the lower secretion of VEGF and ANGPT1 probably established suboptimal conditions for endothelial migration. HGF-1 on the other hand, display a secretion profile similar to FSCs in terms of VEGF, IGFBP3 and ANGPT1 and do significantly enhance endothelial migration. This discrepancy may be explained by the potential contribution of other (yet to be identified) angiogenic factors which influence endothelial migration. With regard to the chemotactic properties of BM-MSCs, similar observations were made by Potapova *et al.* and others, who mentioned a significant increase in HUVECs transmigration caused by stromal cell-conditioned medium [157, 158].

Another important aspect of endothelial migration is directional migration, which plays a key role during wound healing *in vivo*. In order to test the potential impact of DSCs and HGF-1 on the directional migration of HMEC-1, a wound healing assay was performed with conditioned medium. Following 24h of incubation, analysis indicated no notable effect by both DSCs and HGF-1. In

contrast, conditioned medium from BM-MSCs was shown to promote endothelial migration in a so-called scratch test [371, 372]. A potential explanation for this discrepancy could be the presence of Mitomycin C, which was added to eliminate potential bias from endothelial proliferation. Besides its anti-mitotic effect this toxin also exerts anti-angiogenic effects through the induction of VEGF111b, an isoform of VEGF, which inhibits endothelial proliferation, migration and tube formation [373, 374].

In terms of tubulogenesis, functional assays showed a pronounced effect of DPSCs on endothelial tube formation, an outcome which also differed significantly from SCAPs and HGF-1. Earlier studies of Tran-Hung *et al*. and others, reported a similar increase and stabilization of endothelial tubular structures following direct co-culture of HUVECs and DPSCs, indicating a more pericyte-like behavior of DPSCs [177, 375, 376]. Human and murine BM-MSCs on the other hand, are also capable of promoting endothelial tube formation, as was shown by a number of studies [152, 156, 377, 378] . Since ANGPT1 and VEGF play an important role in the induction of tubulogenesis, the aforementioned increase can probably be explained by the angiogenic secretion profile of the different cell populations as DPSCs displayed a notably higher VEGF secretion compared to SCAPs, FSCs and HGF-1.

In the final part of this study, a CAM assay was conducted in order to determine the angiogenic properties of DSCs in an *in vivo* setting. According to earlier reports of Laschke *et al.*, the CAM assay is an ideal model to study vascular development due to its capability to support the ingrowth of blood vessels and its lack of a complete immune system which allows for the assessment of xenografts without rejection [379]. Following incubation with DSCs as well as HGF-1, a characteristic spoke wheel pattern could be distinguished caused by the radial ingrowth of blood vessels [380]. In particular DPSCs and SCAPs significantly enhanced neoangiogenesis. In comparison, BM-MSCs were also found to promote blood vessel ingrowth in a CAM assay, notwithstanding the altered model the authors applied [157, 381]. Despite the expression of several angiogenesis-inhibiting factors such as TIMP, PAI-1, THBS1 and PTX3, these data collectively suggest a predominant pro-angiogenic impact of DSCs, and in particular DPSCs and SCAPs, *in vitro* and *in vivo.*

This study was the first to describe the angiogenic properties of SCAPs and FSCs in an *in vitro* and *in vivo* setting. Furthermore, it compares three different dental stem cells populations, namely DPSCs, SCAPs and FSCs, together with a HGF-1 cell line with regard to their angiogenic expression profile and impact on endothelial cell behavior *in vitro* and *in vivo*. DSCs seemed to have a predominant pro-angiogenic impact on endothelial migration and tube formation, *in vitro* as well as in an *in vivo* set-up. Our results suggest a stronger angiogenic profile and function of DPSCs and SCAPs in comparison to FSCs and HGF-1, encouraging further investigation of both these stem cell populations as therapeutic tools in a wide variety of disorders caused by limited angiogenesis, such as chronic wounds, stroke and myocardial infarction. Moreover, the dental field could also significantly benefit from the angiogenic properties of DSCs, in particular in pulp regeneration and whole tooth engineering, as vascular supply is an important burden to overcome in these applications.

Chapter 5

The regenerative potential of DPSCs and SCAPs in an *in vivo* model of dental pulp regeneration

5.1 Abstract

Within the field of dental tissue engineering, the establishment of adequate tissue vascularization is one of the most important burdens to overcome. As vascular access within the tooth is restricted by the apical foramen, it is of major importance to implement effective vascularization strategies in order to recreate viable components of teeth and periodontal tissues. This study aimed to elucidate whether dental pulp stem cells (DPSCs) and stem cells from the apical papilla (SCAPs) are able to sustain their previously demonstrated angiogenic properties in an *in vivo* model of dental pulp regeneration. DPSCs and SCAPs were suspended in a self-assembling peptide hydrogel and injected in 3D-printed, conical hydroxyapatite (HA) scaffolds, which were subcutaneously transplanted into immunocompromised mice. Following 12 weeks of transplantation, histological and ultrastructural analysis pointed out the formation of vascularized, pulp-like tissue as well as osteodentin in all constructs containing stem cells. However, despite the detection of substantial levels of vascular endothelial growth factor (VEGF) *in vitro*, stem cell constructs did not contain a significantly higher amount of blood vessels in comparison to the control constructs. Although this study was the first to report the formation of vascularized, pulp-like tissue in standardized 3D-printed HA scaffolds containing DPSCs and/or SCAPs, these results suggest the potential osteogenic/odontogenic differentiation of the stem cells rather than the promotion of angiogenesis in this setting and time frame.

5.2 Introduction

Dental pulp tissue is a highly innervated and vascularized soft connective tissue which, while encased in dentin, appears to be vulnerable to external insults such as trauma, chemical irritation or microbial infection [294]. The treatment of permanent, immature necrotic teeth in particular remains to be a challenge, as any factor that interferes with normal pulp physiology may conflict with the completion of root development [190, 191]. Although current regenerative endodontic procedures (REP), involving root canal disinfection followed by the induction of a blood clot in order to promote the release of growth factors and to attract residing stromal cells, have yielded favorable results, the associated technical and biological limitations prompt the need for alternative treatment options [191, 194]. Next to the nature of the regenerated tissue, proper vascularization of the tooth is an often recurring concern in cell homing-based approaches as vascular supply is limited by the apical foramen [210, 224, 382, 383]. However, a recent study of Iohara *et al.* indicated the regeneration of pulp tissue in canals with only 0.7 mm apices when using a dental stem cell-based method [216, 217]. A more pronounced volume of regenerated dental pulp tissue with a higher capillary density was also found in comparison to a cell homing-based approach [218]. As these data are in accordance with data from our group, indicating pro-angiogenic properties of DPSCs and SCAPs, this study aims to elucidate the sustained angiogenic properties of DPSCs and SCAPs in an *in vivo* model of dental pulp generation.

Although the ectopic root transplantation model, involving the use of emptied human root canals, has been widely applied, the size and shape of root canals is prone to variability [228, 229, 384, 385]. Three-dimensional (3D)-printing or additive manufacturing, i.e. the inkjet-based production of 3D-structures by printing liquid binders onto loose powders, on the other hand, provides inorganic scaffolds with a custom-made design, controllable chemistry and porosity. As 3D-printing is a high-throughput fabrication process which allows the use of a wide variety of biomaterials, such as bioactive glasses, polylactic acid (PLA), polyethylene glycol (PEG) or hydroxyapatite (HA), it has a great clinical potential in the field of organ and tissue replacement [386, 387]. Mannoor *et al.*, for example, generated an anatomically correct bionic ear via 3D-printing of a cell-

seeded hydrogel matrix [388]. Furthermore, Khalyfa *et al.* reported the production of craniofacial segments based on calcium phosphate powders [389]. Given the broad availability of biomaterials and the ability to produce custom-made scaffolds according to the patient's needs, 3D-printing also shows great promise in the field of regenerative dentistry [390]. As the biocompatibility and effectiveness of HA have already been demonstrated *in vivo*, this study applies HA conical scaffolds in order to minimize scaffold-related variability and mimic the tooth morphology. DPSCs and SCAPs will be supported with a commercially available self-assembling peptide hydrogel, which has already been used in models of pulp regeneration and is proven to support DPSC viability [229, 293, 384]. Scaffolds are characterized by means of scanning electron microscopy (SEM) and VEGF secretion. Following 12 weeks of transplantation into SCID mice, the tissue content and blood vessel density will be analyzed by means of histological stainings and electron microscopy.

5.3 Materials and Methods

5.3.1 Cell culture

DSCs, i.e. DPSCs and SCAPs, were isolated according to the explant method described in chapter 2 (2.3.1), 3 (3.3.1) and 4 (4.3.1). In order to minimize patient-related variability and the amount of animals required for the ectopic transplantation model, DPSCs and SCAPs (passage 1) of three different donors were pooled and maintained in Minimal essential medium, alpha modification (αMEM, Sigma-Aldrich, St. Louis, USA) supplemented with 2 mM L-glutamine (Sigma-Aldrich) 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma-Aldrich) and 10% FBS (BiochromAG, Berlin, Germany) (further referred to as standard DSC culture medium). The pooled stem cells were cryopreserved in liquid nitrogen for further experiments. All experiments were performed with thawed, passage 2-3 DPSCs and SCAPs.

5.3.2 Scaffold production and characterization

In order to mimic the size and shape of a tooth root, 3D-printed conical HA scaffolds with a standardized length of 4.9 mm and a representative 'apical' opening of 1.7 mm were used in this study (Fig. 5.1) [210, 214, 215]. All scaffolds were custom-made by Sirris (Seraing, Belgium). Briefly, additive manufacturing was used in order to create the scaffolds in a layer by layer fashion from a paste containing HA/TCP powder, resins and a UV photoinitiator. Polymerization of each layer was achieved by means of UV light, after which the scaffolds are cleaned. All scaffolds were thermally treated in order to remove any organic material. Finally, the scaffolds were sintered to increase their density. In order to assess the surface characteristics of the scaffolds, SEM analysis was performed.

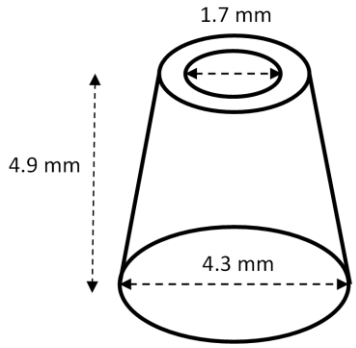


Figure 5.1: Dimensions of 3D-printed hydroxyapatite conical scaffolds. Height: 4.9 mm; Base diameter: 4.3 mm and opening diameter: 1.7 mm.

5.3.3 VEGF secretion

To evaluate whether DSCs were able to survive and maintain their VEGF secretion within the scaffolds and the supporting hydrogel, an ELISA was performed on conditioned medium of scaffolds containing 50,000 DPSCs. As the viability and survival of DPSCs is maintained in 0.2% Puramatrix™ (BD, Franklin Lakes, NJ, USA) according to Cavalcanti *et al.*, different 3D-printed hydroxyapatite conical scaffolds were injected with a 1:1 solution of phosphate-buffered saline (PBS) and a suspension of 50,000 pooled DPSCs (passage 3) in 0.4% Puramatrix™ [293]. The constructs were maintained in standard DSC culture medium for four weeks. Following two and four weeks of culturing, the constructs were incubated with standard DSC culture medium containing 0.1% FBS for 48h in order to obtain conditioned medium. A VEGF ELISA (Raybiotech, Norcross, GA, USA) was performed on the conditioned medium according to the manufacturer's instructions.

5.3.4 Ectopic transplantation model

In order to evaluate the regenerative potential of DPSCs and SCAPs, the aforementioned constructs were transplanted into severe combined immunodeficient (SCID) mice as described before [228]. Constructs containing pure hydrogel, 25,000 pooled DPSCs, 25,000 pooled SCAPs or a 1:1 mixture of 25,000 pooled DPSCs and pooled SCAPs were subcutaneously transplanted into the dorsum of 32 8-week-old, female SCID Hairless Outbred (SHO™) mice

(Crl:SHO-Prkdc^{scid}Hr^{h}, Charles River, Wilmington, MA, USA). Following 12 weeks of transplantation, the scaffolds were removed from the mice and processed for light microscopy and ultrastructural analysis. Before sectioning, pictures were taken with a stereomicroscope (Wild M3Z Stereomicroscope, Heerbrugg, Switzerland) equipped with a Nikon digital net camera DN100 in order to analyze macroscopic signs of blood vessel ingrowth.

5.3.5 Histological stainings

Samples (24 out of 32 resected transplants, 6 per experimental condition) were fixed in 4% paraformaldehyde (PFA) for 48h at room temperature. Following fixation, the base of the conical scaffolds was removed with a surgical saw, after which the resulting cylinders were dehydrated in graded ethanol and embedded in paraffin. After a decalcification period of 6-24h in decalcifying solution (Thermo Scientific™ Richard-Allan Scientific™, Waltham, MA, USA), paraffin was melted again in order to allow impregnation of the tissue. When the paraffin was set, serial sections of 7 µm were made. Sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome, after which they were scanned with a Mirax digital slide scanner (Carl Zeiss Vision, Aalen, Germany). Blood vessels were quantified in a double-blinded fashion by means of Panoramic Viewer software (3DHISTECH Ltd., Budapest, Hungary). Area calculations were also performed with the aforementioned software.

5.3.6 Ultrastructural analysis

Samples (8 out of 32 resected transplants, 2 per experimental condition) were fixed with 2% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.3) at 4°C. Following fixation, the base of the conical scaffolds was removed with a surgical saw, after which the resulting cylinders were treated with 2% osmium tetroxide in 0.05 M sodium cacodylate buffer (pH 7.3) for 1h at 4°C in order to achieve post-fixation. After a staining period of 20 min with 2% uranyl acetate in 10% acetone, the samples were dehydrated with ascending concentrations of acetone. The dehydrated samples were then impregnated overnight in a 1:1 mixture of acetone and araldite epoxy resin at room temperature. Following impregnation, the samples were embedded in araldite epoxy resin at 60°C after

which they were decalcified in decalcifying solution (Thermo Scientific™ Richard-Allan Scientific™) for a period of 6-24h. The decalcified samples were refilled with araldite and cut into semi-thin (0.5 µm) and thin sections (70 nm) with a Leica EM UC6 microtome (Leica, Wetzal, Germany). Semi-thin tissue sections were stained with thionine methylene blue and visualized by means of a Nikon Eclipse 80i microscope equipped with a DS-5 M digital camera. Thin sections were transferred to 0.7% formvar-coated copper grids (Aurion, Wageningen, The Netherlands) and contrasted using a Leica EM AC20 (Leica) with 0.5% uranyl acetate and a stabilized solution of lead citrate. Transmission electron microscopic (TEM) analysis was performed with a Philips EM208 S electron microscope (Philips, Eindhoven, The Netherlands). Digital images were obtained with a Morada Soft Imaging System camera and the corresponding iTEM-FEI software (Olympus SIS, Münster, Germany).

5.3.7 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 Software (GraphPad Software, La Jolla, CA, USA). Data distribution was assessed by means of a D'Agostino & Pearson normality test. As $n < 8$ in each group, data distribution was considered to be non-parametric. Data were compared with a Kruskall-Wallis test combined with a Dunns post-hoc test. Differences were considered to be statistically significant at P-values ≤ 0.05. All data were expressed as mean ± standard deviation (s.d.). * = P-value < 0.05; ** = P-value < 0.01; *** = P-value < 0.001.

5.4 Results

5.4.1 Characterization of 3D-constructs

The first part of the study focused on the characterization of the 3D-printed conical HA scaffolds and the analysis of DPSCs behavior within the constructs. Scanning electron microscopy (SEM) was applied to obtain a clear overview of the scaffolds' structure and surface characteristics (Fig. 5.2). Next to clear layers of deposited HA (arrow, Fig. 5.2B), a pseudo-porous surface could also be distinguished (arrows, Fig. 5.2C). However, in-depth analysis of the scaffolds indicated rather dense HA layers, without distinct pores (Fig. 5.2D).

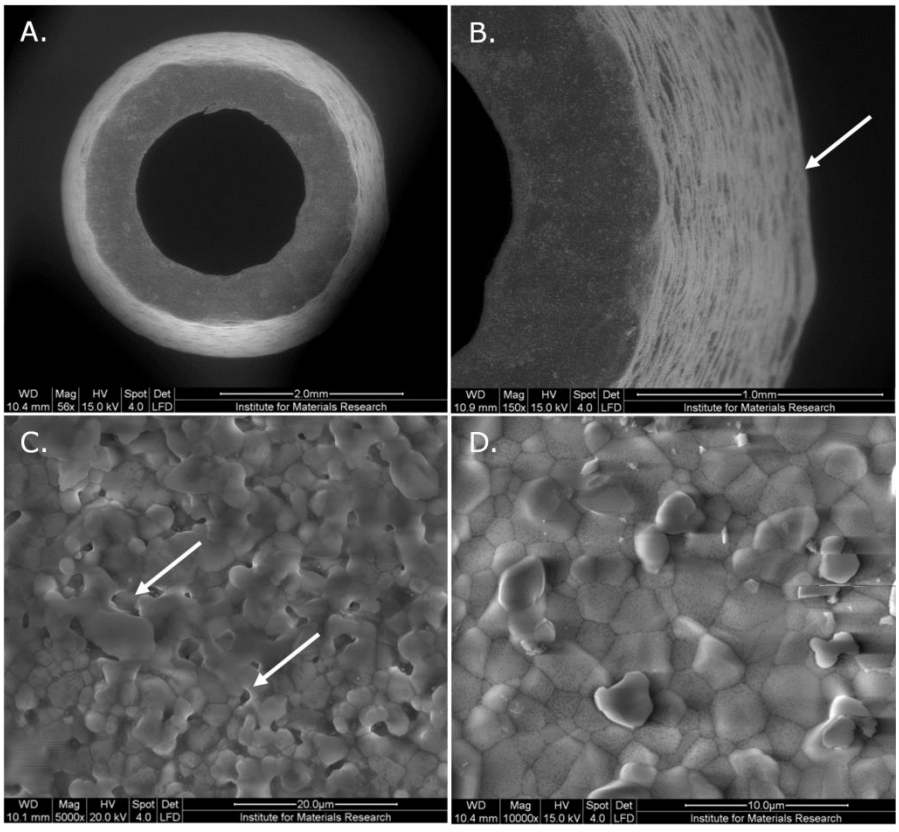


Figure 5.2: SEM characterization of 3D-printed hydroxyapatite scaffolds. A. Overview. Scale bar = 2.0 mm. B. Clear layers of hydroxyapatite (arrow). Scale bar = 1.0 mm. C. Pseudo-porous hydroxyapatite surface (arrows). Scale bar = 20 µm. D. Dense hydroxyapatite layer. Scale bar = 10 µm.

Besides scaffold characterization, the behavior of DPSCs within the 3D-constructs, i.e. a Puramatrix™ cell suspension within the HA scaffold, also needed to be evaluated. The survival and the angiogenic properties of the stem cells within the constructs was monitored by means of a VEGF ELISA. Following two weeks of culturing, the conditioned medium of the constructs clearly contained a substantial amount of VEGF. The secretion of this angiogenic factor increased even further after four weeks of culturing (Fig. 5.3).

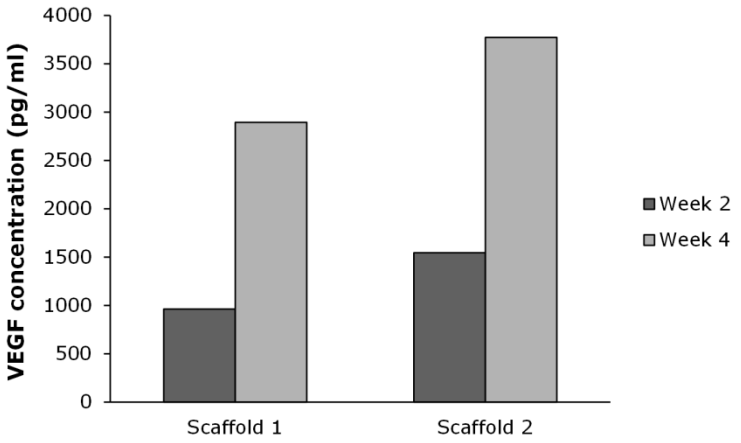


Figure 5.3: VEGF secretion of dental pulp stem cells in 3D-constructs. Dental pulp stem cells suspended in a final concentration of 0.2% Puramatrix in two 3D-printed hydroxyapatite scaffolds showed a clear secretion of VEGF following two weeks of culturing. This concentration increased after four weeks into culture. This assay was performed one time on two different scaffolds containing pooled DPSCs.

5.4.2 Macroscopic analysis of implanted 3D-constructs

Following 12 weeks of implantation in SCID mice, the 3D-constructs were removed and pictures were taken to allow macroscopic characterization. Although blood vessel ingrowth was present in most samples, clear differences were not only apparent between experimental groups, i.e. controls (Fig. 5.4.A, B), DPSCs (Fig. 5.4C, D), SCAPs (Fig. 5.4E, F) and DPSCs + SCAPs (Fig. 5.4 G, H), but also within samples of the same condition.

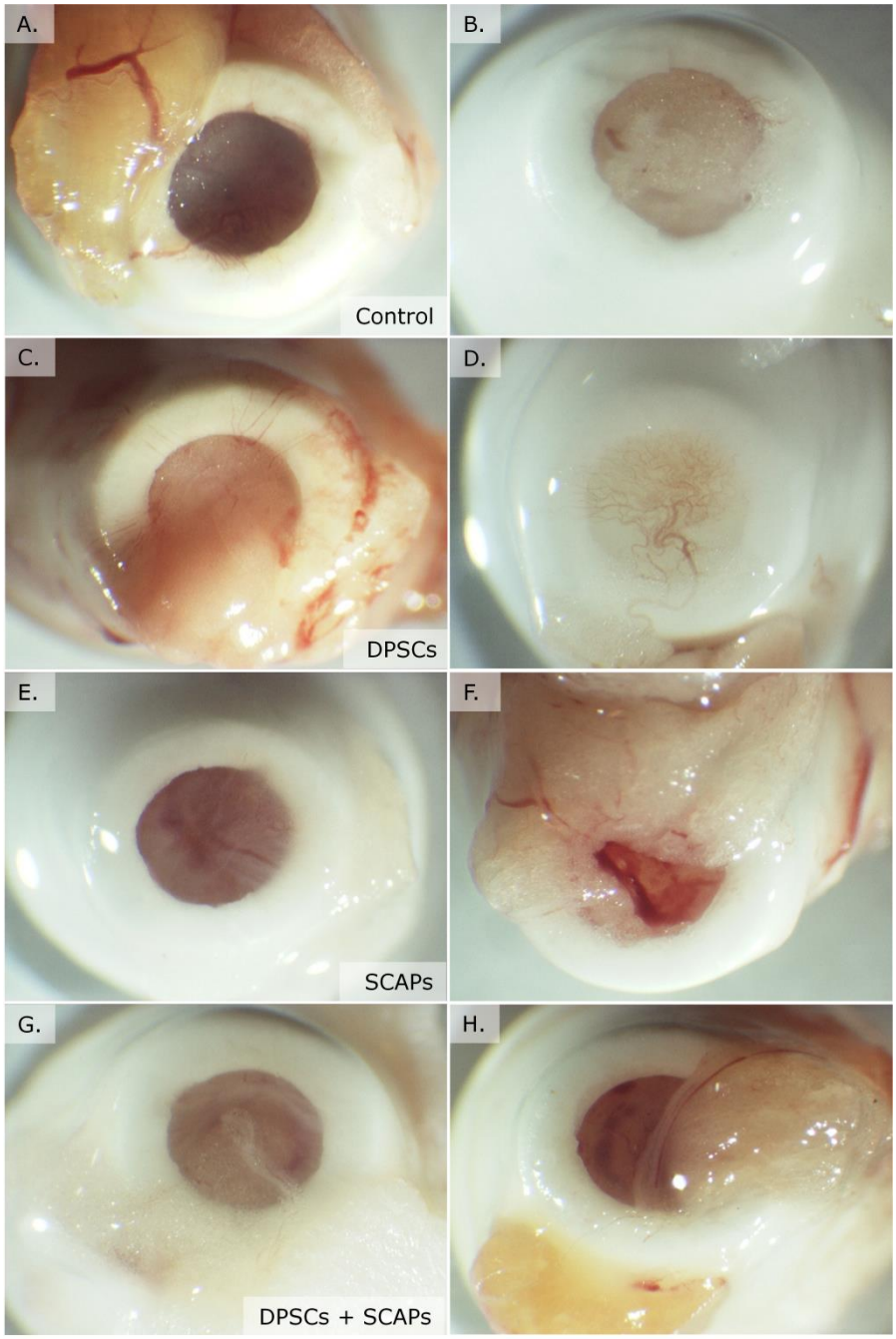


Figure 5.4: Macroscopic view of conical scaffolds following 12 weeks of transplantation. A,B. Control condition. C, D. Dental pulp stem cells (DPSCs). E, F. Stem cells from the apical papilla (SCAPs). G, H. DPSCs + SCAPs. n = 8/ experimental condition.

5.4.3 Histological characterization of 3D-constructs

Of each experimental condition, six samples were embedded in paraffin for in-depth histological analysis. As certain constructs were broken after transplantation or cut in the wrong orientation, they were not suitable for further processing or analysis and thus excluded from the study. Of the remaining constructs, 1 out of 4 in the DPSCs group and 2 out of 6 in the SCAPs group contained less than 10% tissue and were considered to be empty. This led to the following sample sizes: control = 3; DPSCs = 3; SCAPs = 4; DPSCs + SCAPs = 4 (Fig. 5.5).

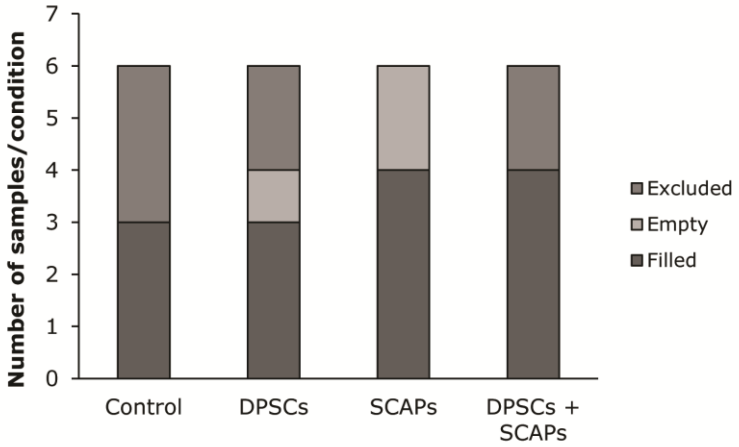


Figure 5.5: Sample distribution in each experimental condition. Excluded = certain samples were excluded from the study due to technical difficulties with sample processing. Empty = Empty 3D-construct were excluded from further quantification and analysis. Filled = Control: 3; DPSCs = 3; SCAPs: 4; DPSCs + SCAPs: 4.

Following a Masson's trichrome staining in order to characterize the connective tissue content of the filled constructs, microscopical analysis indicated the formation of a vascularized, pulp-like tissue in all the constructs containing stem cells (Fig. 5.6H, K, N). Although the regenerated tissue had a structure which was similar to actual pulp tissue (Fig. 5.6A-C), it clearly contained less collagen (Fig. 5.6H, K, N). The control samples, on the other hand, also comprised vascularized, fibrous tissue, albeit a lower percentage and with a different organizational structure (Fig. 5.6D-F). While the outer border of the tissue within the stem cell constructs showed strongly organized, concentric layers of collagen

and, in certain cases, osteodentin formation (Fig. 5.6I, L, O + insets), only one out of three control samples showed loosely arranged, concentric layers of collagen (Fig. 5.6F + inset).

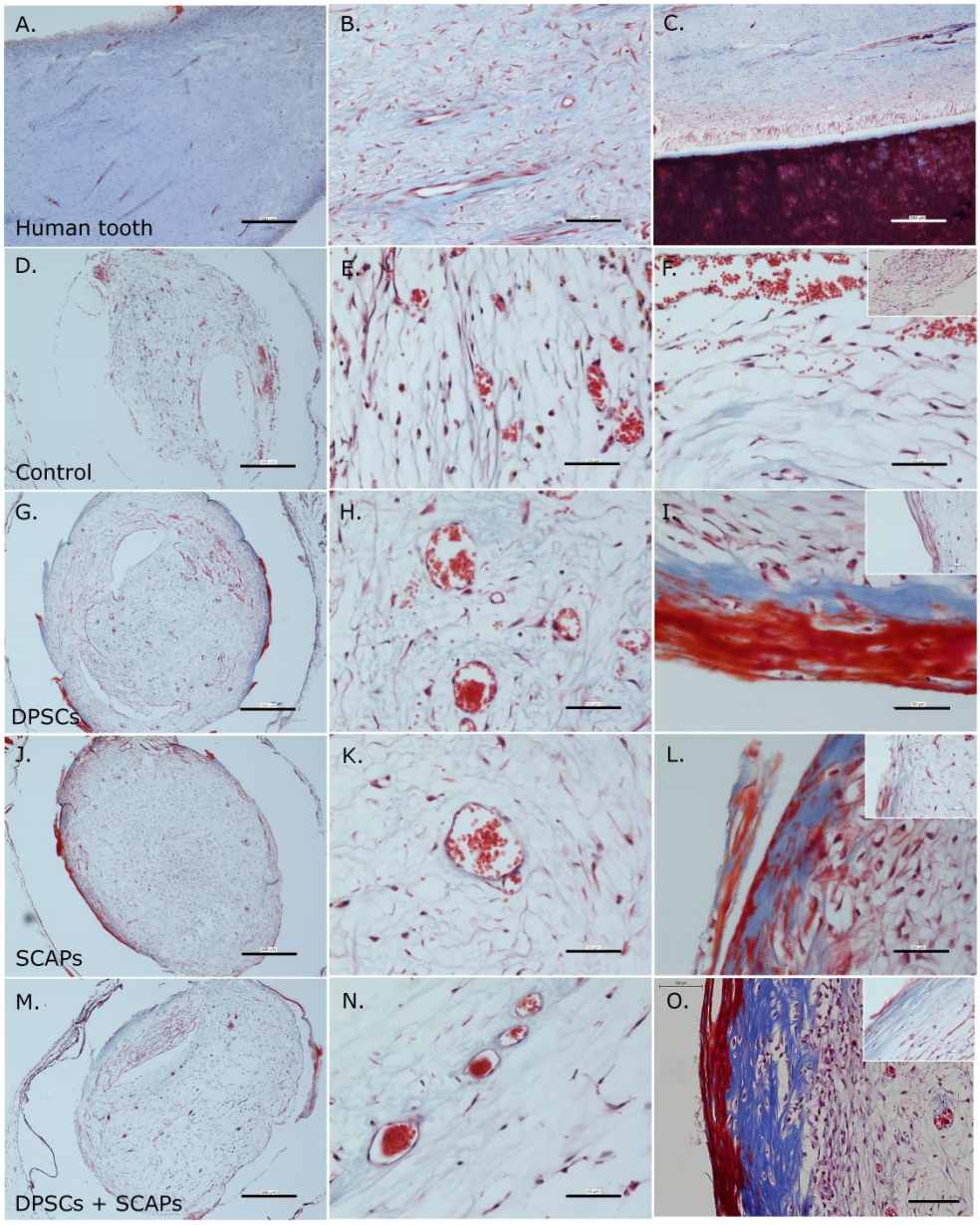


Figure 5.6: Histological characterization of 3D-constructs. A-C. Human tooth. D-F. Control. G-I. DPSCs. J-L. SCAPs. M-O. DPSCs + SCAPs. In all stem cell constructs, vascularized pulp-like tissue was formed (H, K, N), which had a notably lower collagen content in comparison to the human tooth (A-C). Vascularized fibrous tissue was also found within the control constructs (E), although the filling rate and organizational structure was different (D, F) as the outer border of the tissue within the stem cell constructs contained strongly organized, concentric layers of collagen and osteodentin (I, L, O + insets). Scale bars = 500 µm (D, G, J, M); 200 µm (A, C); 100 µm (inset F, O); 50 µm (B, E, F, H, I, K, L, N). Analysis was conducted on constructs with at least 10% tissue content. Control = 3; DPSCs = 3; SCAPs = 4; DPSCs + SCAPs = 4.

In order to obtain a clear view of the differential filling rate of the scaffolds as well as their vascular supply, a H&E staining was performed. The following analyses only included constructs that contained more than 10% tissue. As already mentioned, initial microscopical examination indicated a lower amount of tissue within the control condition (Fig. 5.7A) in comparison to the stem cell conditions (Fig. 5.7D, G, J). This observation was confirmed statistically, pointing out a significantly lower percentage of tissue in the control condition compared to the scaffolds containing stem cells, in particular SCAPs (Fig. 5.7M). With regard to blood vessel content, macroscopic inspection of the scaffolds already indicated signs of blood vessel ingrowth. Further microscopical analysis demonstrated the presence of small as well as larger (sometimes ruptured) blood vessels in all experimental conditions (Fig. 5.7, pictures). While containing a significantly lower amount of tissue, the control condition in particular seemed to contain a significantly high amount of blood vessels (Fig. 5.7N).

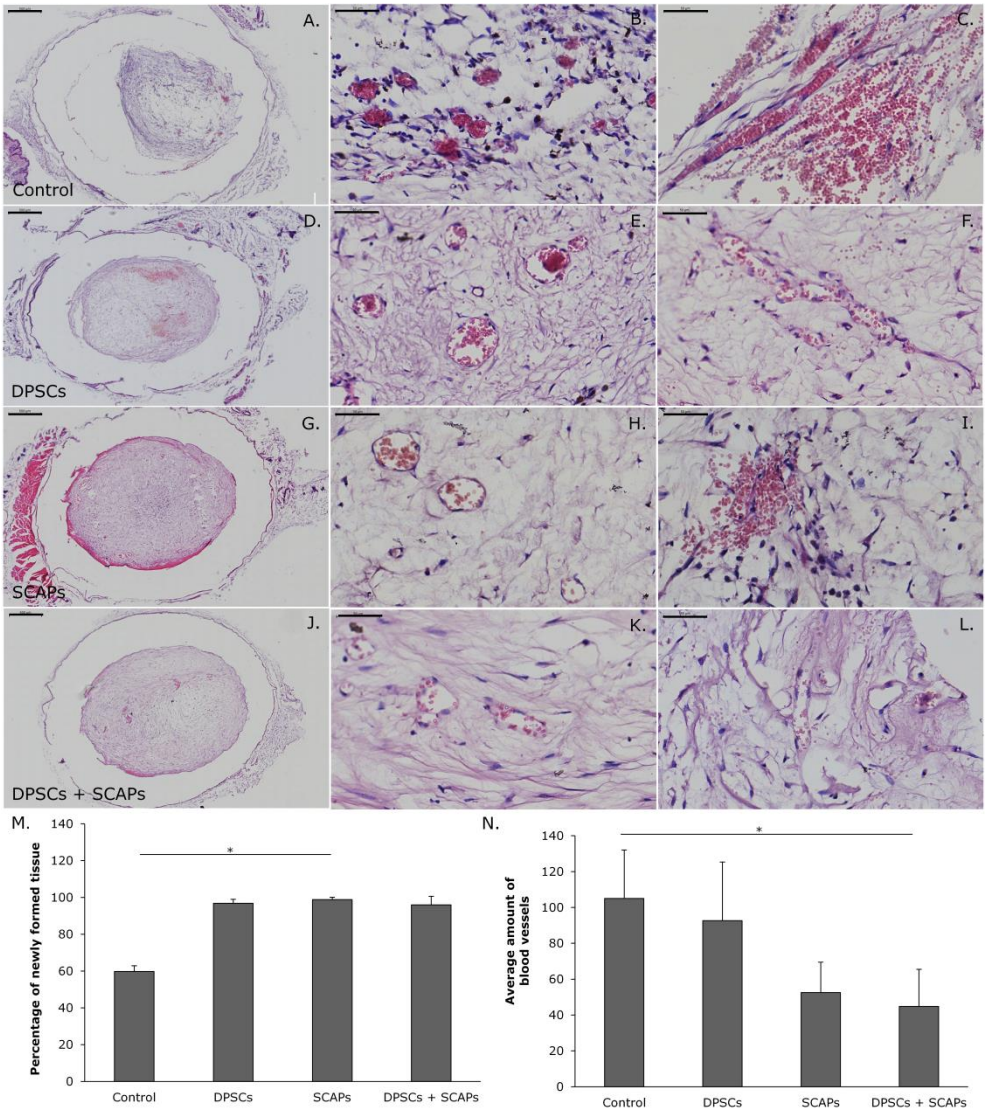


Figure 5.7: Tissue and blood vessel content of 3D-constructs. A-C. Control (n = 3). D-F. DPSCs (n = 3). G-I. SCAPs (n = 4). J-L. DPSCs + SCAPs (n = 4). All experimental conditions contained blood vessels of variable shape and size. Scale bars = 500 µm (A, D, G, J); 50 µm (B, C, E, F, H, I, K, L). M. Percentage of tissue within 3D-constructs. Control constructs contained a significantly lower amount of tissue in comparison to SCAPs constructs. N. Average amount of blood vessels. Control constructs contain a significantly higher amount of blood vessels in comparison to DPSCs + SCAPs constructs.

5.4.4 Semi-thin tissue sections and ultrastructural analysis of 3D-constructs

Of each experimental condition, two samples were processed as (semi-)thin tissue sections for in-depth ultrastructural analysis. Following thionine methylene blue staining of the semi-thin sections, microscopical analysis indicated one control construct to be empty (Fig. 5.8A, inset). The second control construct, on the other hand, was partly filled with loosely arranged fibrous tissue containing numerous blood vessels (Fig. 5.8A, E). While both scaffolds containing DPSCs were entirely filled with connective tissue, the organization of the tissue markedly differed. One construct comprised a more densely organized tissue (Fig. 5.8B inset, F), whereas the other one contained loosely arranged fibrous tissue (Fig. 5.8B, F inset). Similar observations were made in the scaffolds harboring SCAPs, although the constructs were only partly filled with tissue (Fig. 5.8C, G). Active collagen secretion was also apparent in one sample (Fig. 5.8G). The constructs containing DPSCs as well as SCAPs were characterized by densely organized connective tissue containing blood vessels and distinct patches of presumably mineralized tissue (Fig. 5.8D, H). The latter was also apparent in the DPSCs constructs, with a clear deposition at the border of the newly formed tissue against the HA scaffold (Fig. 5.8J-L). The cells within the tissue were mostly linearly arranged against this mineralized tissue (Fig. 5.8J), although in certain regions cellular protrusions were visible within this (osteo)dentin-like tissue (arrows, Fig. 5.8K, L). Nerve tissue containing myelinated nerve fibers was also detected in the tissue surrounding the outer rim of both control constructs (Fig. 5.8I). This was also the case in the all other experimental conditions, although surrounding nerve tissue could only be detected in one out of two constructs containing DPSCs.

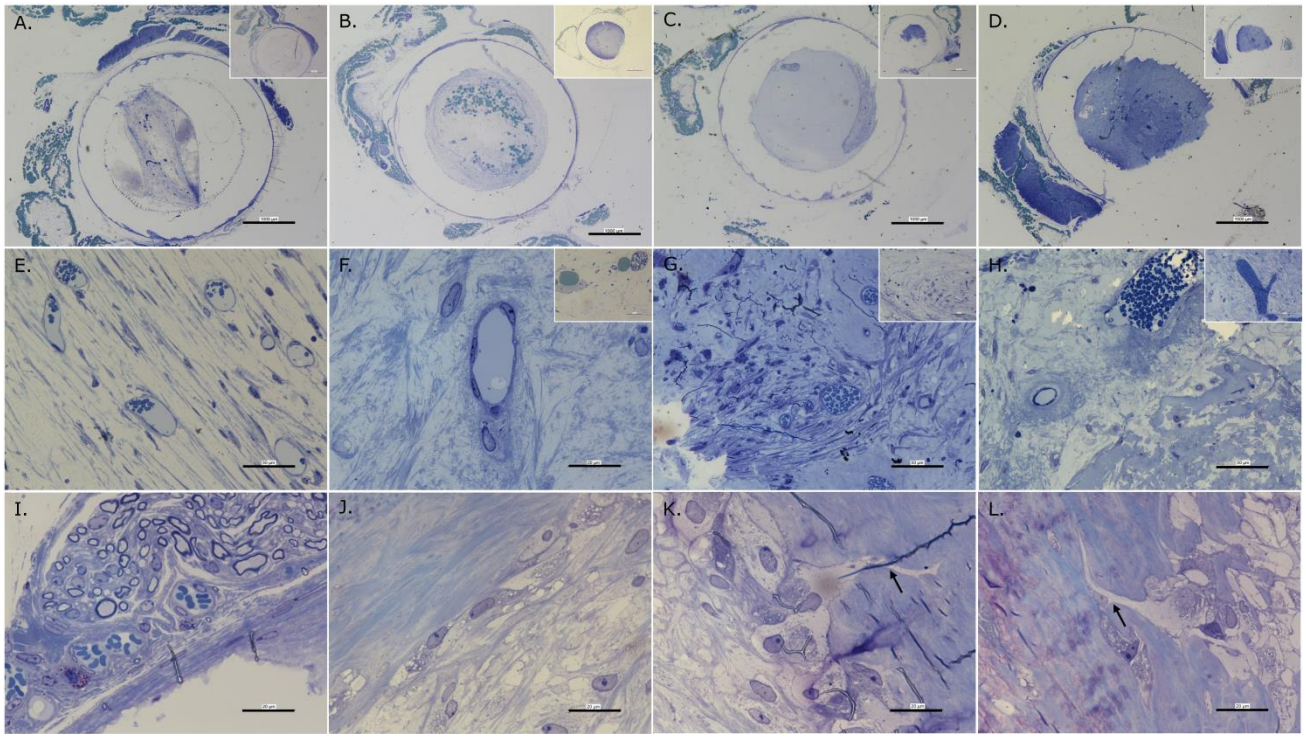


Figure 5.8: Semi-thin sections of conical scaffolds following 12 weeks of implantation. A, E Control condition. B, F. DPSCs. C, G. SCAPs. D, H: DPSCs + SCAPs. All constructs were filled with connective tissue, although the amount and organizational structure significantly differed. I. Myelinated nerves were observed in the tissue surrounding the constructs. J-K (Osteo)dentin-like tissue was clearly present in DPSCs constructs and sometimes contained cellular protrusions (arrows). Scale bars = 1000 µm (A-D); 50 µm (E-H); 20 µm (I-L).

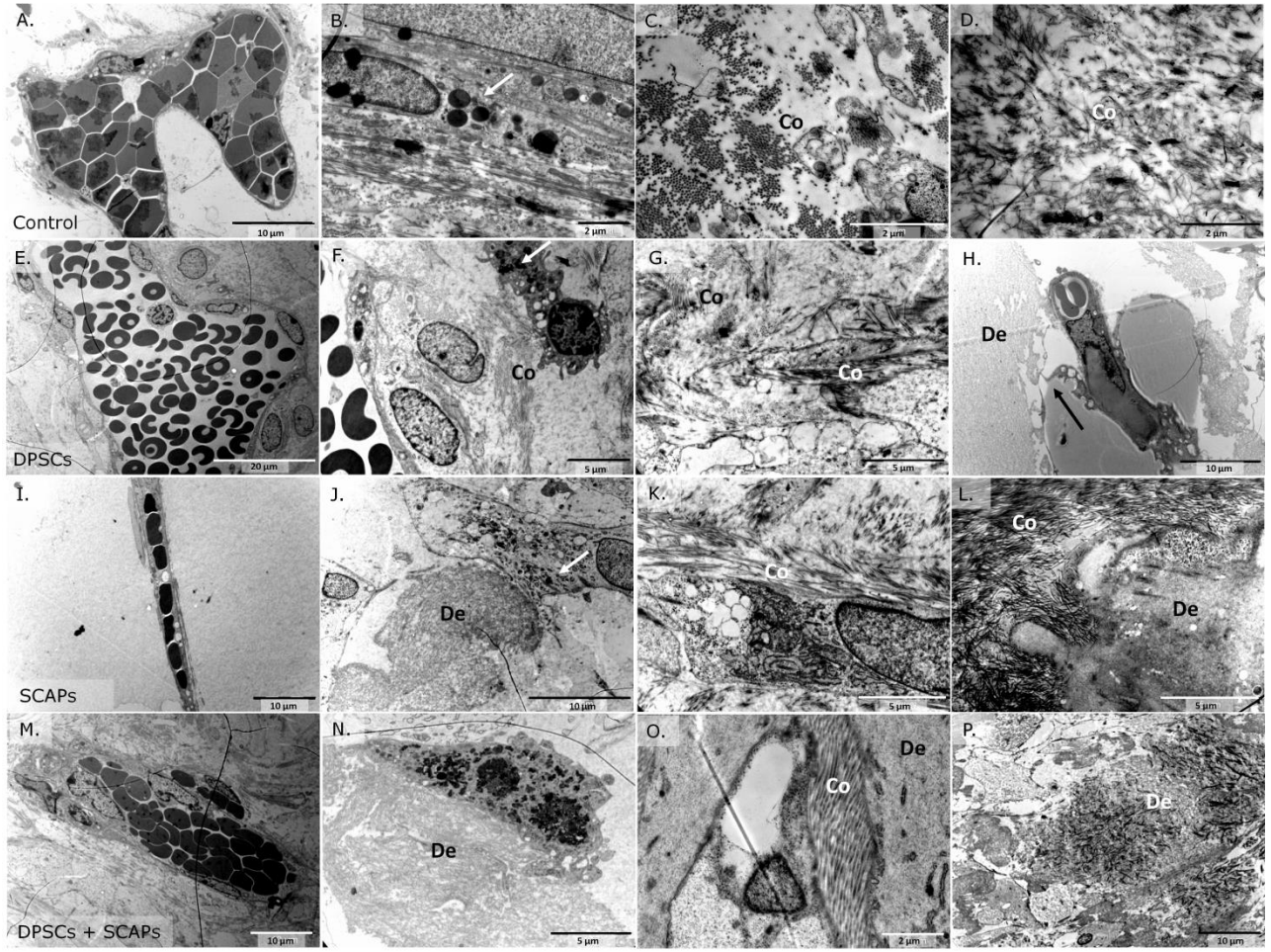

A.
Control
10 µm
B.
2 µm
C.
Co
2 µm
D.
Co
2 µm
E.
DPSCs
20 µm
F.
Co
5 µm
G.
Co
Co
5 µm
H.
De
10 µm
I.
SCAPs
10 µm
J.
De
10 µm
K.
Co
5 µm
L.
Co
De
5 µm
M.
DPSCs + SCAPs
10 µm
N.
De
5 µm
O.
De
Co
2 µm
P.
De
10 µm

Figure 5.9: Ultrastructural analysis of 3D-constructs. A-D. Control. E-H. DPSCs. I-L. SCAPs. M-P. DPSCs + SCAPs. Mature blood vessels of variable shapes and size were found in all experimental conditions (A, E, I , M). One DPSC construct also showed signs of new blood vessel formation (black arrow, H). Samples contained metabolically active cells, some of which comprised electron-dense vesicles (white arrow; B, F, J). Active collagen secretion was also present in all constructs (Co; C, D, F, G, K, L, O). Deposits of collagen, hydroxyapatite and mineralized tissue were only present in the samples containing stem cells (De; H, J, L, N, O, P). Scale bars = 20 μm (E); 10 μm (A, H, I, J, M ,); 5 μm (F, G, K, L, N); 2 μm (B, C, D, O). Analysis was performed on two samples of each experimental condition.

Ultrastructurally, mature blood vessels of variable shapes and sizes were observed in all 3D-constructs (Fig. 5.9A, E, I, M). In one DPSC construct, signs of new blood vessel formation were also detected (black arrow, Fig. 5.9H). All constructs contained metabolically active cells, some of which contained electron-dense vesicles (white arrow, Fig. 5.9B, F, J). All samples showed signs of active collagen secretion (Co, Fig. 5.9C, D, F, G, K, L, O). Tissue deposits containing hydroxyapatite needles and collagen in various stages of mineralization were clearly present in the constructs containing stem cells (De, Fig. 5.9H, J, L, N, O, P). This was not the case in the filled control sample, which only contained patches of collagen secretion (Co, Fig. 5.9C, D).

5.5 Discussion

Within the field of dental tissue engineering, the establishment of adequate tissue vascularization is an important hurdle to overcome. As vascular supply within the tooth is limited by the apical foramen, the size of the tooth root apex required for proper revascularization is an often recurring concern in regenerative endodontic procedures relying on (stem) cell homing [210]. However, when using a dental stem cell-based method, recent reports mentioned the regeneration of dental pulp tissue with a high capillary density in root canals with small apical sizes (0.7 mm) [218]. As already described in chapter 4, DSCs, in particular DPSCs and SCAPs, display pro-angiogenic properties *in vitro* as well as in an *in vivo*-like setting. In order to determine whether these stem cells sustain their pro-angiogenic impact *in vivo*, an ectopic root transplantation model of dental pulp regeneration was performed in immunocompromised mice.

Over the past five years, the ectopic root transplantation model, i.e. the transplantation of human root canals injected with a scaffold containing DSCs, has proven to be an exemplary model for dental pulp regeneration [228-230, 384, 385]. However, since the size and shape of human root canals are prone to variability, standardized 3D-printed HA conical scaffolds are applied in this study in order to mimic the shape of a tooth. As PuraMatrix™ has been successfully used in the aforementioned animal model and it is known to support the survival of DPSCs and to create a permissive environment for angiogenesis, 3D-printed scaffolds containing PuraMatrix™ and DPSCs, SCAPs or DPSCs + SCAPs, were transplanted for 12 weeks in SCID mice [229, 293, 384, 391, 392].

Following 12 weeks of transplantation histological analysis pointed out the formation of vascularized, pulp-like tissue in all stem cell constructs containing more than 10% tissue. Although the regenerated tissues were similar to actual pulp tissue, they clearly contained less collagen. Similar findings were also reported by Rosa *et al.* and others (using PuraMatrix™ and silk fibroin as a scaffold respectively) , indicating more densely organized extracellular matrix in control human pulps [229, 385]. This enhanced matrix formation was also

reported by Dissanayaka *et al.*, when transplanting human root canals containing co-cultures of DPSCs and HUVECs [384].

Despite being contained within a scaffold consisting of dense layers of hydroxyapatite, DPSCs were still able to secrete substantial amounts of VEGF *in vitro. In vivo*, however, the constructs containing DPSCs and/or SCAPs did not display a significantly higher rate of tissue vascularization in comparison to the control condition. While the latter comprised 40% less tissue, it contained a significantly higher number of blood vessels, in particular in comparison to the DPSCs + SCAPs condition. Four weeks of transplantation, however, did not result in the formation of any vascularized, pulp-like tissue in sealed human root canals containing PuraMatrix^{TM}, according to Dissanayaka *et al.* [384]. Rosa *et al*., on the other hand, observed a minimal amount of poorly organized tissue in open root canals containing the same hydrogel after five weeks of transplantation [229]. As we removed and analyzed the scaffolds 12 weeks after transplantation, this suggests that the time frame of transplantation might be a determining factor for the attraction of endogenous cells and the population and/or vascularization of the scaffold structure. Since angiogenesis is a self-limiting biological process, the angiogenic potential of the tissue constructs may have plateaued during these 12 weeks and thereby stimulated the inherent hard tissue-forming capacity of DPSCs and SCAPs, as demonstrated by the patches of mineralized tissue formation in all stem cell constructs. The formation of hard tissue might have also been promoted by the presence of angiogenic growth factors, as VEGF and basic fibroblast growth factor (bFGF) are known to promote the osteogenic/odontogenic differentiation of (dental) MSCs [252, 393-396]. Another aspect which has to be kept in mind is the number of cells related to the size of the scaffold, since cell seeding density can be a defining factor for the differentiation potential of MSCs in general [397-400]. However, in order to detect and confirm the presence of differentiated DPSCs and/or SCAPs immunocytochemical stainings against markers such alkaline phosphatase (ALP), osteocalcin and dentin sialophosphoprotein need to performed, which was unfortunately not possible due to a too aggressive decalcification protocol.

Next to being strongly vascularized, dental pulp is also a highly innervated soft connective tissue. However, in this study myelinated nerve fibers were only

detected in the tissue closely surrounding the constructs. Although it is difficult to compare these results with previous reports as they only studied the tissue within the constructs, the presence and ingrowth of nerve fibers has merely been mentioned by Iohara *et al.*, following only two weeks of *in situ* transplantation of a selected subpopulation of DPSCs in pulpectomized dog teeth [216, 326]. As these nerve fibers originated from apical tissue, these results indicate that the attraction or regeneration of nerve fibers requires specific cues from the oral environment, which are seemingly absent in the subcutaneous environment of the ectopic root transplantation model.

In conclusion, this study was the first to report the formation of vascularized, pulp-like tissue in standardized, 3D-printed HA scaffolds containing DPSCs and/or SCAPs following 12 weeks of transplantation in immunocompromised mice. Despite the substantial secretion of VEGF *in vitro*, the stem cell constructs did not contain a significantly higher amount of blood vessels in comparison to the control condition, which was probably due to osteogenic/odontogenic differentiation. In order to gain more insight into the angiogenic process over time and to generate a balance between tissue vascularization and stem cell differentiation, further research is required regarding the regenerative capacity of DPSCs and SCAPs at intermediate time points such as four and eight weeks. Nevertheless, these data encourage further investigation of the use of 3D-constructs containing DPSCs or SCAPs within the field of regenerative dentistry.

Chapter 6

General discussion and summary

Adequate tissue vascularization is not only a restricting factor for the survival of engineered and transplanted tissues, but it also plays a crucial role in ischemic diseases such as stroke and myocardial infarction (MI). As the formation of blood vessels is considered to be a well-coordinated biological process regulated by a tight balance of stimulating and inhibiting proteins, therapeutic angiogenesis is often accomplished by the delivery of pro-angiogenic agents, such as growth factors [147, 401]. However, to date large, randomized, placebo-controlled and phase I and II clinical trials have only provided limited evidence for their clinical efficacy, urging the need for efficient treatment protocols [401, 402]. Over the past decade, several stem cell-based therapies have been postulated as a more regenerative approach for revascularization, in cardiovascular medicine as well as in tissue engineering [168, 194, 403, 404]. Bone marrow-derived mesenchymal stem cells (BM-MSCs), for example, have shown great promise in animal models of MI and hindlimb ischemia [351-353]. However, conflicting outcomes were found in long-term clinical trials on the stem cell-based treatment of MI [405-409]. The increasing controversy regarding the exact mechanism of action of these stem cells sparked the search for alternative sources of adult stem cells.

Within the adult human body, stem cell populations can be distinguished in a variety of tissues, such as umbilical cord, adipose tissue and teeth [17, 295, 297, 298]. The latter not only harbor dental pulp stem cells (DPSCs), but also stem cells from the apical papilla (SCAPs) and dental follicle precursor cells (FSCs) [17-19, 237]. Generally speaking, dental stem cells (DSCs) are considered to be mesenchymal-like stem cells, characterized by a classical trilineage differentiation potential into adipogenic, chondrogenic and osteogenic lineages and the expression of stem cell markers such as CD29, CD44, CD73 and CD90 [3, 15].

This dissertation aimed to elucidate the angiogenic properties of different dental stem cell populations *in vitro* and *in vivo.* First of all, the effect of different isolation procedures on the stem cell properties of DPSCs was evaluated, after which the 'stemness' of DPSCs, SCAPs and FSCs was confirmed and compared. The angiogenic properties of the aforementioned DSCs were investigated by means of expression profiling, functional *in vitro* tests and a chorioallantoic

membrane assay (CAM). Finally, the pro-angiogenic impact of DPSCs and SCAPs was tested in an *in vivo* model of dental pulp regeneration. The results obtained in this study are summarized and reviewed in the following paragraphs.

What is the effect of different isolation procedures on the stem cell properties of dental pulp stem cells?

DPSCs can be obtained from extracted third molars by means of two widely applied isolation protocols, namely enzymatic digestion (DPSCs-EZ), which involves the digestion of the extracellular matrix with collagenase I and dispase in order to acquire a single cell suspension, or the explant method (DPSCs-OG), a protocol based upon the plastic adherence and subsequent outgrowth of cells out of tissue fragments. As the preferred isolation approach is still subject of considerable debate, we evaluated and compared both isolation protocols with regard to their influence on the stem cell properties and multilineage differentiation capacity of DPSCs in **chapter 2**.

Despite DPSCs-EZ being characterized as a more heterogeneous cell population, there were no significant differences found between both cell populations at the ultrastructural level. DPSCs-EZ and DPSCs-OG also showed a similar ability to form colonies and to proliferate. Both cell populations expressed analogous levels of mesenchymal stem cell markers, such as CD44, CD90 and CD105. The expression of CD117, however, was prone to donor-related variability. With regard to the trilineage differentiation potential of DPSCs, analysis indicated an incomplete formation of adipocytes by DPSCs-EZ as well as DPSCs-OG. Osteogenic and chondrogenic differentiation, on the other hand, was distinct in both stem cell populations.

In contrast to earlier reports comparing different isolation approaches for DPSCs, this study found no pronounced differences between DPSCs-EZ and DPSCs-OG. Huang *et al.*, for example, indicated a higher proliferation rate for DPSCs-EZ [265]. When isolating SHEDs by means of enzymatic digestion, these cells were found to express higher levels of CD34 and to have a more distinct hard tissue-forming potential [309]. The latter was also confirmed by Karamzadeh *et al.* for

DPSCs-EZ, next to higher levels of CD105 and CD146 expression by DPSCs-OG [318].

As the *in vitro* microenvironment may be an important factor determining the characteristics and properties of MSCs, this could probably be an explanation for the aforementioned discrepancies [410]. Next to culture medium additives, such as growth factors or (fetal bovine) serum, oxygen tension and plastic adherence could also have an effect on the gene expression profile or differentiation capacity of MSCs [410-414]. In addition, cell passaging also notably affects the stem cell properties of MSCs, as already mentioned by Patel *et al.* and others [320, 415]. Overall, one should assume that in order to objectively compare two groups or populations of stem cell, the cells should at least be expanded and cultured within the same medium.

In this study, both DPSCs-EZ and DPSCs-OG were derived from the same donors and cultured in the same growth medium, thereby eliminating potential bias from culture-induced differences in morphology and differentiation potential between both cell populations. Both isolation methods yielded stem cell populations with pronounced hard tissue-forming capacities, which suggests their use as a potential treatment strategy for bone and cartilage injuries.

Do different dental stem cell populations have similar stem cell characteristics?

Over the past decade, dental tissues and their residing stem cell populations, e.g. DPSCs, SCAPs and FSCs, have been widely studied and characterized. Given their straightforward isolation from extracted third molars, together with their proliferative and immunomodulatory advantages over BM-MSCs, DSCs have grown into an attractive source of mesenchymal-like stem cells with a prospective use in numerous clinical applications [15, 175, 305, 416]. However, one should definitely take into account potential differences regarding the developmental origin of different dental stem cell populations as it is a defining factor in their intrinsic capacity to repair and regenerate (dental) tissues [24, 417, 418]. Therefore, in **chapter 3**, we aimed to compare and confirm the 'stemness' of the aforementioned stem cell populations and to adequately assess

tissue-related differences by looking at colony formation, immunophenotype and multilineage differentiation potential.

With regard to immunophenotype characterization, analysis indicated the uniform expression of the traditional panel of mesenchymal stem cell markers by all three dental stem cell populations. As already mentioned in chapter 2, the small percentage of CD117-positive cells is likely due to donor-related variability. Surprisingly, only a subset of DSCs expressed neural crest marker Snail, suggesting subpopulations of dental stem cells with different developmental origins. As the dental pulp is already known to contain different stem cell niches, it may be useful to determine the existence and origin of these niches in all dental tissues by means of different developmental markers [181, 301, 419, 420].

Concerning the trilineage differentiation potential of DSCs, it was already mentioned in chapter 2 that DPSCs might not be an ideal source of stem cells to generate fat tissue. The results obtained in this study indicate similar findings for SCAPs and FSCs. In contrast to a number of studies mentioning the successful osteogenic differentiation of FSCs, FSCs were not able to completely differentiate into cells of osteogenic lineage [19, 347, 348, 421]. Although these reports all mention ALP activity and the (presumed) presence of calcified nodules after applying varying induction media for relatively short periods of time, it should be emphasized that an ultrastructural analysis is required to adequately assess the morphological changes underlying the differentiation process as was also the case in this study. Another aspect which should be kept in mind is the ability of FSCs to differentiate into cementoblasts, given their developmental origin. In other words, when studying the hard tissue-forming capacity of FSCs, one should morphologically and molecularly differentiate between bone and cementum [184, 350, 422-424]. Following chondrogenic differentiation, cartilage fragments were only observed in the extracellular space of differentiated DPSCs and SCAPs. While the successful chondrogenic differentiation of DPSCs has been reported previously, limited data are available with regard to the chondrogenic differentiation potential of SCAPs and FSCs [15, 329, 341, 349, 350, 425].

Overall, there were no substantial differences in the stem cell properties of DPSCs, SCAPs and FSCs, although FSCs displayed an incomplete osteogenic and chondrogenic differentiation potential under the given experimental conditions. When considering the selection of these cells for clinical applications, these differences should be taken into account.

What are the paracrine angiogenic properties of dental stem cells *in vitro* and *in vivo*?

Despite elaborate *in vitro* and *in vivo* characterization and numerous studies suggesting their potential role in the repair or regeneration of diseased and damaged dental tissues, little is known concerning the angiogenic properties of DSCs. With regard to DPSCs, Shi *et al.* already mentioned their perivascular origin due to their expression of perivascular marker CD146, alpha smooth muscle actin and a pericyte-associated antigen 3G5 [300]. A couple of studies also reported on the ability of DPSCs to differentiate into endothelial or pericyte-like cells *in vitro*, although proof of *in vivo* functionality remains limited at this stage [179, 181, 182, 329]. In terms of paracrine angiogenic properties, a number of studies demonstrated the expression of angiogenic growth factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF), either under basal circumstances or after injury or hypoxia [174-177]. With regard to the presumably more immature dental stem cell populations, i.e. SCAPs and FSCs, literature indicates the expression of endostatin, VEGF, bFGF and hypoxia-inducible factor 1 alpha (HIF1α)) [15, 184, 342]. A recent study also mentioned the ability of SCAPs to increase endothelial tube formation following direct co-culture with human umbilical cord vein endothelial cells (HUVECs) [426].

In **chapter 4**, we aimed to further elucidate the paracrine angiogenic properties of DPSCs, SCAPs and FSCs, not only at the level of marker expression but also at the level of functional behavior *in vitro* and *in vivo*.

The results obtained in the first part of this study demonstrate the differential expression of pro-angiogenic as well as anti-angiogenic factors at mRNA and protein level by DSCs and human gingival fibroblasts (HGF-1). Variability in

expression levels was not only observed between different DSCs, indicating tissue-related differences, but also between donor samples of the same stem cell population.

In a second part of the study, different aspects of the angiogenic process were mimicked by means of *in vitro* assays in order to evaluate the impact of DSCs on the functional behavior of human microvascular endothelial cells (HMEC-1). Despite the secretion of several proliferation-stimulating proteins, such as VEGF, DSCs did not increase endothelial proliferation in a MTT assay. As the latter is primarily considered to be a viability test, it might be useful to perform a Bromodeoxyuridine (BrdU) ELISA in order to objectively assess cellular proliferation. While DPSCs and SCAPs had a positive impact on endothelial migration in a transwell migration assay, no significant influence was observed in a wound healing assay. Besides the absence of a chemotactic gradient, the addition of the anti-mitotic substance Mitomycin C could also be an explanation for this discrepancy, as already mentioned in chapter 4 [373, 374]. As 24h is a relatively short period to inhibit cell proliferation, the omission of Mitomycin C combined with a staining against proliferation marker Ki67 might be a another way to perform a wound healing assay while monitoring potential bias of cell proliferation. In terms of endothelial tube formation, DPSCs caused a pronounced increase in tubulogenesis. In contrast to a recent study of Yuan *et al.*, indicating an increase in endothelial tubes following direct co-culture of SCAPs and HUVECs, no significant augmentation was provoked by SCAPs [426]. Although one should definitely take into account tissue-related differences between different endothelial cell populations, this might also suggest that DSCs, in certain cases, not only require paracrine factors, but also direct cell contact with endothelial cells to exert their angiogenic effects.

In order to confirm whether DSCs could sustain their angiogenic properties in an *in vivo*-like setting, a CAM assay was performed in the last stage of the study. Analysis indicated that DPSCs and SCAPs had a positive impact on neoangiogenesis.

Despite the expression of several anti-angiogenic factors such as TIMP, PAI-1, THBS1 and PTX3, these data collectively suggest a predominant pro-angiogenic

impact of DPSCs and SCAPs, *in vitro* and *in vivo.* As vascular supply is not only an important burden to overcome in ischemic diseases but also in regenerative dentistry, applications such as dental pulp regeneration and whole tooth engineering might significantly benefit from the angiogenic properties of DSCs.

Do DPSCs and SCAPs sustain their pro-angiogenic impact in an *in vivo* model of dental pulp regeneration?

Despite being a strongly vascularized and innervated connective tissue serving many physiological functions, dental pulp is vulnerable to external insults such as caries, infections and trauma, which emphasizes the need for effective treatment protocols. Despite the impressive body of positive outcomes, the treatment of necrotic, immature permanent teeth remains a challenge in the field of regenerative endodontics. As vascular access within the human tooth is restricted by the apical foramen, the establishment of sufficient tissue vascularization is one of the most important hurdles to overcome. Recent advances in dental stem cell research have provided increasing insight into their regenerative potential and their prospective use in the formation of viable, vascularized dental tissues [228-230, 384]. As already mentioned in chapter 4, DPSCs and SCAPs in particular appear to have pronounced pro-angiogenic properties, which led us to focus on the ability of these stem cells to regenerate dental pulp tissue and promote angiogenesis *in vivo* in **chapter 5.**

Despite being enclosed by a scaffold consisting of dense layers of hydroxyapatite, DPSCs were still able to secrete substantial amounts of VEGF *in vitro*. Following 12 weeks of transplantation, vascularized pulp-like tissue was detected in all stem cell constructs containing more than 10% tissue. Next to organized, concentric layers of collagen, the regenerated tissue also contained patches of osteodentin. In terms of vascularization, there was no increase in the vascular supply of the stem cell constructs in comparison to the control condition.

These results may be explained by the time frame of transplantation applied in this study. As 12 weeks is a quite extended time period this might have not only been determining for the ingrowth of endogenous cells in the control scaffold,

but it could also have caused the angiogenic potential of the stem cell constructs to plateau, as angiogenesis is a self-limiting biological process. These conditions, together with the presence of hard tissue-promoting angiogenic growth factors such as VEGF and bFGF and the osteoinductive capacity of hydroxyapatite, may have created an environment which promoted odontogenic/osteogenic differentiation of DSCs rather than the attraction of blood vessels. In order to confirm the presence of differentiated stem cells within the tissue constructs immunocytochemical stainings against markers such as alkaline phosphatase, osteocalcin and dentin sialophosphoprotein need to be performed. However, in retrospect the decalcification process appeared to be too aggressive, thereby diminishing the immunoreactivity of the isolated tissues. Definitive conclusions are thus hard to draw at this point.

A less aggressive decalcification protocol and the use of labeled stem cells could facilitate the detection of the stem cells within the scaffolds. For example, the cells could be labeled with iron oxide particles, which would allow visualization of the cells within scaffolds by means of magnetic resonance imaging (MRI) [427]. Another option would be labeling the cells with green fluorescent protein (GFP), which would facilitate the detection and the characterization of the transplanted cells when conducting immunofluorescent stainings against multiple markers. This would also allow a more elaborate ultrastructural analysis of the tissue constructs, as the GFP-labeled cells could be detected by means of gold particle-labeled antibodies. Furthermore, an adaptation of the transplantation protocol itself, i.e. the application of a shorter transplantation time such as four or eight weeks, might gain more insight into the angiogenic potential of the stem cells in these settings.

Taken together, this study was the first to report the *in vivo* formation of vascularized pulp-like tissue by DPSCs and SCAPs in 3D-printed, hydroxyapatite scaffolds. Given the determining impact of the cellular microenvironment, adjustments of the transplantation protocol are required in order to generate a balance between the angiogenic properties and the hard tissue-forming capacity of the stem cells.

Future considerations

As previously mentioned, dental tissues are a readily available source of mesenchymal-like stem cells as the extraction of third molars, premolars and deciduous teeth is considered to be common orthodontic practice and the isolation of DSCs is a relatively straightforward procedure. The retention of their stem cell features after cryopreservation, their low immunogenicity and immunomodulatory properties led to an increasing interest in the clinical application of DSCs [305, 428-440]. However, little is known about the *in vivo* immunomodulatory properties of DSCs. Tomic *et al.*, for example, mentioned the formation of granulomatous tissue characteristic of xenotransplant rejection following the transplantation of human DPSCs and FSCs in BALB/c mice [438]. Zhao *et al.*, on the other hand, observed a clear improvement of inflammation after transplantation of rat DPSCs in a mouse model of colitis [440]. There were also no signs of immune rejection after injection of dental pulp stem cells from exfoliated deciduous teeth (SHEDs) in a dog model of muscular dystrophy [441]. With regard to dental pulp regeneration, however, the outcome of allogeneic DSCs transplantation is currently not known as most ectopic root transplantation models are conducted in immunocompromised mice and most *in situ* models apply autologous DSCs [189, 216, 217, 228-233, 235, 384, 385]. Since the use of autologous DSCs at the time of therapeutic necessity also has its practical drawbacks as it requires the extraction of a tooth, more research is required regarding the immunomodulatory behavior of allogeneic DSCs *in vivo* and potential graft-versus-host responses, not only against the cells itself but also their supporting scaffolds. Only in that way, one can really contemplate the use of DSCs in a clinical setting. In particular, the ability of DSCs to promote angiogenesis *in vitro and in vivo* reported in this dissertation opens up a wide range of possibilities for DSC-based regenerative therapies. In myocardial infarction (MI), for example, cell-based therapies have been postulated as a regenerative approach for cardiac repair since maladaptive remodeling of the heart tissue is partly attributed to the loss of cardiomyocytes and the limited endogenous repair capacity of the heart. A successful cell-based therapy would not only reduce scar tissue formation but would also restore the amount of viable cardiomyocytes within the heart and, more importantly, promote

angiogenesis [442]. Given the electrophysiological and angiogenic properties of DPSCs *in vitro* and *in vivo*, these cells might be a potent candidate therapy for cardiac tissue repair and/or regeneration [443, 444]. Recent work of Di Scipio *et al.* already reported the ability of rat DPSCs to home towards injured cardiomyocytes [445]. These data confirm earlier work of Gandia *et al.*, indicating improved functional parameters in a rat model of MI following intramyocardial injection of DPSCs and thus suggesting a beneficial role of these cells in cardiac tissue repair [178]. However, to date, the exact mechanisms involved remain unclear as hDPSCs are assumed to exert cardioprotective effects in a paracrine manner. The specific microenvironment at the time of transplantation and the transplantation period itself might be a determining factor in the cardiac differentiation potential of DPSCs. Furthermore, the underlying molecular mechanisms of action are still unidentified, i.e. which growth factors comprise the cardioprotective phenotype of DPSCs and what is their influence on residing cardiomyocytes, cardiomyocyte precursor cells and cardiac fibroblasts actively involved in scar tissue formation.

As the current thrombolytic therapy can only be administered for a limited period of time, cerebral ischemia or stroke is another ischemic disorder which demands novel, alternative treatment options [446, 447]. The neurogenic and angiogenic potential of DSCs makes them ideal candidates for cell-based therapy to broaden the aforementioned therapeutic window. However, to date only one study reported the application of DPSCs in an animal model of ischemic stroke. Leong *et al.* indicated an enhanced post-stroke recovery four weeks after the intracerebral administration of DPSCs. According to the authors, this recovery was probably mediated by cell-dependent paracrine mechanisms instead of neural replacement, indicating a potential role for angiogenic growth factors. However, the exact mechanisms remain to be elucidated as the study lacked a real quantification of vascular supply as well as any molecular analysis such as real-time PCR [448].

Although these results all emphasize the therapeutic potential of D(P)SCs in a number of clinical applications, the first step in the implementation of DSC-based regenerative therapy in a daily clinical setting would still be the

processing and conservation of clinical-grade DSCs in a dental stem cell banking system [210, 449].

When contemplating the upscale production and banking of (dental) stem cells, one should definitely take into account the increasing controversy regarding the addition of animal sera to culture media, which is considered common practice nowadays. Besides the batch-to-batch variety in quality and protein concentration, the potential risk of transmitting infections or eliciting a host immune response are also critical concerns [450-452]. Although several successful attempts have been made to expand DSCs in serum-free conditions, depending on the panel of added growth factors stem cell differentiation may be induced [453-456]. Karbanová *et al.*, for example, mentioned the increased expression of endothelial markers after the addition of VEGF and insulin transferrin sodium selenite (ITS) to a serum-free DPSCs culture [181]. Neurogenic differentiation of DPSCs after the use of a commercially available serum replacement has also been reported [457]. To date, an extensive body of research has been published regarding the use of (autologous) human serum or other blood derivatives as suitable alternatives in MSCs cultures [414, 458-464]. The extensive amount required for stem cell expansion, as well as donor-related variability and the limited availability of these blood derivatives poses an important problem in the search for appropriate and optimal culture conditions for the up-scale expansion of (dental) stem cells [451, 452, 465, 466].

When working with (autologous) stem cells, not only the quality of the preserved cells and tissues should be monitored, but donor- and/or patient-related variability also has to be kept in mind. For example, the patient's general dental health, i.e. the presence of inflammation or deep caries, could have an effect on the intrinsic biological behavior of residing stem cells, such as proliferation rate and mineralization potential [321, 323, 467, 468]. Pressure and tension caused by orthodontic tooth movement not only mediate bone remodeling and potential root resorption, but they can also induce differentiation of residing DSCs [324, 325, 469]. A final factor that certainly needs to be considered is the donor's age. Given the emergence of mesenchymal stem cells (MSCs) as a potential treatment in various disease settings, multiple studies already reported on the age-related decline in number and function of MSCs present in adult organs.

With regard to DSCs in particular, age-related effects on proliferation, migration as well as regenerative potential were found [322, 326, 470]. In contrast, Atari *et al*. and others showed that age is apparently not a crucial factor for the maximal proliferation potential and the expression of stem cell-related genes by DPSCs [471, 472]. These findings suggest that the implementation of a public dental stem cell banking system not only requires careful evaluation and legislation at different levels but it also demands further characterization of confounding factors such as medium additives, age and (oral) health before any clinical need can be met.

With regard to the use of biomaterials and scaffolds, three dimensional (3D) printing or additive manufacturing has opened up a wide range of possibilities in the field of regenerative dentistry and beyond. This technique not only provides custom-made scaffolds with a controllable chemistry and design but it also allows the use of a wide variety of biomaterials such as bioactive glasses, polylactic acid (PLA), polyethylene glycol (PEG) or hydroxyapatite (HA) [386, 387, 390]. Besides the production of maxillofacial segments, additive manufacturing also facilitates the fabrication of dental crowns and prostheses [389, 390, 473-475]. In terms of dental tissue engineering, Kim *et al.* reported the formation of periodontal tissue following orthotopic transplantation of a 3D-printed poly-ε-caprolactone/HA incisor scaffold in rats [476]. Ectopic transplantation of porous, root-shaped HA/TCP scaffolds containing FSCs, on the other hand, merely led to the formation of mineralized matrix [248]. Similar results were reported by Sonoyama *et al.*, indicating the formation of dentin in root-shaped HA/TCP scaffolds with SCAPs after *in situ* transplantation in swine [236]. The complete regeneration of dental tissues within custom-made scaffolds would be a first step in the creation of 'viable' implants and a great leap forward in the field of personalized dentistry.

In conclusion, the results presented in this PhD dissertation indicate that the choice of isolation protocol does not affect the stem cell properties of DPSCs. The tissue of origin, however, is a determining factor for the differentiation potential of DPSCs, SCAPs and FSCs. Our results also showed that DSCs, in particular DPSCs and SCAPs, display pro-angiogenic properties which are sustained *in vivo*, as they were capable to regenerate vascularized pulp-like

tissue. Although the stem cells were suspected to differentiate into hard-tissue forming cells, these data indicate that DPSCs and SCAPs are promising candidates for cell-based therapies and (dental) tissue engineering applications.

Chapter 7

Nederlandse samenvatting

Adequate weefseldoorbloeding is niet alleen een bepalende factor voor de overleving van getransplanteerde en geregenereerde weefsels, maar speelt ook een cruciale rol bij tal van ischemische aandoeningen zoals een beroerte of myocardiaal infarct (MI). Aangezien de vorming van nieuwe bloedvaten wordt beschouwd als een gecoördineerd biologisch proces dat sterk wordt gereguleerd door stimulerende en remmende eiwitten, wordt therapeutische angiogenese vaak bewerkstelligd met behulp van pro-angiogene agentia, zoals groeifactoren. Tot op heden is er echter weinig bewijs voor de klinische werkzaamheid van deze eiwitten, wat de nood voor meer efficiënte behandelingen benadrukt. Therapieën gebaseerd op het gebruik van stamcellen, bijvoorbeeld, worden beschouwd als een meer regeneratieve invalshoek voor het bevorderen van bloedvoorziening, zowel bij cardiovasculaire toepassingen als bij weefselregeneratie. Mesenchymale stamcellen afkomstig van het beenmerg (BM-MSCs) werden al succesvol toegediend in diermodellen voor ischemische aandoeningen, maar toepassingen in klinische studies leidden tot eerder dubbelzinnige resultaten. Omwille van deze teleurstellende uitkomst en de toenemende onduidelijkheid over het exacte werkingsmechanisme, werd er gezocht naar alternatieve bronnen van volwassen stamcellen.

In het menselijk lichaam zijn er tal van weefsels die stamcellen bevatten, zoals de navelstreng, vetweefsel en spieren. Ook tanden bevatten verschillende stamcelpopulaties, zoals stamcellen van de tandpulpa (DPSCs), de apicale papilla (SCAPs) en precursorcellen van het tandfollikel (FSCs). Algemeen kan men stellen dat tandstamcellen (DSCs) zich gedragen als mesenchymale stamcellen (MSCs), met andere woorden ze brengen stamcelmerkers zoals CD29, CD44, CD73 en CD90 tot expressie en ze zijn in staat te differentiëren tot vetcellen, botcellen en kraakbeencellen.

Deze doctoraatsthesis had als doel de angiogene eigenschappen van DSCs verder te onderzoeken, zowel *in vitro* als *in vivo*. In eerste instantie werden de mogelijke effecten van verschillende isolatiemethodes op de karakteristieke eigenschappen van DPSCs geëvalueerd, waarna het stamcelkarakter van DPSCs, SCAPs en FSCs met elkaar werd vergeleken en bevestigd. Vervolgens werden de angiogene eigenschappen van bovengenoemde DSCs onderzocht met behulp van expressie-analyse, functionele *in vitro* testen en een chorioallantois

membraan assay (CAM). In een laatste fase werden de angiogene eigenschappen van DPSCs en SCAPs bestudeerd in een diermodel voor tandpulpa regeneratie. De resultaten van deze studie worden samengevat en besproken in onderstaande paragrafen.

Wat is het effect van verschillende isolatiemethodes op de karakteristieke eigenschappen van stamcellen uit de tandpulpa?

DPSCs kunnen op twee verschillende manieren geïsoleerd worden uit wijsheidstanden, namelijk via enzymatische digestie (DPSCs-EZ) of via de zogenaamde uitgroeimethode (DPSCs-OG). Terwijl enzymatische digestie bestaat uit het afbreken van extracellulaire matrix met behulp van een enzymencocktail, is het protocol van de uitgroeimethode gebaseerd op de eigenschap van mesenchymale stamcellen om aan een plastic oppervlak te hechten. Aangezien er nog altijd veel discussie is over het feit welke isolatiemethode men dient toe te passen, werden in **hoofdstuk 2** beide methodes vergeleken met betrekking tot hun mogelijke invloed op de karakteristieke eigenschappen en het differentiatiepotentieel van DPSCs.

Ondanks het feit dat DPSCs-EZ werden gekarakteriseerd als een meer heterogene stamcelpopulatie, werden er geen significante verschillen gevonden tussen DPSCs-EZ en DPSCs-OG op ultrastructureel niveau. Beide stamcelpopulaties vertoonden ook een vergelijkbare celdeling en de eigenschap om kolonies te vormen. Zowel DPSCs-EZ als DPSCs-OG brachten vergelijkbare niveaus van klassieke mesenchymale stamcelmerkers tot expressie, zoals CD44, CD90 en CD105. De expressie van CD117 werd echter gekenmerkt door donorvariabiliteit. Verder werden er geen verschillen gevonden in het differentiatiepotentieel van beide stamcelpopulaties; beiden vertoonden een onvolledige differentiatie tot vetcellen. Differentiatie tot bot- en kraakbeencellen was duidelijk zichtbaar in DPSCs-EZ en DPSCs-OG.

In tegenstelling tot voorgaande studies met betrekking tot verschillende isolatiemethodes voor DPSCs, werden er geen opmerkelijke verschillen gevonden tussen stamcellen geïsoleerd met behulp van de twee meest voorkomende isolatiemethodes. Een mogelijke verklaring hiervoor is de micro-

omgeving van de stamcellen, aangezien deze een bepalende factor kan zijn voor de eigenschappen van MSCs. Cultuurmedia, zuurstofspanning en zelfs aanhechting op plastic oppervlakken kunnen een invloed hebben op de genexpressie en differentiatiecapaciteit van MSCs. Algemeen kan men stellen dat een objectieve vergelijking van twee stamcelpopulaties slechts mogelijk is wanneer de stamcellen in dezelfde omstandigheden en cultuurmedia gekweekt zijn.

In deze studie zijn beide stamcelpopulaties afkomstig van dezelfde donoren, waarna ze gekweekt werden in hetzelfde cultuurmedium om op die manier donor- en cultuur-geïnduceerde verschillen op vlak van morfologie en differentiatiepotentieel tussen beide populaties te vermijden. Uit de resultaten van deze studie blijkt dat zowel DPSCs-EZ als DPSCs-OG een uitgesproken capaciteit tot bot - en kraakbeenvorming hebben, wat erop wijst dat deze stamcellen als mogelijke therapie gebruikt kunnen worden voor het herstel van bot - en kraakbeendefecten.

Hebben verschillende populaties van tandstamcellen vergelijkbare karakteristieke eigenschappen?

De voorbije tien jaar werden de verschillende weefsels van de tand en de bijbehorende stamcelpopulaties, namelijk DPSCs, SCAPs en FSCs, uitgebreid bestudeerd en gekarakteriseerd. Omwille van de eenvoudige isolatie uit wijsheidstanden, betere celdeling en immunomodulerende eigenschappen in vergelijking met mesenchymale stamcellen uit het beenmerg, werden deze cellen al snel beschouwd als een aantrekkelijke bron van mesenchymale stamcellen voor potentieel gebruik in tal van klinische toepassingen. Aangezien het weefsel van herkomst bepalend is voor de intrinsieke capaciteit van de stamcellen om weefsels te herstellen en te regenereren, moeten mogelijke verschillen te wijten aan deze afkomst zeker in rekening worden gebracht. In **hoofdstuk 3** werd er dan ook een vergelijking gemaakt tussen de verschillende soorten tandstamcellen (DSCs) om op die manier hun stamcelkarakter te bevestigen en mogelijke oorsprong gerelateerde verschillen op vlak van kolonievorming, eiwitexpressie en differentiatiepotentieel te ontdekken.

Fenotypische analyse wees uit dat de drie stamcelpopulaties een uniforme expressie vertoonden van de klassieke mesenchymale stamcelmerkers. Zoals reeds aangehaald in hoofdstuk 2, werd ook hier de expressie van CD117 gekenmerkt door donor-gerelateerde variabiliteit. Slechts een deel van de tandstamcellen bracht het zogenaamde neurale lijst-eiwit Snail tot expressie, wat mogelijk wijst op het bestaan van meerdere subpopulaties van tandstamcellen met een verschillende embryonale oorsprong.

In hoofdstuk 2 werd reeds vermeld dat DPSCs waarschijnlijk niet de ideale stamcellen zijn voor de regeneratie van vetweefsel. Vergelijkbare observaties werden nu ook gemaakt bij SCAPs en FSCs. In tegenstelling tot eerdere studies, waren FSCs niet in staat tot volledige differentiatie tot botcellen. Er moet echter benadrukt worden dat een analyse op ultrastructureel niveau, hetgeen niet werd uitgevoerd in bovengenoemde studies, essentieel is om het succes van een differentiatieprotocol te bepalen. Bovendien moet men rekening houden met het feit dat FSCs een embryonale aanleg hebben tot het vormen van cementum. Bij het bestuderen van het differentiatiepotentieel van FSCs, dient er dus zowel op morfologisch als moleculair vlak een onderscheid te worden gemaakt tussen bot en cementum. Op vlak van chondrogene differentiatie werd de aanwezigheid van kraakbeenfragmenten enkel opgemerkt bij DPSCs en SCAPs. In tegenstelling tot DPSCs, waarvan de chondrogene differentiatie uitgebreid beschreven is, is er slechts een beperkte hoeveelheid data beschikbaar met betrekking tot de kraakbeenvormende capaciteit van SCAPs en FSCs.

Uit deze studie blijkt dus dat er geen substantiële verschillen zijn in de stamceleigenschappen van DPSCs, SCAPs en FSCS, hoewel FSCs in de gegeven omstandigheden niet in staat zijn tot volledige chondrogene en osteogene differentiatie. Deze resultaten suggereren dat, ondanks het feit dat DSCs als mesenchymale stamcellen worden beschouwd, er toch enkele verschillen zijn tussen de verschillende stamcelpopulaties waarmee men rekening dient te houden bij klinische toepassingen.

Wat zijn de paracriene, angiogene eigenschappen van tandstamcellen in vitro en in vivo?

Ondanks de uitgebreide literatuur met betrekking tot de eigenschappen en het regeneratief potentieel van DSCs, is er weinig geweten over de angiogene eigenschappen van deze stamcellen. DPSCs, bijvoorbeeld, zijn in staat te differentiëren tot endotheelcellen of pericyten in vitro, al is er tot op heden een beperkte hoeveelheid bewijs voor de in vivo functionaliteit van deze cellen. Verder brengen DPSCs ook verschillende angiogenese-gerelateerde groeifactoren tot expressie, zoals VEGF, bFGF en PDGF. SCAPs en FSCs, stamcelpopulaties waarvan verondersteld wordt dat ze iets meer immatuur zijn, brengen volgens de literatuur enkel endostatine, VEGF, bFGF en HIF1α tot expressie. SCAPs zijn echter ook in staat tot het bevorderen van tubulogenese van HUVECs.

In **hoofdstuk 4** werd er dan ook gefocust op de paracriene angiogene eigenschappen van DPSCs, SCAPs en FSCs, zowel op vlak van eiwitexpressie als op vlak van functioneel gedrag *in vitro* en *in vivo*.

In eerste instantie werd aangetoond dat DSCs en gingivale fibroblasten een differentiële expressie vertonen van zowel pro-angiogene als anti-angiogene factoren op mRNA en eiwitniveau. Er was niet alleen sprake van expressievariabiliteit tussen de verschillende stamcelpopulaties, maar ook tussen donorstalen van dezelfde stamcelpopulatie. Om de impact van DSCs en gingivale fibroblasten op de functie en het gedrag van endotheelcellen (HMEC-1) te evalueren, werden er *in vitro* testen uitgevoerd die de verschillende aspecten van angiogenese nabootsten. Ondanks het feit dat DSCs verschillende celgroei-bevorderende eiwitten tot expressie brachten, werd er geen stijging in endotheliale proliferatie waargenomen tijdens een MTT assay. In tegenstelling tot de transwell migratie test, waarin er een significante stijging in endotheliale migratie werd veroorzaakt door DPSCs en SCAPs, werd er geen invloed van de DSCs op endotheliale migratie in een wondheling test gedetecteerd. Verder werd er enkel een significante toename in endotheliale tubulogenese veroorzaakt door DPSCs, in tegenstelling tot eerdere studies die deze eigenschap ook toewezen aan SCAPs wanneer ze in co-cultuur werden gebracht met HUVECs. Dit laatste

zou er op kunnen wijzen dat DSCs in bepaalde gevallen direct celcontact vereisen om angiogenese te kunnen beïnvloeden. Om na te gaan of DSCs ook in staat zijn angiogenese in vivo te beïnvloeden, werd er een CAM assay uitgevoerd. Uit de analyse bleek dat zowel DPSCs als SCAPs een positieve invloed hadden op de vorming van nieuwe bloedvaten.

Deze resultaten tonen aan dat, ondanks de expressie van verschillende anti-angiogene factoren, DPSCs en SCAPs een significant positieve invloed hebben op angiogenese, zowel *in vitro* als *in vivo*. Aangezien bloedvoorziening niet alleen belangrijk is in ischemische aandoeningen maar ook in de regeneratieve tandheelkunde, hebben toepassingen zoals tand(pulpa) regeneratie veel baat bij de angiogene eigenschappen van DSCs.

Zijn DPSCs en SCAPs in staat hun angiogene eigenschappen te behouden in een diermodel voor tandpulpa regeneratie?

Ondanks het feit dat de tandpulpa een sterk gevasculariseerd en bezenuwd weefsel is met tal van fysiologische functies, is deze ook zeer gevoelig voor cariës, infecties en trauma. Met name de behandeling van necrotische, immature, permanente tanden blijft een grote uitdaging in het domein van de regeneratieve endodontie. Aangezien de bloedvoorziening van de tand beperkt wordt door het foramen apicale, is het bewerkstelligen van een adequate bloedvoorziening één van de belangrijkste obstakels om te overwinnen. De toenemende belangstelling voor het onderzoek naar DSCs heeft echter voor meer inzicht gezorgd in het potentieel gebruik van deze stamcellen bij de vorming van levensvatbare, gevasculariseerde tandweefsels. Aangezien in hoofdstuk 4 werd aangetoond dat DPSCs en SCAPs pro-angiogene eigenschappen bezitten, werd er in **hoofdstuk 5** gefocust op de capaciteit van deze cellen om *in vivo* tandpulpa weefsel te regenereren en angiogenese te bevorderen.

Na vier weken *in vitro* waren DPSCs nog steeds in staat tot expressie van substantiële hoeveelheden VEGF, ondanks het feit dat de cellen omgeven waren door meerdere lagen hydroxyapatiet. Wat betreft *in vivo*, werd er twaalf weken na de transplantatie gevascularisseerd, op pulpa-gelijkend weefsel gedetecteerd

in alle constructen die stamcellen bevatten. Naast georganiseerde, concentrische lagen van collageen, bevatte het geregenereerde weefsel ook gebieden met duidelijke vorming van osteodentine. De mate van bloedvoorziening in de stamcelconstructen was echter niet significant hoger in vergelijking met de controles.

De resultaten kunnen mogelijk verklaard worden door de langere transplantatietijd die toegepast werd in deze studie. Aangezien 12 weken een vrij lange periode is kan dit niet alleen bepalend zijn geweest voor de aantrekking van endogene cellen in de controle constructen, maar het kan ook voor een afvlakking van het angiogeen potentieel van de stamcelconstructen hebben gezorgd, gezien het feit dat angiogenese een zelfbeperkend biologisch proces is. Deze omstandigheden kunnen samen met de aanwezigheid van botgroeibevorderende angiogene eiwitten zoals VEGF en bFGF en de osteoinductieve capaciteit van hydroxyapatiet een omgeving hebben gecreëerd die bevorderend was voor de osteogene/odontogene differentiatie van DSCs in plaats van de aantrekking van bloedvaten. Om de aanwezigheid van gedifferentieerde cellen in de gevormde weefsels te bevestigen dienen er immunocytochemische kleuringen te worden uitgevoerd. Dit laatste was echter niet mogelijk omwille van een te agressief ontkalkingsprotocol dat de immunoreactiviteit van de weefsels heeft aangetast. Het is dus niet vanzelfsprekend om op dit moment definitieve conclusies te trekken.

De detectie van de stamcellen in de constructen zou zeker worden vergemakkelijkt door het gebruik van een minder agressief ontkalkingsprotocol en gelabelde stamcellen. Zo kunnen de cellen bijvoorbeeld gelabeld worden met behulp van ijzer partikels, waardoor ze gemakkelijk gevisualiseerd kunnen worden met MRI. Een labeling met GFP in combinatie met immunocytochemische kleuringen tegen verschillende merkereiwitten zou dan weer de detectie en karakterisatie van de getransplanteerde cellen vergemakkelijken. Deze labeling zou ook een veel uitgebreidere ultrastructurele analyse toelaten aangezien de GFP-gelabelde cellen gedetecteerd kunnen worden met goudpartikel-gelabelde antilichamen. Verder zou een aanpassing van het transplantatieprotocol, met andere woorden een kortere transplantatietijd van vier of acht weken, een optimaal evenwicht kunnen

creëren tussen bloedvoorziening en stamceltransplantatie, en op die manier het regeneratief potentieel van DPSCs en SCAPs dus maximaal benutten.

Enkele overwegingen voor de toekomst

Aangezien de extractie van wijsheidstanden, kiezen en melktanden vaak voorkomt in de praktijk en de isolatie van DSCs een relatief eenvoudige procedure is, worden tandweefsels beschouwd als een zeer toegankelijke bron van mesenchymale stamcellen. Zowel het behoud van karakteristieke eigenschappen na cryopreservatie als immunomodulerende eigenschappen, maakt van DSCs een aantrekkelijke, potentiële bron voor stamceltherapie in tal van aandoeningen. Er is echter weinig geweten over de *in vivo* immunomodulerende eigenschappen van DSCs. In één enkele studie werd er een afstotingsreactie geobserveerd na de transplantatie van humane DSCs in muizen, terwijl andere studies dan weer een succesvolle allogene transplantatie van DSCs vermelden. Wat betreft tandpulpa regeneratie is de uitkomst van allogene stamceltransplantatie nog niet gekend aangezien de meeste ectopische worteltransplantatie modellen gebruik maken van immuungecompromitteerde muizen en *in situ* modellen gebruik maken van autologe stamcellen. Bovendien heeft het gebruik van autologe DSCs, wanneer therapeutisch vereist, ook zijn nadelen, aangezien het de extractie van een bestaand element vraagt. Er dient dus meer onderzoek te gebeuren naar het immunomodulerende gedrag van allogene DSCs *in vivo* en naar de mogelijke reacties van de ontvanger tegen de getransplanteerde cellen en hun ondersteunende scaffolds. Enkel op die manier kan men de klinische toepassing van DSCs overwegen.

Met name de mogelijkheid van DSCs om angiogenese te bevorderen, zoals gerapporteerd in dit proefschrift, zorgt voor heel wat therapeutische mogelijkheden. In myocardiaal infarct (MI) bijvoorbeeld, zou een succesvolle celtherapie niet alleen zorgen voor een reductie van het littekenweefsel maar ook het aantal cardiomyocyten herstellen en, nog belangrijker, angiogenese stimuleren. Gezien de elektrofysiologische en angiogene eigenschappen van DPSCs *in vitro* en *in vivo* zijn deze cellen een ideale kandidaattherapie voor het herstel en/of de regeneratie van hartweefsel. Eerdere studies toonden reeds aan dat (rat) DPSCs in staat zijn om naar cardiomyocyten te migreren en een

verbetering van de functionele parameters te veroorzaken na injectie in een MI ratmodel. De exacte werkingsmechanismen die aan de basis liggen van deze verbetering zijn wel nog niet duidelijk, al wordt verondersteld dat DPSCs op een paracriene manier te werk gaan. De micro-omgeving van de cellen en de transplantatie tijd kunnen echter ook bepalend zijn voor het cardiaal differentiatiepotentieel van DPSCs, net zoals de groeifactoren die mogelijk worden uitgescheiden door de cellen en hun effecten op cardiomyocyten, hartstamcellen en hartfibroblasten.

Cerebrovasculair accident (CVA) of beroerte is een andere ischemische aandoening waarin DPSCs een therapeutische rol kunnen spelen. Tot op heden is er slechts één studie die een opmerkelijke verbetering na het toedienen van DPSCs in een CVA diermodel vermeldt. Volgens de auteurs werd deze verbetering vermoedelijk gemedieerd door paracriene factoren, wat een mogelijke rol voor angiogene eiwitten suggereert. De exacte werkingsmechanismen blijven echter onduidelijk aangezien de bloedvoorziening niet werd gekwantificeerd en een moleculaire analyse ontbrak.

Hoewel deze resultaten het therapeutisch potentieel van D(P)SCs in tal van klinische toepassingen benadrukken, is de eerste stap in de implementatie van DSCs-gebaseerde therapie als dagelijkse klinische toepassing nog steeds het verwerken en bewaren van klinisch geschikte DSCs in een stamcelbank.

Wanneer men echter de productie en conservatie van (tand)stamcellen overweegt, dient er zeker rekening te worden gehouden met de toenemende controverse rond de toevoeging van dierlijke sera in cultuurmedia. Naast batch-to-batch variatie in kwaliteit en eiwitconcentratie, vormen het potentiële risico op infecties en het opwekken van een immuunrespons in de gastheer ook een probleem. Ondanks het feit dat er al enkele succesvolle pogingen ondernomen zijn tot het kweken van DSCs in serumvrije omstandigheden, kan de toevoeging van vervangende groeifactoren ook differentiatie van de stamcellen induceren. Tot op heden is er uitgebreid onderzoek gedaan naar het gebruik van (autoloog) humaan serum en andere bloedproducten als mogelijke alternatieven voor mesenchymale stamcelculturen. Het feit dat deze culturen een grote hoeveelheid serum vereisen, in combinatie met de beperkte beschikbaarheid en de donor-

gerelateerde variabiliteit van deze producten, vormt een groot probleem in de zoektocht naar gepaste en optimale cultuuromstandigheden voor de expansie van (tand)stamcellen op grote schaal.

Wanneer er wordt gewerkt met (autologe) stamcellen is niet alleen de kwaliteit van cellen en weefsels belangrijk maar ook mogelijke donor-gerelateerde variabiliteit dient in het achterhoofd te worden gehouden. De aanwezigheid van ontsteking of cariës kan bijvoorbeeld een effect hebben op het intrinsieke biologische gedrag van de stamcellen, zoals celgroei en mineralisatiepotentieel. De druk en kracht die wordt uitgeoefend tijdens orthodontische procedures heeft niet alleen een invloed op het omliggende bot en de tandwortel, maar kan ook differentiatie van DSCs induceren. Tot slot is de leeftijd van de donor ook een belangrijke factor waarmee men rekening dient te houden. Er zijn immers al verschillende studies die een leeftijdsgerelateerde afname in aantal en functie van MSCs in verschillende volwassen organen vermelden. Wat betreft DSCs, werden er effecten op de celgroei, migratie en het regeneratief potentieel geobserveerd. Er zijn echter ook studies die het tegendeel beweren, namelijk dat leeftijd geen cruciale factor is voor de expressie van stamcelmerkers of celproliferatie. Deze resultaten suggereren dat de implementatie van een openbare stamcelbank niet alleen een zorgvuldige evaluatie en wetgeving vereist, maar ook verder onderzoek naar mogelijke beïnvloedende factoren zoals mediumadditieven, leeftijd en gezondheid, vooraleer enige klinische toepassing mogelijk is.

Wat betreft het gebruik van verschillende biomaterialen en scaffolds, biedt 3D-printing heel wat verschillende mogelijkheden zowel in de regeneratieve tandheelkunde als in andere domeinen. Met behulp van 3D-printing kunnen er namelijk niet alleen op maat gemaakte scaffolds geproduceerd worden maar deze techniek biedt ook de mogelijkheid om verschillende biomaterialen te gebruiken, zoals PLA, PEG of hydroxyapatiet. Naast de productie van botsegmenten, kunnen er ook tandkronen en protheses geproduceerd worden met 3D-printen. Met betrekking tot de regeneratie van tandweefsels zijn er verschillende studies die de regeneratie van paradontaal ligament en de productie van gemineraliseerd weefsel vermelden in HA/TCP scaffolds. Tot op heden werd er echter nog geen tandpulpa geregenereerd in deze scaffolds. De

volledige regeneratie van tandweefsels in op maat gemaakte scaffolds zou niet alleen een eerste stap zijn in de creatie van 'levensvatbare' implantaten maar zou ook een grote stap voorwaarts zijn binnen de gepersonaliseerde tandheelkunde.

Tot slot tonen de resultaten in dit doctoraatsproefschrift aan dat de keuze van isolatiemethode geen invloed heeft op de karakteristieke eigenschappen van DPSCs. Het weefsel van herkomst is echter wel een bepalende factor voor het differentiatiepotentieel van DPSCs, SCAPs en FSCs. Onze resultaten toonden ook aan dat DSCs, in het bijzonder DPSCs en SCAPs, angiogene eigenschappen bezitten die worden behouden in vivo, aangezien ze in staat zijn tot de vorming van gevascullariseerd, op pulpa-gelijkend weefsel. Hoewel de stamcellen werden verondersteld te differentiëren naar odontogene/osteogene cellen, tonen de resultaten van deze studie aan dat DPSCs en SCAPs goede kandidaten zijn voor zowel celtherapieën als de regeneratie van (tand)weefsels.

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Curriculum vitae

Petra Hilkens werd geboren op 6 februari 1988 in Genk. In 2006 behaalde ze haar diploma Algemeen Secundair Onderwijs (ASO) in de afstudeerrichting Latijn-Wetenschappen aan de Stedelijke Humaniora te Dilsen. In hetzelfde jaar startte ze haar universitaire opleiding aan de Universiteit Hasselt, waar ze in 2009 haar bachelor diploma in de Biomedische Wetenschappen behaalde. Aansluitend behaalde ze haar master diploma in de Biomedische Wetenschappen in 2011. Haar eindwerk , getiteld: 'The angiogenic properties and enodothelial differentiation potential of human dental pulp stem cells', werd uitgevoerd in het Biomedisch Onderzoeksinstituut (BIOMED) van de Universiteit Hasselt in de groep van Prof. dr. Ivo Lambrichts. In oktober 2011 startte ze haar doctoraat als FWO aspirant. De daaropvolgende jaren was ze actief als lid in het onderwijsteam van verschillende vakken in de opleiding Biomedische Wetenschappen en Geneeskunde. Verder volgde ze verschillende cursussen in het kader van de Doctoral School for Medicine en Life Sciences, zoals biosafety, good scientific conduct and lab book taking, statististiek, effective scientific communication en academic English. Bovendien heeft ze actief deel genomen aan het schrijven van een FWO doctoraatsbeurs en een FWO beurs voor postdoctoraal onderzoeker geschreven. Tenslotte ontving ze een prijs voor de beste posterpresentatie tijdens het PhD symposium 'Recent advances in neuronal and cardiac tissue engineering: from lab to clinic' aan de Universiteit Hasselt in 2014.

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Petra, mei 2015

Don't think, it complicates things.
Just feel, and if it feels like home, then follow its path.