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master in de biomedische wetenschappen: klinische moleculaire wetenschappen

Masterproef

Exploring and comparing the angiogenic properties of different dental stem cell populations

Promotor :
Prof. dr. Ivo LAMBRICHTS
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Masterproef voorgedragen tot het bekomen van de graad van master in de biomedische wetenschappen , afstudeerrichting klinische moleculaire wetenschappen

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Preface

The field dental stem cells already gained my attention a few years ago. In my second bachelor year, I wrote a report on this exciting topic and stem cells were a central part of my education from then on. Therefore, I am very grateful to have been given the opportunity to work with dental stem cells during my senior internship. Moreover, the group of Morphology has shown me the importance of working together as a team. Since science is not just black and white, it is important to share opinions, to help and support each other. These 8 months we have been an enriching experience and will help me to start a career in scientific research.

First of all, I would like to thank my promotor Prof. dr. Ivo Lambrichts, whose scientific passion and enthusiasm were very inspiring. Moreover, he never hesitated to get us involved in some of his other research projects.

Secondly, I would like to thank my daily supervisor, Petra, who indeed has been there for me every single day of my internship. I am grateful for everything you taught me, for the help with my thesis and for all the patience you had. Your guidance was of great value to me. My sincere congratulations for training me as a student during your first year as a PhD student. I am convinced that you will complete your PhD project with great success.

I am very grateful to have had dr. Annelies Bronckaers as my second supervisor. Her guidance and criticism have helped me a lot during my internship and will definitely help me in the future as well. Moreover, you have taught me that it is important not always to be serious in the lab, but to work in a relaxed way with room for laughter from time to time.

The other members of the Morphology group were always ready to answer my questions or to provide help and advice. Therefore, I would like to thank Pascal Gervois, dr. Wendy Martens and dr. Tom Struys.

We are very grateful to Prof. dr. Constantinus Politis from Ziekenhuis Maas en Kempen, Bree, for providing us with patient material.

I would like to thank Marc Jans, for preparing our EM samples and helping us with the imaging, and Jeanine, who was always there to give us advice on immunostainings.

Behind the scene, my mother was always there to support me and listen to my laboratory stories with sincere interest. She has always been there for me.

I would also like to thank my boyfriend who was always ready to help me and calm me down or make me laugh in times of stress.

Finally, a big thank you to the other senior students for the fun moments, the numerous coffee breaks and all the laughter we had together.

Abbreviations

BM MSC	Bone-marrow mesenchymal stem cell
CAM	Chorioallantoic membrane
CM	Conditioned medium
FBS	Fetal bovine serum
FSC	Dental follicle stem cell
DPSC	Dental pulp stem cell
DSC	Dental stem cell
DSCM	Dental stem cell medium
EC	Endothelial cell
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
EZ	Enzymatic digestion isolation
HGF-1	Human gingival fibroblast 1 cell line
HSC	Hematopoietic stem cell
IGFBP-3	Insulin growth factor binding protein 3
MSC	Mesenchymal stem cell
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OG	Outgrowth isolation
PC	Pericyte
PDLC	Periodontal ligament stem cell
RT-PCR	Reverse-transcriptase polymerase chain reaction
SCAP	Stem cell from the apical papilla
SECM	Standard endothelial cell medium
SMC	Smooth muscle cell
TEM	Transmission electron microscopy
VEGF	Vascular endothelial growth factor

Abstract

Therapeutic angiogenesis is very promising in regenerative medicine and the treatment of pathologies associated with insufficient vascularization. However, current revascularization strategies have led to rather disappointing results. Since angiogenesis is key to the development and repair of the tooth, this study explored and compared the angiogenic potential of 3 stem cell populations derived from human third molars: dental pulp stem cells (DPSCs), dental follicle stem cells (DFSCs) and stem cells from the apical papilla (SCAPs), with the emphasis on their paracrine actions. A human gingival fibroblast cell line (HGF-1) was also included. For therapeutic applications, stem cells need to be isolated from their microenvironment and expanded *ex vivo.* Thus, the effects of 2 main isolation methods on stem cell behavior were compared for DPSCs.

DSCs were isolated by means of an explant (OG) or enzymatic digestion (EZ) method. Stem cells were examined for morphology by means of phase-contrast and transmission electron microscopy, for surface marker expression by means of immunocytochemical stainings and FACS analysis, for their colony formation potential in a CFU-F assay and for their proliferation properties by means of an MTT assay. Angiogenic factors secreted by the DSCs and the HGF-1 were identified in a Human Angiogenesis Array, followed by validation and quantification by means of ELISA, RT-PCR and *in situ* stainings. Functional *in vitro* assays with a Human Microvascular Endothelial Cell line (HMEC-1) were performed to gain more insight in the effect of the DSCs and the HGF-1 on the different steps of the angiogenic process. The effect of DSCs and the HGF-1 on endothelial cell (EC) proliferation, migration and tube formation were assessed in an MTT assay, a transwell system and a Matrigel assay respectively. Finally, a chorioallantoic membrane (CAM) assay was applied to investigate the angiogenic potential of DSCs *in ovo*.

DPSCs could be easily isolated and expanded *in vitro* by means of an EZ and OG method. DPSCs-EZ were more heterogeneous compared to DPSCs-OG with extensive vacuolization at ultrastructural level. Higher levels of the markers CD44 and CD90 were found for DPSCs-OG compared to DPSCs-EZ. The various DSC populations and the HGF-1 differentially express both pro- and anti-angiogenic factors, such as IGFBP-3, IL-8, TIMP-1 and VEGF, on mRNA and protein level. VEGF was even shown to be present *in situ* in all dental tissues. Functional *in vitro* assays showed induction of EC migration and tube formation, but not EC proliferation by all DSCs. No effects were found in the CAM assay. Based on marker expression, ultrastructure and suitability for therapeutic applications, an OG isolation method was preferred over an EZ technique. Secondly, it was shown that the human third molar may serve as a single source for 3 different stem cell populations with angiogenic potential. These results indicate that therapeutic angiogenesis with DSCs would provide opportunities to treat many patients, simply with cells gathered from their own or other individuals' teeth.

Samenvatting

Therapeutische angiogenese biedt hoop in de regeneratieve geneeskunde en het behandelen van aandoeningen veroorzaakt door onvoldoende vascularisatie. De huidige revascularisatietherapieën zijn echter onsuccesvol. Aangezien angiogenese een belangrijke rol speelt in de ontwikkeling en het herstel van de tand, vergelijkt deze studie de angiogene eigenschappen van 3 populaties tandstamcellen (DSCs): tandpulpa stamcellen (DPSCs), tandfollikel stamcellen (FSCs) en stamcellen van de apicale papil (SCAPS), met de nadruk op hun paracriene effecten. Een humane gingivale fibroblast cellijn (HGF-1) werd eveneens geïncludeerd. Voor therapeutische doeleinden, moeten stamcellen geïsoleerd worden uit hun omgeving en *in vitro* geëxpandeerd worden. Daarom werd het effect van 2 courante isolatiemethoden op de stamceleigenschappen van DPSCs onderzocht.

DSCs werden geïsoleerd via een uitgroei (OG) of enzymatische digestiemethode (EZ) en werden onderzocht op basis van morfologie via fase-contrast of elektronenmicroscopie, markerexpressie via immunocytochemie en FACS analyse, kolonievorming via een CFU-F assay en proliferatie via een MTT assay. Angiogene factoren gesecreteerd door DSCs en HFG-1 cellen werden geïdentificeerd in een Humane Angiogenese Array, gevolgd door validatie en kwantificatie via ELISA, RT-PCR en *in situ* immunohistochemie. Functionele *in vitro* testen met een Humane Microvasculaire endotheelcellijn (HMEC-1) werden uitgevoerd om inzicht te verwerven in het effect van DSCs en de HGF-1 op de verschillende stappen van het angiogeneseproces. Het effect van DSCs op HMEC-1 proliferatie, migratie en netwerkvorming werd onderzocht in een MTT assay, een Transwell systeem en een Matrigel assay respectievelijk. Een chorioallantoische membraan (CAM) assay werd uitgevoerd om de angiogene eigenschappen van DSCs *in ovo* te bestuderen.

DPSCs konden efficient geïsoleerd en geëxpandeerd worden met behulp van zowel de EZ en OG methode. De DPSCs-EZ waren meer heterogeen t.o.v de DPSCs-OG met vacuolisatie zichtbaar op ultrastructureel niveau. Een hogere expressie van de markers CD44 en CD90 werd aangetoond voor de DPSCs-OG t.o.v. de DPSCs-EZ. In deel 2 van deze studie werd aangetoond dat de verschillende DSCs en de HGF-1 een variabele expressie vertonen van pro- en anti-angiogene factoren zoals IGFBP-3, IL-8, TIMP-1 en VEGF op mRNA of eiwitniveau. VEGF werd bovendien aangetoond in de tandweefsels *in situ*. Functionele *in vitro* assays wezen aan dat DSCs HMEC-1 migratie en netwerkvorming induceren, maar geen effect hebben op HMEC-1 proliferatie. In de CAM assay werden geen effecten gevonden.

Op basis van markerexpressie, ultrastructuur en geschiktheid voor klinische toepassingen werd de OG methode verkozen boven de EZ methode. Deze studie toont aan dat de menselijke tand een potentiële bron is van 3 stamcelpopulaties met angiogene eigenschappen. Therapeutische angiogenese met DSCs biedt mogelijkheden tot het behandelen van vele patiënten met behulp van cellen afkomstig van hun eigen tanden of deze van andere individuen.

1. Introduction

1.1 Angiogenesis

1.1.1 The vascular network

The vascular network not only provides the body with oxygen and nutrients, but is also a route for waste disposal and immune surveillance [1, 2]. The formation of the blood vessel system occurs via 3 main mechanisms: vasculogenesis, angiogenesis and arteriogenesis [2-4]. Early in the embryonic development, angioblasts differentiate into endothelial cells (ECs) during **vasculogenesis**. This process leads to the formation of immature non-functional blood vessels, which then expand to a more mature network via **angiogenesis** [2, 4, 5]. Finally, **arteriogenesis** augments blood flow by increasing the luminal diameter of existing arteries [4]. This study will focus on angiogenesis and its regulating factors.

1.1.2 The process of angiogenesis

Angiogenesis is not only important during embryogenesis, but after birth it is also the main mode of blood vessel formation, e.g. in organ growth, wound healing and the menstrual cycle. This complex multistep event leads to the assembly of new blood vessels by the branching or elongation of pre-existing vessels and is mediated by an interplay between various cells and growth factors [3, 4, 6].

Angiogenesis starts with vasodilatation and an increase in permeability of existing vessels. Pericytes (PCs) and smooth muscles cells (SMCs) detach and the extracellular matrix (ECM) surrounding the vessels is degraded by enzymes, such as matrix metalloproteinases, leading to vessel destabilization. Subsequently, ECs start to proliferate and migrate towards the angiogenic stimulus [1]. The ECs assemble into solid tubes, in which a lumen is created afterwards. The elongation proceeds in a directional way until an adjacent vessel is encountered. Fusion of the contacting vessels by anastomosis can then occur, giving rise to a continuous lumen to allow blood flow. Once the ECs have assembled into vessels, they become quiescent, allowing long-term survival and differentiation. Finally, vessel remodeling and maturation occurs with attachment of PCs and SMCs for additional support and strength. Deposition of a basement membrane leads to the establishment of a mature vessel phenotype [1, 3, 4].

1.1.3 The regulation of angiogenesis

Angiogenesis is a tightly regulated process, controlled by a balance of pro- and anti-angiogenic factors, with specific factors involved in each step (Fig.1, Suppl.S.1.1). Normally, the angiogenic inhibitors dominate, however, stimuli such as hypoxia and nutrient deprivation induce the production of pro-angiogenic factors, tilting the angiogenic balance towards vessel formation, a process which is known as the angiogenic switch [2, 6-9].

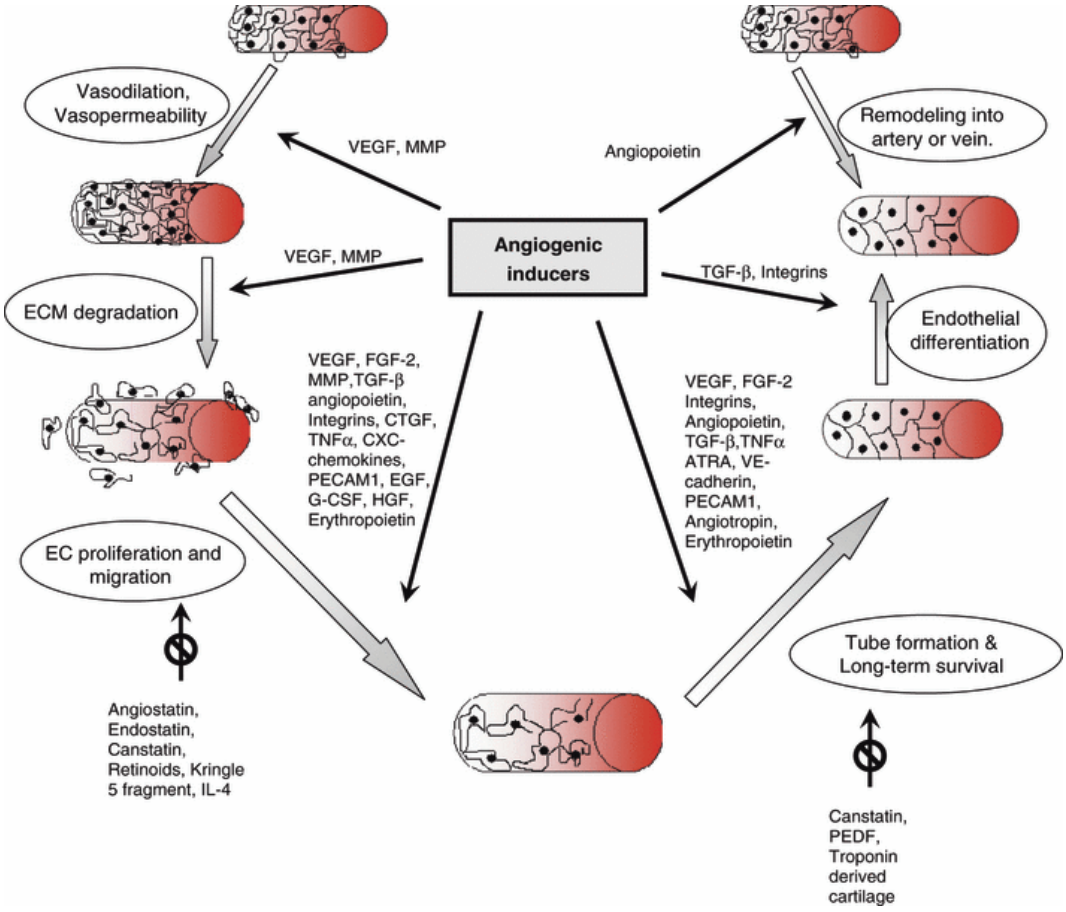

Figure 1: The process of angiogenesis and the main angiogenic factors involved. Angiogenesis is a multistep process involving vasodilatation, an increase in permeability, degradation of the ECM, EC proliferation and migration, tube formation, EC survival and differentiation, and vessel remodeling. Specific pro- and anti-angiogenic factors are involved in each step. Adapted from: Bhadada et al. *Fundamental & Clinical Pharmacology* 2011, 25: 29–47

Unfortunately, the angiogenic equilibrium often gets disturbed, resulting in excessive or insufficient blood vessel formation and contributing to numerous pathologies (Suppl.S.1.2). Excessive angiogenesis plays a role in e.g. cancer metastases and rheumatoid arthritis. Insufficient angiogenesis, on the other hand, leads to chronic wounds, myocardial ischemia and stroke. Moreover, the main cause of failure in the field of tissue engineering is lack of vascularization, which leads to necrosis of tissue grafts [3, 4, 7, 10].

Readjusting the angiogenic balance by the administration of pro- or anti-angiogenic factors seems a logical solution. Indeed, anti-angiogenic factors have been widely explored in e.g. cancer research, with promising results [11]. The administration of angiogenesis stimulating proteins or genes, on the other hand, has led to rather disappointing results [12, 13]. In addition, phase I and II clinical trials have shown that pro-angiogenic gene and protein therapy cause risks for e.g. growth of latent tumors and hemangioma formation [4, 10, 13-15].

Cell-based therapy is suggested as a better alternative, providing a sustained source of a variety of pro-angiogenic factors. However, finding the appropriate cell type is difficult. Besides stimulating angiogenesis, suitability for large scale clinical use is also a prerequisite.

Several cell types are under investigation for therapeutic angiogenesis, e.g. EC progenitors, SMC progenitors, bone marrow mononuclear cells, embryonic stem cells, skeletal myoblasts and mesenchymal stem cells (MSCs) [12, 16, 17]. Unfortunately, results obtained in animal experiments and the first human clinical trials have been inconsistent and not as robust as expected [18]. Nevertheless, the most promising candidate of the currently tested cell types are MSCs [10, 19-21].

1.2 Stem cells

Stem cells have the unique capacity to self-renew and to differentiate into multiple cell types, allowing maintenance of their stem cell pool and the generation of specific tissues respectively. Due to these characteristics, stem cells have raised high hopes in medicine and tissue engineering [22-24]. Stem cells can be classified according to their potency as totipotent, pluripotent, multipotent and unipotent, or according to their developmental hierarchy as embryonic and adult stem cells. The zygote or fertilized egg is **totipotent**, because it can give rise to all embryonic and extra-embryonic tissues. **Pluripotent** stem cells, such as embryonic stem cells, can generate germ cells and all cells of the 3 germ layers. Embryonic stem cells can be isolated from the inner cell mass of the blastocyst and possess a high plasticity and long replicative lifespan. However, risks of rejection, teratoma formation and ethical issues limit their use [24-27]. Adult stem cells, such as neuronal stem cells, are **multipotent** stem cells and are located in specific niches of all postnatal organs. They can be activated from their resting state to replace damaged cells of the tissue they reside in, but have limited differentiation and self-renewal capacities. Although new concepts of trans-differentiation and cell fusion expand their plasticity and therapeutic potential, there is still controversy about this subject [22, 23, 28-31]. Finally, **unipotent or monopotent** stem cells only produce one specific cell lineage, e.g. epidermal skin stem cells [24, 28, 29]. Stem cells not only exert beneficial effects by replacing damaged cells via differentiation, but they also stimulate endogeneous repair by means of paracrine actions. In this aspect MSCs have superior properties.[20, 32, 33]

1.2.1 Mesenchymal stem cells

MSCs are a heterogeneous population of adult stem cells of mesodermal origin, traditionally isolated from the bone marrow. However, they can be found in perivascular niches of virtually all tissues, e.g. skeletal muscles, the kidney, the adipose tissue and the umbilical cord (Fig.2) [32-39]. Differences exist between MSCs derived from different niches, which could indicate possible tissue specific functions. Therefore, characterization criteria have been defined by The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) in 2006. MSCs are plastic adherent and express the MSC markers CD73, CD90 and CD105, but are negative for CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR. Finally, they display adipogenic, chondrogenic and osteogenic differentiation potentials [40, 41].

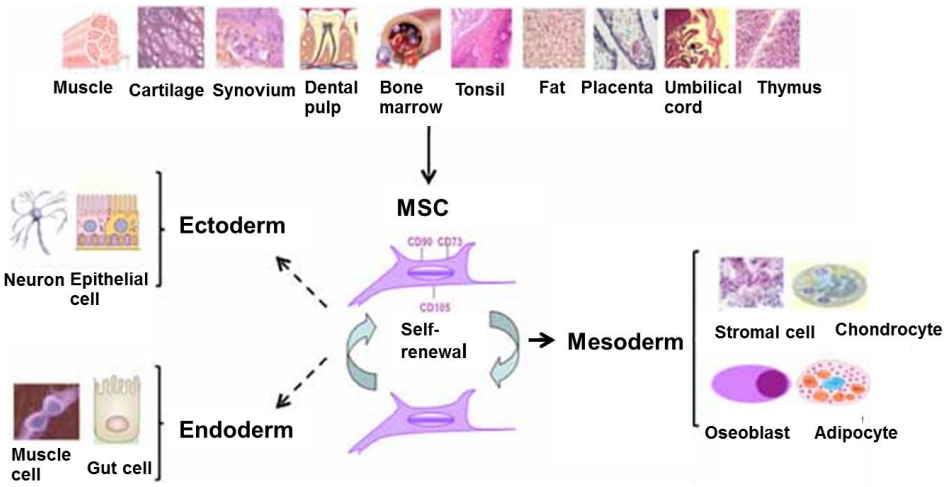

Figure 2: MSCs and their characteristics. MSCs can be found in virtually all organs of the body. They show self-renewal, have specific markers expression profiles, can give rise to all mesodermal lineages *in vitro and in vivo* (full lines) and even ecto- or endodermal cell types *in vitro* (dotted lines), which is known as transdifferentiation. Adapted from: Kuhn et al., *Journal of Cellular Physiology*. 2010, 222: 268–277.

MSCs have gained much attention because of their exceptional paracrine actions, mediating immunomodulation and promoting endogenous repair. Furthermore, MSCs have the ability to home to sites of inflammation or tissue damage, making them suitable for cell-based therapy [33, 42]. Trans-differentiation into cardiomyocytes, hepatocytes and neuronal cells has also been reported, further increasing their therapeutic potential [43]. Since the traditional bone marrow niche yields only low MSC numbers and since the isolation procedures cause high morbidity, easy accessible sources with more therapeutic applicability are now being explored, e.g. human teeth [42, 44, 45].

1.3 The tooth

In humans, teeth are essential in mastication, speech and have esthetic functions. Humans possess a set of milk or deciduous teeth during childhood and a subsequent set of permanent teeth. The dentition can be classified according to shape and function as incisors, canines, premolars, and molars. Each tooth can anatomically be subdivided into crown, root and neck and the five main parts of the tooth are the dental pulp, dentin, enamel, cementum and the periodontal ligament.

The crown of each tooth is covered by a hard, acellular tissue, called **enamel**. Enamel is the most highly mineralized material of the body and is composed of aligned prisms of hydroxyapatite crystals with residues of organic matrix (Fig.3A). It is produced by ameloblasts, which degenerate once the tooth has erupted. The **dental pulp,** on the other hand, is the soft tissue located in the center of the tooth, which does have some regenerative capacities. It is highly vascularized and contains nerves. The pulp is surrounded by a layer of **dentin**. This resilient tissue is composed of 70% of hydroxyapatite crystals and is synthesized by odontoblasts. A characteristic feature of dentin is its dentin tubuli, containing cytoplasmatic extensions of the odontoblasts (Fig.3B). At the tooth root, hard bone-like **cementum** covers dentin, which is produced by cementoblasts.

The **periodontal ligament** is responsible for the anchoring of the tooth in the sockets of the alveolar bone and has a sensory function (Fig.3C). It is composed of dense collagen fibers and fibroblasts, allowing shock absorbance during mastication. Another supportive tissue surrounding the tooth is the **gingiva,** which is the part of the oral mucosa continuous with the periodontal ligament [46, 47] .

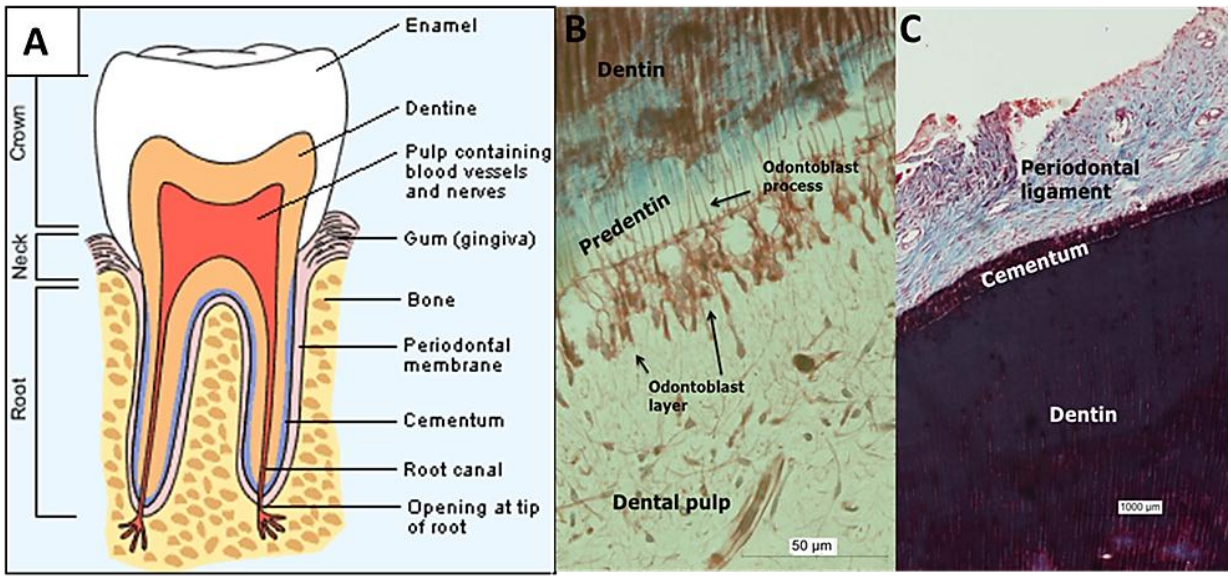

Figure 3: The anatomy of the tooth. A) Each tooth is contains a crown, root and neck area and is composed of dental pulp, dentin, enamel, cementum and the periodontal ligament tissues. B) The dental pulp is surrounded by dentin, in which odontoblasts and their processes give rise to the characteristic dentin tubuli. C) The tooth root is mainly composed of dentin covered with cementum. Residuals of the periodontal ligament are also present.

Since the tooth is composed of multiple tissues, its morphogenesis is a very complex process, involving multiple steps. Teeth are of ectodermal origin and develop by reciprocal interactions between the oral epithelium and cranial neural crest-derived mesenchyme [48]. Although induction of tooth development already starts during embryogenesis, its morphogenesis is not completed until the age of 18 to 21, and therefore, the presence of stem cells is highly suggested. Indeed, several niches of dental stem cells (DSCs) have been identified in human teeth [48-52].

1.3.2 Dental stem cells

DSCs are mesenchymal-like stem cells, located in several perivascular niches of the human tooth. These adult stem cells show good expansion properties and are multipotent with adipogenic, osteogenic, chondrogenic and even cardiogenic and neurogenic potentials [49-51, 53]. Moreover, DSCs are ideal for clinical applications as their use is free of ethical issues and they can be easily obtained since extracted human teeth are routinely discarded as medical waste, e.g. in routine wisdom tooth extractions after orthodontic treatment. Furthermore, it has been shown that DSCs are suited for long term cryopreservation [49, 54]. Even though no FDA-approved therapies using DSCs are available, companies for DSC banking already exist [55, 56].

Immunomodulatory effects have been shown for DSCs, creating the possibility for allogenic use and expanding their range of applications [44, 53, 54, 57, 58]. Four subpopulations of DSCs have been identified in human permanent teeth: dental pulp stem cells (DPSCs), dental follicle stem cells (FSCs), stem cells from the apical papilla (SCAPs) and periodontal ligament stem cells (PDLSCs). These subtypes can be found in different niches of the developing tooth at different time points (Fig.6). Although the DSC populations are all derived from the same organ, differences in their characteristics exist, reflecting their functions *in vivo* (Suppl.S.1.3) [49-53].

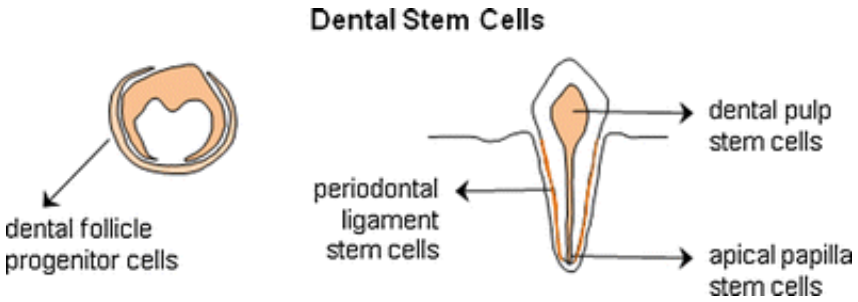

Figure 4: The different DSC populations. Four subpopulations of DSCs can be found in different niches of the permanent tooth: DPSCs, FSCs, SCAPs and PDLCs. The FSCs are present more early in tooth development than the other subpopulations. Adapted from: Inanc et. al. , 2011, *Stem Cell Rev.* 7(3):683-92.

a. Dental pulp stem cells

The presence of stem cells in pulp tissue is highly suggested, since severe tooth damage reaching into the dental pulp induces a limited regeneration process. Indeed, Gronthos *et al*., 2000 were the first to isolate stem cells from the human dental pulp of third molars [55]. Since the dental pulp is essential for tooth viability and strength, pulp regeneration with DPSCs is promising in dentistry to prevent tooth loss [59, 60]. Stem cells from the dental pulp of children's milk teeth, known as stem cells from human exfoliated deciduous teeth (SHEDS), are possibly more immature than DPSCs [49]. However, they are beyond of the scope of this project.

b. Stem cells from the apical papilla

Stem cells have also been isolated from the apical papilla of the developing tooth apices. The apical papilla and the dental pulp are derived from the same precursor structure, namely the dental papilla. However, the apical papilla arises from the apical part of the dental papilla. SCAPs are thus thought to be the precursors of root odontoblasts and play a role in root formation. Therefore, SCAPs might have potential for root and apex regeneration Moreover, because of its collateral circulation, the apical papilla may survive during pulp necrosis, making it beneficial for pulp regeneration [60-62].

c. *Dental follicle stem cells*

The dental follicle is a loose connective tissue sac surrounding the developing tooth, involved in root formation and tooth eruption. It is a source of FSCs, which are thought to give rise to the periodontal ligament, part of the cementum and the alveolar bone and, therefore, are promising for periodontal or bone regeneration [45, 63, 64]. Since FSCs are the precursors of periodontal ligament stem cells, these latter will not be included in this study [45, 65].

1.3.3 Dental stem cells in therapeutic angiogenesis

Not only does angiogenesis play an important role in the development and repair of the dental tissues, but the stem cell populations of the tooth are also located in perivascular niches [66]. Therefore, this study proposes DSCs for vascular regeneration. Since the dental pulp a highly vascularized tissue and angiogenesis is essential in pulp healing, DPSCs seemed the most promising DSC type for therapeutic angiogenesis [55, 67-71]. Although EC differentiation by DPSCS has been previously reported, our lab was not able to reproduce any of these results [72]. However, DPSCs are also known to secrete a wide range of pro-angiogenic factors, such as VEGF and FGF-2 [67, 73-75]. Indeed, previous research in our laboratory has also shown that DPSCs have angiogenic effects via paracrine actions, mainly by the secretion of VEGF and IL-8. DPSCs were shown to induce EC migration, but no effect on EC proliferation was found, which is an essential step in the angiogenesis process. *In vivo* experiments by other groups support our findings, since DPSCs have been shown to induce neovascularization after *in vivo* transplantation in an acute myocardial infarction and a mouse hind limb ischemia model. However, the DPSCs were reported to be in proximity of the newly formed vessels and no vessel incorporation was found [69, 74].

We will explore if the other DSC populations have additional or superior pro-angiogenic properties with respect to DPSCs, with the focus on their paracrine actions. Although the angiogenic potential of the other subtypes has never been explored, it is strongly suggested. The DSC populations are all located in perivascular niches and express the PC marker CD146, the vascular remodeling factor CD105 and even express the angiogenic factor FGF-2 [50, 51, 62]. In addition, SCAPs and FSCs are more immature than DPSCs [49-51]. The apical papilla and the dental pulp are both derived from the dental papilla, however, the apical papilla arises from the apical area. During tooth morphogenesis, vessels firstly enter the apical area in clusters coinciding with the future positions of the roots. Moreover, SCAPs have been shown to be essential for dental pulp revascularization after pulp necrosis or tooth transplantation [76]. The dental follicle, on the other hand, is the first structure of the developing tooth that is infiltrated with a vascular supply and contains a very high concentration of blood vessels [46]. Angiogenic properties are thus highly suggested for both SCAPS and FSCs.

1.4 Research plan and objectives

For use in therapeutic applications, stem cells need to be isolated from their microenvironment and expanded *ex vivo* to obtain sufficient cell numbers. Not only the therapeutic potential of DSCs in angiogenesis, but also their behavior *in vitro* needs to be investigated, including the effect of the isolation procedure and the *in vitro* cell expansion. The first part of this study will focus on the effect of isolation procedures on DPSCs, since different isolation methods have been shown to give rise to different stem cell populations [77, 78]. An explant outgrowth (OG) technique will be compared with an enzymatic digestion (EZ) method. OG isolation is based on the migration and plastic adherence properties of MSCs, allowing migration of these stem cell out of small tissue pieces. In the EZ method, the extracellular matrix is enzymatically degraded, releasing single cells from the tissue. The morphology of the resulting DPSCs will be analyzed and their proliferation properties will be examined by means of an MTT assay. Their marker expression profiles will be investigated via FACS and immunocytochemistry and colony formation will be assessed. The most suited isolation technique will be used to isolated stem cells for the angiogenesis part of this project.

For the angiogenesis part, a human gingival fibroblast cell line (HGF-1) will be included. HGFs are thought to be involved in enhanced angiogenesis in chronic periodontitis, via the secretion of IL-8 and MCP-1 [79]. Moreover, in contrast to the skin, wound healing within the gingiva and oral mucosa is characterized by little inflammation and rapid scarless healing. These properties make the HGF-1 suitable control cells in this part of the study [80, 81].

The angiogenic potential of DPSCs, SCAPs, FSCs and the HGF-1 will be explored to see if the 3 latter have additional or superior pro-angiogenic properties with respect to DPSCs. We hypothesize that the different DSC populations have complementary effects by the secretion of diverse angiogenic factors, affecting all steps of the angiogenesis process and leading to a more robust angiogenic effect. Therefore, the main objectives are to identify and compare the angiogenic factors secreted by the different DSC populations and to explore their angiogenic potential and some of the mechanisms involved *in vitro* and *in ovo*. The angiogenic factors secreted by the different DSC populations and the HGF-1 will be identified in a Human Angiogenesis Array. Validation of the identified factors will be performed by means of RT-PCR and ELISA. Further, it will be investigated if these different cell types promote EC proliferation, migration and tube formation *in vitro*, which are essential steps in angiogenesis. Finally, a chorioallantoic membrane (CAM) assay will be used to investigate the angiogenic properties of the DSCs and the HGF-1 *in ovo*.

Therapeutic angiogenesis with DSCs would provide a way to treat patients suffering from ischemia, chronic wounds or stroke, simply with cells gathered from their own or even other individuals' teeth. Since insufficient vascularization often leads to graft necrosis, DSCs would be of great value in the field of tissue engineering, including regenerative dentistry.

2. Materials and methods

2.1 Dental stem cell isolation

DSCs were isolated from human third molars, extracted for orthodontic or therapeutic reasons. The molars were obtained from Ziekenhuis Maas en Kempen, Bree, with informed consent of the donors. The age of the patients varied from 16 to 18. Immediately after extraction, dental tissues were gathered under sterile conditions. The dental follicles and apical papilla were separated from the mineralized tooth with a forceps. To access the dental pulp, the molars were mechanically fractured and the pulp tissue was removed. The dental tissues were then transported to the laboratory for stem cell isolation at 37°C in standard dental stem cell medium (DSCM). DSCM consisted of α-MEM (Sigma, Bornem, Belgium) supplemented with 2 mM L-Glutamine (Sigma), 100 U per ml penicillin 100 mg per ml streptomycin (Sigma) and 10 % fetal bovine serum (FBS, Biochrom AG, Berlin, Germany). The dental tissues were washed in fresh culture medium to remove blood remnants or debris and were then mechanically minced into fragments of 1 to 2 mm^3.

2.1.1 Explant isolation or outgrowth technique (OG)

Tissue fragments were cultured in 6-well plates in DSCM and incubated at 37°C with 5% CO_2 in a humidified atmosphere. The explants were monitored daily under a Nikon Eclipse inverted microscope TS100 and after a few days outgrowing cells were visible. Medium changes were performed every 3 to 4 days. After 10 to 14 days, confluence was reached and the cells were harvested with 0.05% trypsine/EDTA (Sigma) and sub-cultured for future experiments or cryopreserved for later use. Aliquots of approximately 500 000 cells were transferred to freezing medium, composed of α-MEM, 20% FBS and 10% dimethylsulfoxide (DMSO, Sigma).

2.1.2 Enzymatic digestion isolation technique (EZ)

Tissue fragments were incubated with 3 mg per ml collagenase type I and 4 mg per ml dispase type II (Sigma) in DSCM for one hour at 37°C. Single cell suspensions were obtained by passing the cells through a 70 µm BD Falcon strainer (BD Biosciences, Erembodegem, Belgium). Subsequently, the cells were put into culture at 37°C with 5% CO_2 in a humidified atmosphere until confluence was reached. The cells were harvested with 0.05% trypsine/EDTA and sub-cultured for future experiments or cryopreserved.

2.2 Cell lines

2.2.1 Human gingival fibroblast 1 cell line

A HGF-1 cell line (CRL-2014) was purchased from the company ATCC (Molsheim, Cedex, France). The HGF-1 were cultured in the DSCM at 37°C in a humidified atmosphere with 5% CO_2. Medium changes were performed every 2 to 3 days and at confluence the cells were subcultured or cryopreserved in α-MEM supplemented with 20% FBS and 10% DMSO.

2.2.2 Human microvascular endothelial cell line 1 (HMEC-1)

A human microvascular endothelial cell line was purchased from the Centre for Disease Control and Prevention, Atlanta, Georgio, US [82]. The cells were grown at 37°C in a humidified atmosphere with 5% CO_2 in standard endothelial cell medium (SECM), consisting of MCDB-131 (Invitrogen, Merelbeke, Belgium) supplemented with 1 mM L-Glutamine, 100 U per ml penicillin 100 mg per ml streptomycin, 10% FBS, 10ng/ml recombinant human endothelial growth factor (hEGF, Gibco, Paisley, UK) and 1µg per ml hydrocortisone (Sigma). Medium changes were performed every 2 to 3 days and at confluence the cells were subcultured or cryopreserved. HMEC-1 cells were frozen in MCDB-131 supplemented with 1 mM L-Glutamine, 100 U per ml penicillin 100 mg per ml streptomycin, 20% FBS, 10 ng per ml hEGF, 1 µg per ml hydrocortisone and 10% DMSO.

2.3 Morphology

The morphology of the cells was checked daily and pictures were taken on a Nikon eclipse TS100 inverted microscope with a Jenoptik ProgRes C3 camera. The morphology of the cells was also investigated by means of transmission electron microscopy (TEM). For this cells were seeded on Thermanox™ coverslips in 24-well plates at a density of 2500 cells per cm^2 and were prepared for ultrastructural analysis [83]. After fixation with 2% glutaraldehyde in 0.05M sodium cacodylate buffer (pH=7,3) , the samples were post-fixed in 2% osmium tetroxide for 1 hour and stained with 2% uranyl acetate in 10% acetone for 20 minutes. Subsequently, the cells were put through a dehydrating series of graded concentrations of acetone and they were embedded in araldite epoxy resin at 60°C using the pop-off method. Ultrathin sections of 60 nm were made with a Leica EM UC6 microtome, which were mounted on 0.7% formvar-coated 50 mesh copper grids. The samples were contrasted with uranyl acetate and lead citrate and examined with a Philips EM 208 S transmission electron microscope equipped with a Morade Soft Imaging System camera. Images were analyzed with ITEM-FEI software (Olympus, SIS, Munster, Germany).

2.4 Conditioned medium (CM)

Conditioned medium (CM) was made by seeding DSCs (passage 2, 3), HGF-1 (passage 5) at a density of 20000 cells per cm^2 and incubating them in DSCM at 37°C and 5% CO_2. The cells were allowed to adhere overnight, were rinsed in PBS and cultured in DSCM with 0.1% instead of 10% FBS (0.1% DSCM) for 48 hours to induce growth factor secretion [84, 85]. After 48 hours, the CM was harvested and aliquots were stored at -80°C for experimental use.

2.5 Colony formation (CFU-F) assay

To assess colony formation, DSCs were seeded at low densities in 6-well plates provided with DSCM. Densities of 52 and 208 cells per cm^2 were seeded in duplicate. The cells were incubated at 37°C with 5% CO_2 and checked daily under the microscope for colony formation. After 10 days, the cells were fixed in 4% paraformaldehyde in PBS (PFA) and a Toluidine Blue staining (Merck, Darmstadt, Germany) was performed. Pictures were taken with a high resolution digital camera and macroscopic colonies (> 50 cells) were quantified by 3 independent individuals with Image J cell counter Software.

2.6 Fluorescence activated cell sorting (FACS)

Surface marker expression of DPSCs, FSCs and SCAPs (passage 2,3) was investigated by means of FACS analysis, according to a standardized protocol (Suppl.S.2.1). The used primary and secondary antibodies, together with the appropriate isotype controls, are listed in Table 2.1. Samples were analyzed with a Becton Dickinson FACS Calibur. At least 10 000 events were recorded for each sample and data processing was done with Becton Dickinson Cell Quest Pro Software.

Table 2.1: Antibodies FACS analysis

Antigen	Type		Label	Company	Dilution
Primary antibodies					
CD34	21620344	Mouse mIgG1κ	PE	Immunotools, Friesoythe, Germany	1/100
CD44	21270444	Mouse mIgG2b	PE	Immunotools, Friesoythe, Germany	1/100
CD45	12-0459-71	Mouse mIgG1κ	PE	eBioscience, Vienna, Austria	1/100
CD90	11-0909-42	Mouse mIgG1κ	FITC	eBioscience, Vienna, Austria	1/100
CD105	12-1057-41	Mouse mIgG1	PE	eBioscience, Vienna, Austria	1/100
NGFR (p75)	Ab8877	Mouse mIgG1κ	N	Abcam, Cambridge, UK	1/100
STRO-1	MAB1038	Mouse mIgM	N	R&D systems, Oxon, UK	1/50
Secondary antibodies					
Goat anti-mouse	A10543	Goat IgG F(ab')2 frag.	R-PE	Invitrogen, Merelbeke, Belgium	1/300
Rat anti-mouse IgM	11-5890-81	Rat IgG2a κ	FITC	eBioscience, Vienna, Austria	1/300
Isotypes					
Mouse mIgG1/kappa	12-4714-42		PE	eBioscience	1/100
	11-4714-42		FITC	eBioscience	1/100
Mouse mIgG2b	12-4732-42		PE	eBioscience	1/100
Mouse mIgGM	11-5890-81		FITC	eBioscience	1/50

m = monoclonal ; N = non-labeled ; p = polyclonal

2.7 Human angiogenesis array

A human Proteome Profiler™ Angiogenesis array (R&D Systems, Oxon, UK) was performed, allowing parallel determination of the relative expression levels of human angiogenesis-related proteins in duplo. CM of DPSCs, FSCs and SCAPs (passage 2,3) and the HGF-1 (passage 5) was used with inclusion of 0.1% DSCM a control. The array was performed in accordance with the manufacturer's guidelines. Quantification was performed with Image J Dot Blot Analyzer Software. The experiment was repeated for 2 different patient samples.

2.8 Enzyme-linked Immunosorbent Assay (ELISA)

The concentration of specific angiogenic factors in the CM of DPSCs, SCAPs and FSCs (passage 2,3) and the HGF-1 (passage 5) was determined by means of ELISA for CXCL-16 (Quantikine® Human CXCL16 Immunoassay, R&D Systems, Abingdon, UK), IGFBP-3 (RayBio® Human IGFBP-3 ELISA Kit, RayBiotech, Boechout, Belgium) and VEGF (RayBio® Human VEGF ELISA Kit, RayBiotech), in accordance with the manufacturer's guidelines.

2.9 RT-PCR

Cell pellets of approximately 500 000 cells were made for DPSCs, FSCs, SCAPs (passage 2, 3) and the HGF-1 (passage 5). Total cellular RNA was extracted by means of a Qiagen RNeasy® Plus Mini Kit (Maryland, USA) according to the manufacturer's guidelines. cDNA was synthesized from 700 ng of extracted RNA with a Promega Reverse Transcription System Kit (Madison, USA) according to the manufacturer's protocol with a Bio-rad MyClycler thermal cycler 1.065 according to the program in Table 2.2.

Table 2.2: protocol cDNA synthesis

cDNA Mix	
	Per sample (µl):
25 mM MgCl2	4
10 x RT buffer	2
10 mM dNTPs	2
dT primers	1
Rnase inhibitor	0.5
AMV-RT	0.5
cDNA Program	
60 minutes	42°C
5 minutes	85°C
5 minutes	4°C

Reverse-transcriptase polymerase chain reaction (RT-PCR was performed with Taq DNA Polymerase (Roche, Mannheim, Germany) according to the program in Table 2.3 A. Primers were purchased from Eurogentec S.A. (Seraing, Belgium) and are listed in Table 2.3 B.

Samples were loaded on a 1.2% agorose gel (Invitrogen, Merelbeke, Belgium) together with Orange G and a 100bp ladder (Invitrogen). Non-template controls were included.

Table 2.3A: PCR protocol

PCR Mix		
	Per sample (µl):	
MilliQ		18.5
10 x PCR-buffer		2.5
forward primer (25 µM)		1
reverse primer (25 µM)		1
Taq Polymerase		0.75
dNTP mix		0.25
PCR Program		
1 x	5 minutes	94°C
35 x	1 minute	95°C
	1 minute	60°C
	45 seconds	72°C
1 x	10 minutes	72°C
		4°C

Tabel 2.3B: Primers PCR

Test samples					
Gene	**Primer**	**Sequence**	**Size (bp)**	**Tm (°C)**	**Product size (bp)**
VEGF	Forward Reverse	CCT-TGC-TGC-TCT-CC-CC-AC ATC-TGC-ATG-GTG-ATG-TTG-GA	20 20	64 58	280
Housekeeping genes					
Gene	**Primer**	**Sequence**	**Size (bp)**	**Tm (°C)**	**Product size (bp)**
β2-microglobulin	Forward Reverse	CTC-ACG-TCA-TCC-AGC-AGA-GA CGG-CAG-GCA-TAC-TCA-TCT-TT	20 20	62 60	213

2.10 Immunohistochemical and –cytochemical staining

For histological and immunohistochemical analysis dental tissues were fixed in 4% PFA. Dehydration was performed in ascending series of ethanol and butanol. Subsequently, the tissues were embedded in paraffin and serial section of 7 µm were made with a Leitz Wetzlar 1512 Microtome (Leica, Aartselaar, Belgium). For immunocytochemical stainings, cells were seeded on glass coverslips in 24-well plates at a density of 2500 cell per cm^2. The cells were grown in culture medium until 80 to 90% confluence was reached and were then fixed in 4% PFA. Both immunohistochemical and immunocytochemical stainings were performed according to standardized protocols (Suppl.S2.2, S2.3). Immunofluorescence was performed by means of labeled primary or secondary antibodies, listed in Table 2.4. Negative controls were only incubated with the secondary antibody.

Diaminobenzidine (DAB) stainings were performed with a Dako EnVision®+ System-HRP Kit (Dako, Carpinteria, USA) in accordance with the manufacturer's guidelines and a standard protocol (Suppl.S2.4).

Pictures were taken with a Nikon Eclipse 80i Fluorescence Microscope equipped with a Nikon DS-2MBWc digital sight camera or a Nikon Eclipse 80i inverted Microscope equipped with a Nikon DS-5M digital sight camera.

Table 2.4: Antibodies Immunostainings

Antigen	Type		Label	Company	Dilution
Primary antibodies					
CD29	Ab3167	Mouse mIgG1κ	N	Abcam, Cambridge, USA	1/50
CD29	Ab5185	Rabbit pIgG	N	Abcam, Cambridge, USA	1/100
CD117/c-Kit	Sc-168	Rabbit pIgG	N	Santa Cruz, Heidelberg, Germany	1/100
CD146	Ab75769	Rabbit mIgG	N	Abcam, Cambridge, USA	1/100
FGF-2	F6162	Mouse mIgG1	N	Sigma, Staint Louis, USA	1/250
NGFR/p75	Ab8877	Mouse mIgG1	N	Abcam, Cambridge, USA	1/100
$VEGF_{165 \text{ and } 121}$	MAB293	Mouse mIgG2b	N	R&D, Oxon, UK	1/50
STRO-1	MAB1038	Mouse mIgM	N	R&D systems, Oxon, UK	1/50
Secondary antibodies					
Donkey anti-mouse	A31570	Donkey IgG	AF 555	Invitrogen, Paisley, Scotland	1/500
Donkey anti-rabbit	A21206	Donkey IgG	AF 488	Invitrogen, Paisley, Scotland	1/500

m = monoclonal ; N = non-labeled ; p = polyclonal

2.11 MTT proliferation assay

The proliferation properties of DPSCs-OG and DPSCs-EZ were assessed by means of an (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. DPSCs (passage 2,3) were seeded in threefold into 96-well plates at a density of 6250 cells per cm^2. The cells were grown in DSCM at 37°C for 24, 48, and 72 hours, after which an MTT assay was performed.

The effect of DSCs and HGF-1 on EC proliferation was also investigated by means of MTT. HMEC-1 cells were seeded in 96-well plates at a density of 15625 cells per cm^2 in SECM and incubated overnight at 37°C with 5% CO_2 to adhere. On the next day, the medium was replaced by CM of DPSCs, FSCs, SCAPS (passage 2-4) and the HGF-1 (passage 5). 0.1% DSCM was used as a negative control and standard 10% DSCM as a positive control. All conditions were performed in triplet. After 72 hours of incubation an MTT assay was performed.

For the MTT assay, 5mg per ml MTT solution (Sigma) in 0.1% DSCM was added to each well with a final concentration of 0.5 mg per ml. After an incubation of 4 hours, the MTT solution was removed and 0.01M glycine (Sigma) in DMSO (Sigma) was added in each well to dissolve the formazan crystals. The absorbance of the samples was measured with a Benchmark Microplate reader (Bio-rad, Eke, Belgium) at a wavelength of 550 nm.

2.12 Transwell migration assay

The influence of the DSC populations on EC migration was investigated via a Transwell system with the HMEC-1. DPSCs, FSCs and SCAPs (passage 3-6) were seeded in 24-well plates in DSCM at a density of 25000 cell per cm^2 and incubated overnight at 37°C with 5% CO_2 to adhere (lower compartment). The cells were put on 0.1% DSCM for 24 hours to induce growth factor secretion. HMEC-1 cells were seeded on Thincert™ tissue culture inserts of pore size 8µm (Greiner Bio-One, Wemmel, Belgium) in 0.1% DSCM at a density of 1488 cells per cm^2 (upper compartment). The inserts were placed in the wells with the dental stem cells, allowing diffusion of soluble factors. After 24 hours the inserts were fixed in 4% PFA and migrating HMEC-1 cells were stained with 0.1% crystal violet (Merck, Darmstadt, Germany) in 70% ethanol. Subsequently, 2 random pictures were taken for each condition on a Nikon eclipse TS100 inverted microscope with a Jenoptik ProgRes C3 camera. Quantification was performed by means of Carl Zeiss Axiovision 4.6 Software (Carl Zeiss N.V.-S.A., Zaventem, Belgium) and values were expressed as mean area percentage.

2.13 Matrigel tube formation assay

HMEC-1 were seeded on growth factor-reduced Matrigel (BD Bioscience) in 15µ angiogenesis slides (Ibidi, München, Germany) at a density of 40 000 cells per cm^2. The HMEC-1s were seeded in dental 0.1% DSCM or standard DSCM as negative and positive controls respectively. The test samples were seeded in DSC and HGF-1 CM. For each condition 2 representative pictures were taken after 2, 4, 6 and 24 hours on a Nikon eclipse TS100 inverted microscope with a Jenoptik ProgRes C3 camera. For quantification, Image J Software was used. The average length of minimum 5 tubes was calculated for each picture and the average of the 2 representative pictures was calculated.

2.14 Chorioallandtois membrane (CAM) assay

Fertilized white leg horn eggs of embryonic day 3 (E3) were incubated for 3 days at 37°C at constant humidity. To detach the developing CAM from the shell, 3 ml of albumen was removed. Subsequently, a square window was made in the eggshell, exposing the CAM. The window was sealed with cellophane tape and the eggs were returned to the incubator. At E9, sterilized gelatin sponges of 2 mm^3 (Gelfoam Upjohn, Kalamazoo, MI) were placed on top of the CAM. Then, test and control conditions were adapted as shown in Table 2.5. CM of DPSCs (passage 3), CM concentrate (concentrated 5-fold with a Pierce protein concentrator 9kDA MWCO, Thermo Scientific, Erembodegem, Belgium) and DPSCs (passage 3) were used as test samples. DPSCs were first washed in 0.1% DSCM and PBS to remove interfering growth factors from the standard DSCM. After administration of the sponges, the eggs were sealed again with cellophane tape and were returned to the incubator. At E12, the CAM was removed from the eggs and pictures were taken with a Nikon dn 100 Digital Network Camera.

Afterwards, the CAM was fixed in 4% PFA and stored in 70% ethanol for histological processing. For quantification, grids of concentric circles surrounding the sponges were plotted on the pictures and the number of blood vessels intersecting the circles were counted.

Table 2.5: Conditions CAM assay

Test sponge	Control Sponge
10000 DPSCs (10 µl)	PBS (10 µl)
50000 DPSCs (10 µl)	PBS (10 µl)
DPSC CM (5 µl)	0.1% DSCM (5 µl)
CM concentrate (5 µl)	0.1% DSCM concentrate (5 µl)

2.15 Statistical analysis

Graphpad Prism 5 Software (Graphpad Software Inc., California,USA) was used for statistical analysis. Normality of the data was examined by means of a D'Agostino-Pearson omnibus normality test. In case of a Gaussian distribution, a Student t-Test was used to compare 2 groups. One way Anova and a Bonferonni Multiple Comparison Post-hoc Test were used to compare multiple groups. When the data were not arranged according to the Gaussian distribution, a Mann-Whitney Test was performed to compare 2 groups. A Kruskal-Wallis Test was applied with a Dunn's Post Test to compare multiple groups. Statistical significance was reached at p-values ≤ 0.05.

3. Results

3.1 Comparing an EZ and OG isolation technique for DPSCs

3.1.1 Isolation and culture procedures

The efficiency and quality of DPSC isolation by means of an EZ and an OG technique was investigated. Dental pulp tissues from human third molars were mechanically minced into fragments of 1 to 2 mm^3. These pieces were put in culture to allow outgrowth of DPSCs with the OG technique. For the EZ method, the pieces were first digested with a collagenase-dispase solution for one hour at 37°C, passed through a strainer and the resulting single cell suspension was put in culture. The EZ method already gave rise to adherent cells overnight (Fig.5 A,C) and confluence was reached after approximately one week. Microscopical analysis revealed heterogeneous cell populations, containing not only typical fibloblast-like spindle-shaped cells (f) but also polygonal cells with multiple processes (p) (Fig.5 A,C). The OG fragments started to adhere after 2 to 4 days and outgrowing cells with an elongated shape (e) were visible from then on (Fig.5 B,D). The wells reached confluence after 10 to 14 days. OG isolation resulted in a more homogeneous stem cells population with a spindle-shaped morphology and long cytoplasmic processes (Fig.5 B,D).

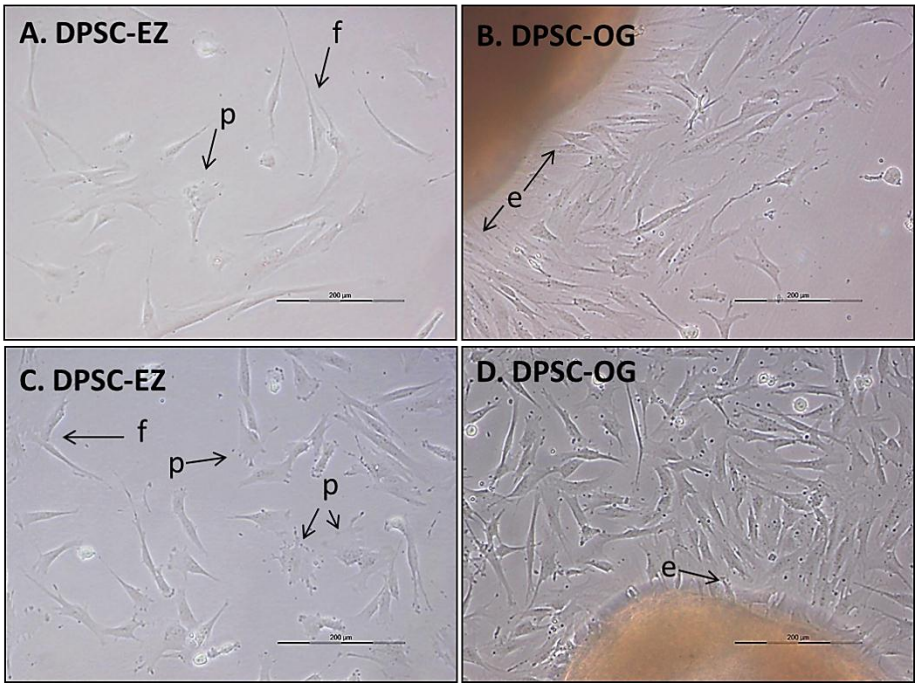

Figure 5: Comparison of DPSC isolation via an EZ and OG method. Already 1 day after the isolation, DPSCs-EZ (passage 0) were visible (A). DPSCs-EZ constituted of a morphological heterogeneous cell population with not only typical fibloblast-like spindle-shaped cells (f), but also polygonal cells with multiple processes (p) (A,C) After 2 to 4 days, DPSCs-OG started to grow out of the explant pieces (passage 0) as elongated cells (e), whereas typical fibroblast-shaped cells were present at further distance from the explants (B,D). The OG method yielded a more homogeneous stem cell population of spindle-shaped cells (B,D). Scale bar = 200µm

3.1.2 Ultrastructural analysis by transmission electron microscopy

The morphological properties of DPSCs-EZ and DPSCs-OG were investigated at ultrastructural level by means of transmission electron microscopy (TEM). No clear differences in ultrastructure were observed between DPSCs-EZ and DPSCs-OG. A normal oval cell shape with a perinuclear organelle-rich zone (p), containing rough endoplasmic reticulum cisternae (r) and mitochondria (m), could be clearly distinguished from of an organelle-poor peripheral zone (o) for both DPSCs-EZ and DPSCs-OG. Large euchromatic nuclei with one or more nucleoli (n) were visible. However, extensive vacuolization throughout the entire cytoplasm (v) was found for DPSCs-EZ, but not for DPSCs-OG.

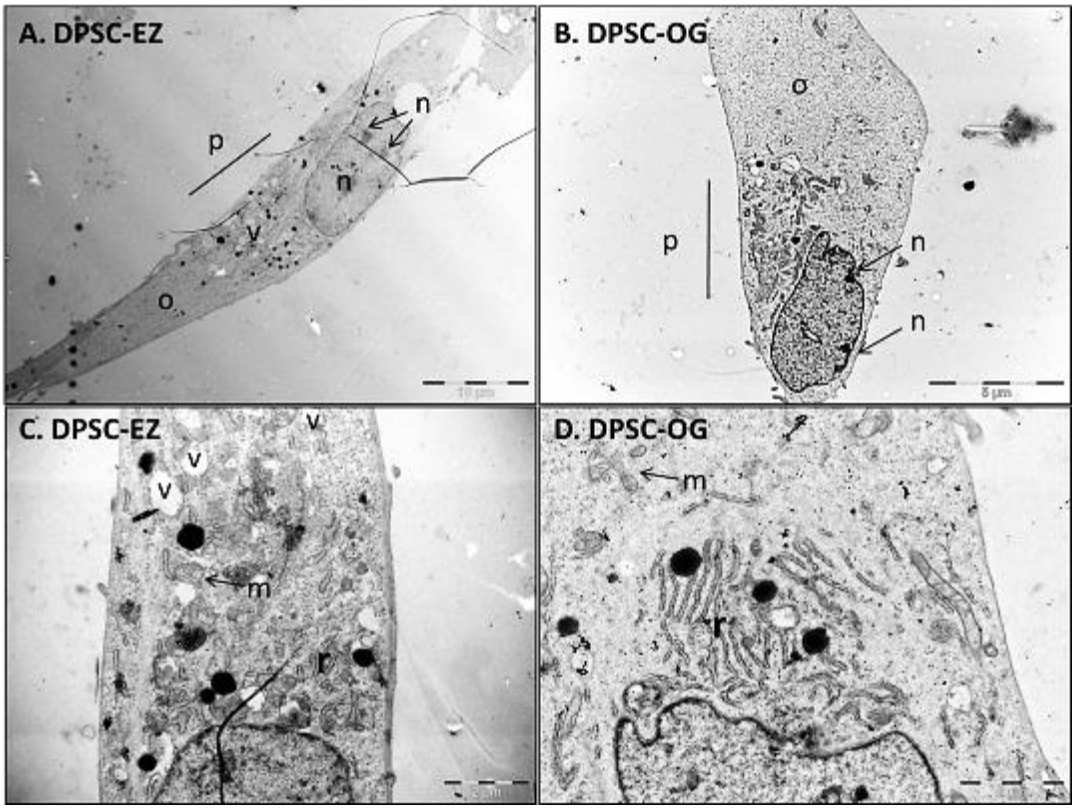

Figure 6: Comparison of DPSCs-EZ and DPSCs-OG at ultrastructural level by means of TEM. No clear differences in ultrastructure were observed between DPSCs-EZ and DPSCs-OG. A normal oval cell shape with a perinuclear organelle-rich zone (p), containing, rough endoplasmic reticulum cisternae (r) and mitochondria (m), could be clearly distinguished from of an organelle-poor peripheral zone (o) for both DPSCs-EZ and DPSCs-OG. Large euchromatic nuclei with one or more nucleoli (n) were visible. However, extensive vacuolization throughout the entire cytoplasm (v) was found for DPSCs-EZ, but not for DPSCs-OG. Scale bar: A = 10µm, B = 5µm, C and D = 2µm

3.1.3 Colony forming unit-fibroblast (CFU-F) assay

A fibroblast colony forming unit (CFU-F) assay was performed, in accordance with Friedenstein *et al*., 1976, as a traditional assay to identify MSCs, based on their properties of plastic adherence and self-renewal or colony formation [(86)]. DPSCs-EZ and DPSCs-OG were seeded at low densities and were cultured for 10 days. Both DPSCs-EZ and DPSCs-OG cells were able to form macroscopic colonies of > 50 cells (Fig.7 A,B,C). No significant differences in colony formation were found between DPSCs-EZ and DPSCs-OG after quantification (Fig.7 D).

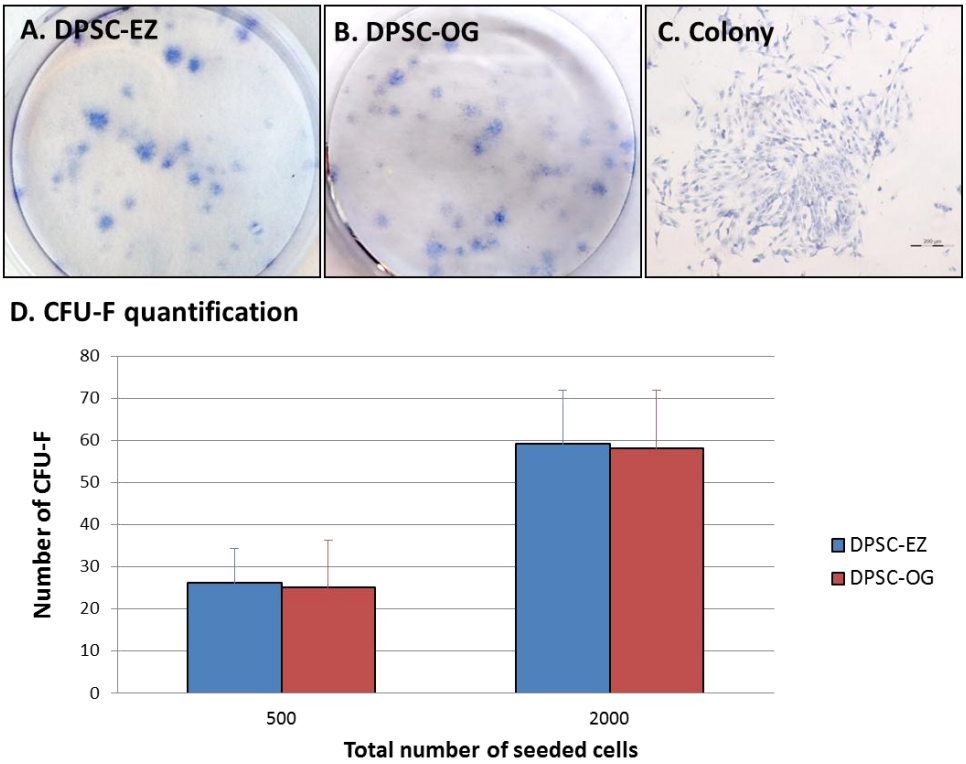

Figure 7: CFU-F assay of DPSCs-EZ and DPSCs-OG. Colony formation was observed for both DPSCs-EZ (A) and DPSCs-OG (B). No differences in colony formation were observed between DPSCs-EZ and DPSCs-OG. Data were analyzed by means of a Mann-Whitney Test. Colony formation was assessed for 4 different patients for both DPSCs-EZ and DPSCs-OG (n = 4). Scale bar: C = 200µm. Data are expressed as mean ± SD.

3.1.4 MTT proliferation assay

An MTT assay was performed to evaluate the proliferation properties of DPSCs-EZ and DPSCs-OG. DPSC proliferation was measured at different time points: 24, 48, 72 and 96 hours after seeding. No significant differences in proliferation were found between DPSCs-EZ and DPSCs-OG (Fig. 8).

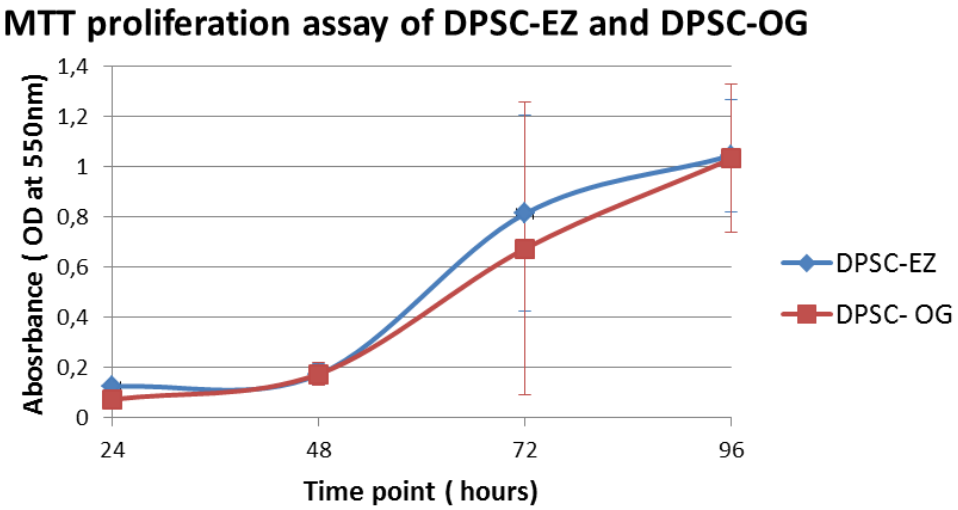

Figure 8: MTT proliferation assay of DPSCs-EZ and DPSCs-OG. DPSCs-EZ and DPSCs–OG were seeded at a density of 6250 cells per cm^2 in a 96-well plate, provided with DSCM After 24, 48, 72 and 96 hours an MTT proliferation assay was performed. No differences in proliferation were observed between DPSCs-EZ and DPSCs-OG. Data were analyzed by means of a Mann-Whitney Test. The assay was repeated for 6 different patients (n = 6) in 3 independent experiments. Data are expressed as mean ± SD.

3.1.5 Surface marker expression analysis by means of FACS

Surface marker expression of DPSCs-OG and DPSCs-EZ was investigated by means of FACS analysis. Both stem cells groups were negative for the hematopoietic stem cell (HSC) markers CD34 and CD45, but were strongly positive for the MSC marker CD90, with significantly higher expression by DPSCs-OG compared to DPSCs-EZ (p-value = 0.043). DPSCs-EZ and DPSCs-OG were mild to strongly positive for the MSC marker CD44, with again a higher expression for DPSCs-OG compared to DPSCs-EZ (p-value = 0.04). However, much patient variability was observed for this marker.

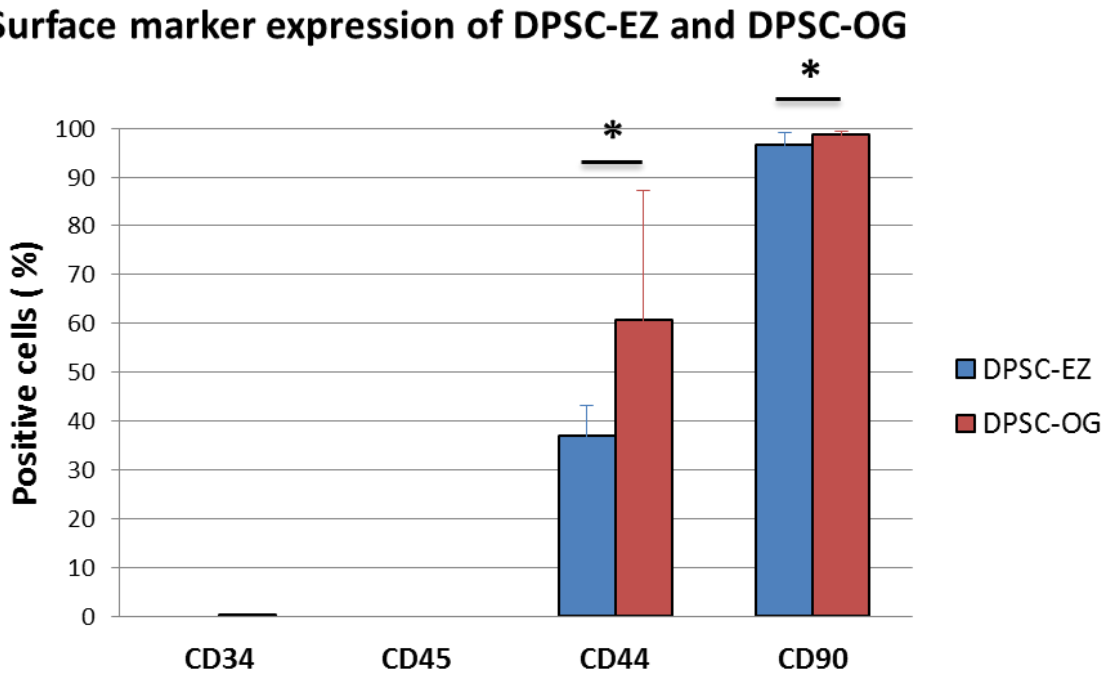

Figure 9: Comparison of surface marker expression profiles of DPSCs-EZ and DPSCs-OG. Marker expression of DPSCs-EZ and DPSCs-OG (passage 2,3) were investigated by means of FACS analysis. DPSCs-EZ and DPSCs-OG were negative for CD34 and CD45, but strongly positive for CD90, with a significantly higher expression of CD90 for DPSCs-OG. Variable expression levels for CD44 were found for both DPSCs-EZ and DPSCs-OG, with significantly higher expression levels for DPSCs-OG. A student t-test was performed to compare marker expression levels. * p-value < 0.05. Marker expression was examined for 9 different patients (n = 9) in 7 independent experiments. Data are expressed as mean ± SD.

3.1.6 Immunocytochemical analysis marker expression

Immunocytochemical stainings for CD29 and CD117 were performed on DPSCs-EZ and DPSCs-OG (Fig.10 A-I). A uniform expression of the MSC marker CD29 was observed for both DPSCs-EZ and DPSCs-OG (Fig.10 A-F). The HSC marker, CD117, was expressed by most (Fig.10 C-E green), but not all patients (Fig.10 A-C green), with variable fluorescence intensity. CD117 was mainly observed perinuclear and in the cell body (white arrows) (Fig.10 D-F). DAB immunostainings were used to verify the expression of CD117. Again, CD117 was found to be expressed in both DPSCs-OG and DPSCs-EZ (brown) (Fig.10 G-I). However, only subfractions of DPSCs were positive for CD117. No clear differences in expression were observed between DPSCs-OG and DPSCs-EZ for CD117.

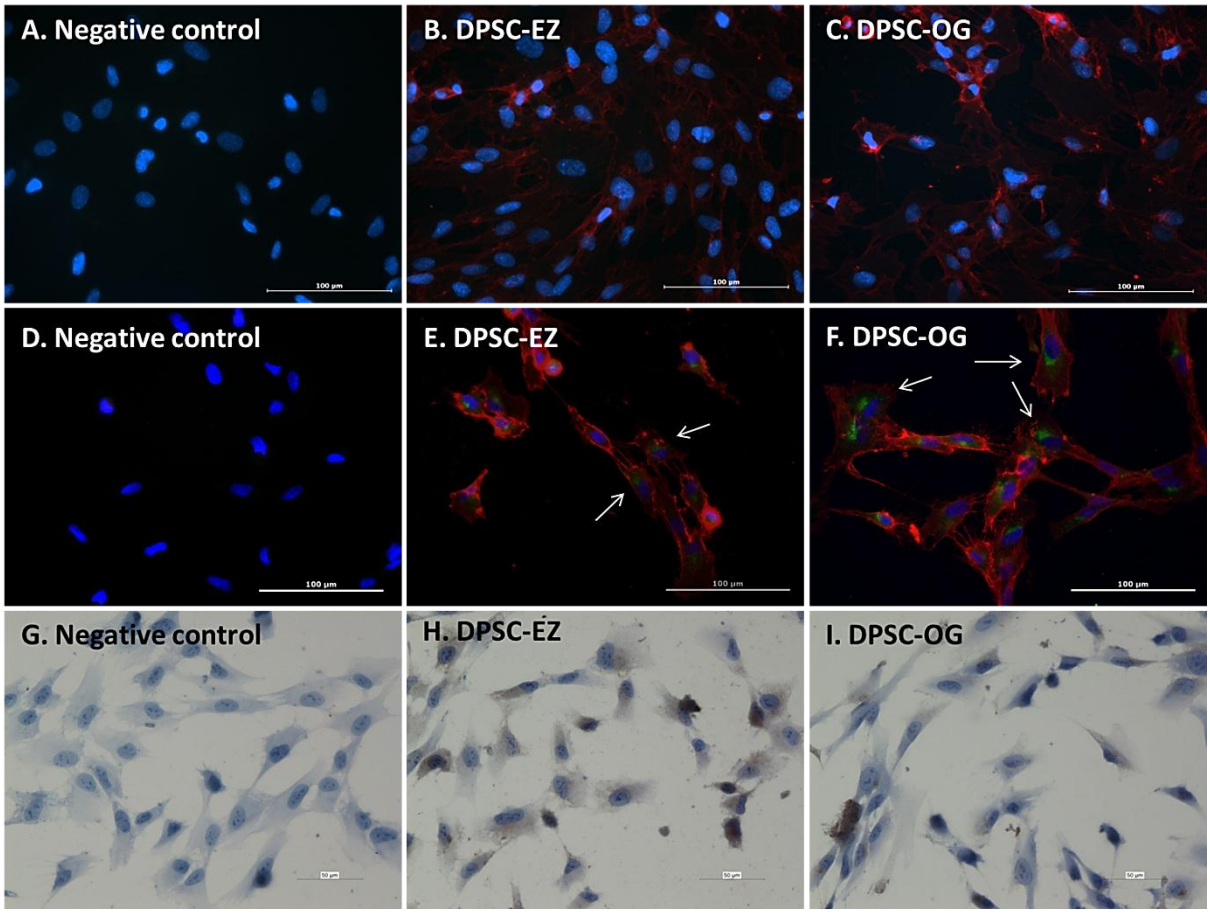

Figure 10: Immunocytochemistry for CD29 and CD117 on DPSCs-EZ and DPSCs-OG. Immunocytochemistry was performed for CD29 (red), CD117 (green) with a DAPI nucleus staining (blue). A uniform expression of the MSC marker CD29 was observed on the cytoplasma membrane of both DPSCs-OG and DPSCs-EZ (A-F). A heterogeneous expression was observed for the fluorescence stainings against the HSC marker CD117, which was expressed by DPSCs of most (D-F), but not all patients (A-C), with variable fluorescence intensity. The expression of CD117 was validated DAB immunocytochemical stainings (G-I). No differences in expression for CD117 were observed between DPSCs-EZ and DPSCs-OG. The fluorescence stainings were repeated for 6 different patients (n = 6) and the DAB stainings for 3 different patients (n = 3) in 6 independent experiments. Scale bar A-F = 100µm, G-I = 50µm.

3.2 Comparing the angiogenic properties of the different DSCs and HGF-1

3.2.1 Morphology of the different DSCs

The morphology of the different DSCs was compared by means of phase-contrast microscopy. Both DPSCs and FSCs showed a typical fibroblast-like morphology with long cytoplasmic processes (Fig.11 A-D). For the FSCs, side populations of cubic cells were also visible during the isolation and at early passages (Fig.11 C). The morphology of the SCAPs was rather polygonal with multiple cytoplasmic processes (Fig.11 E, F).

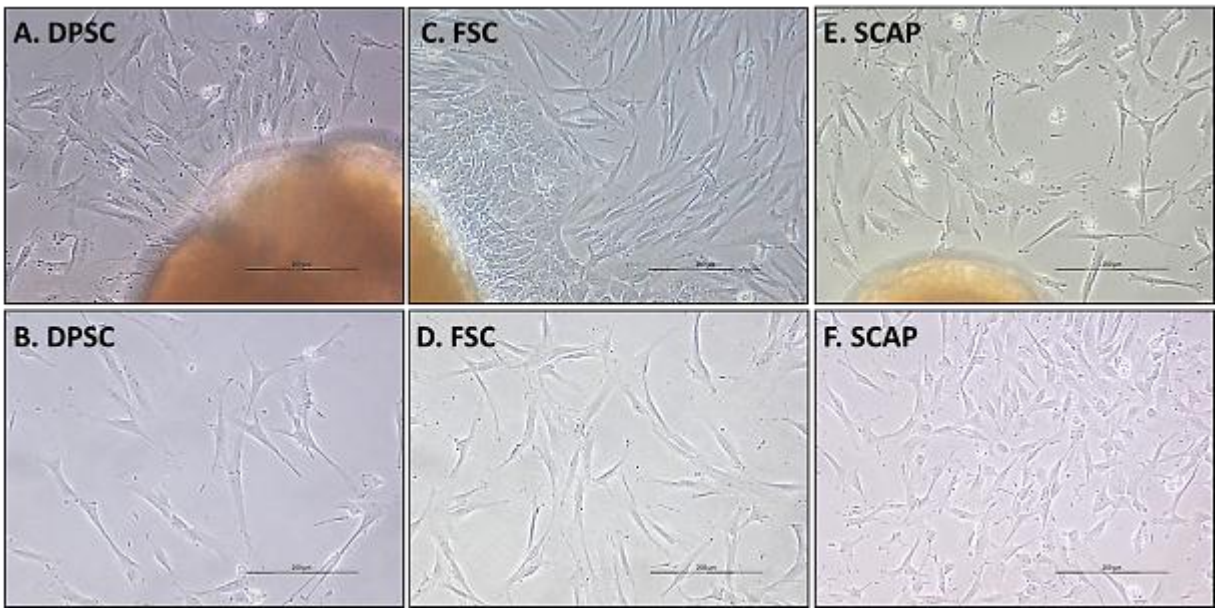

Figure 11: Comparing the morphology of the different DSC populations. A typical fibroblast-like morphology with long cytoplasmic processes was observed for DPSCs and FSCs (A-D). Cubic cells were also visible for the FSCs at early passages (C). The morphology of the SCAPs was rather polygonal with multiple cytoplasmic processes (E-F). Scale bar = 200µm

3.2.2 Colony formation

A CFU-F assay was also performed to compare the plastic adherence and self-renewal properties of the different DSCs. DPSCs, FSCs and SCAPs were able to form macroscopic colonies and no differences in colony formation were found after quantification between the different DSCs. A trend towards lower CFU-F numbers for DPSCs compared to FSCs and SCAPs at a density of 52 cells per cm^2 was observed, but this was not present at 208 cells per cm^2.

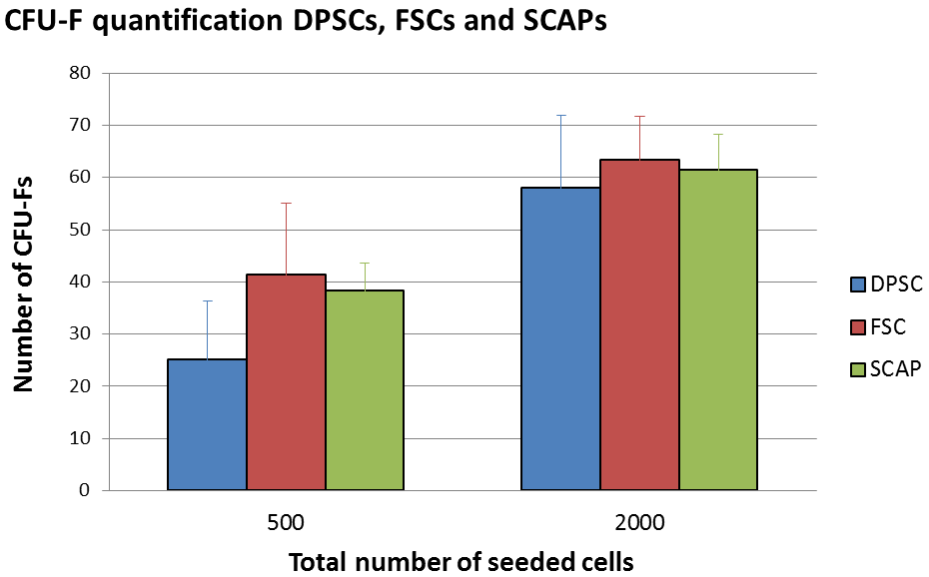

Figure 12. CFU-F assay of DPSCs, FSCs and SCAPs. DPSCs, FSCs and SCAPs were able to form macroscopic colonies and no differences in colony formation were found after quantification. A trend towards lower CFU-F numbers was found for DPSCs compared to FSCs and SCAPs at a density of 52 cells per cm^2, but not at 208 cells per cm^2. Data were analyzed by means of a Kruskall-Wallis test and a Dunn's Post Test. CFU-F assays were performed on 4 patient samples for DPSCs (n = 4) and 5 different patients for FSCs and SCAP (n = 5). Data are expressed as mean ± SD.

3.2.3 FACS analysis markers

Marker expression profiles of DPSCs, FSCs and SCAPs were compared by means of FACS analysis. All 3 DSC populations were negative for the HSC markers CD34 and CD45. Mild to high expression levels were observed for the MSC marker CD44. All groups were strongly positive for the MSC markers CD105 and CD90. No significant differences in marker expression were observed between DPSCs, FSCs and SCAPs.

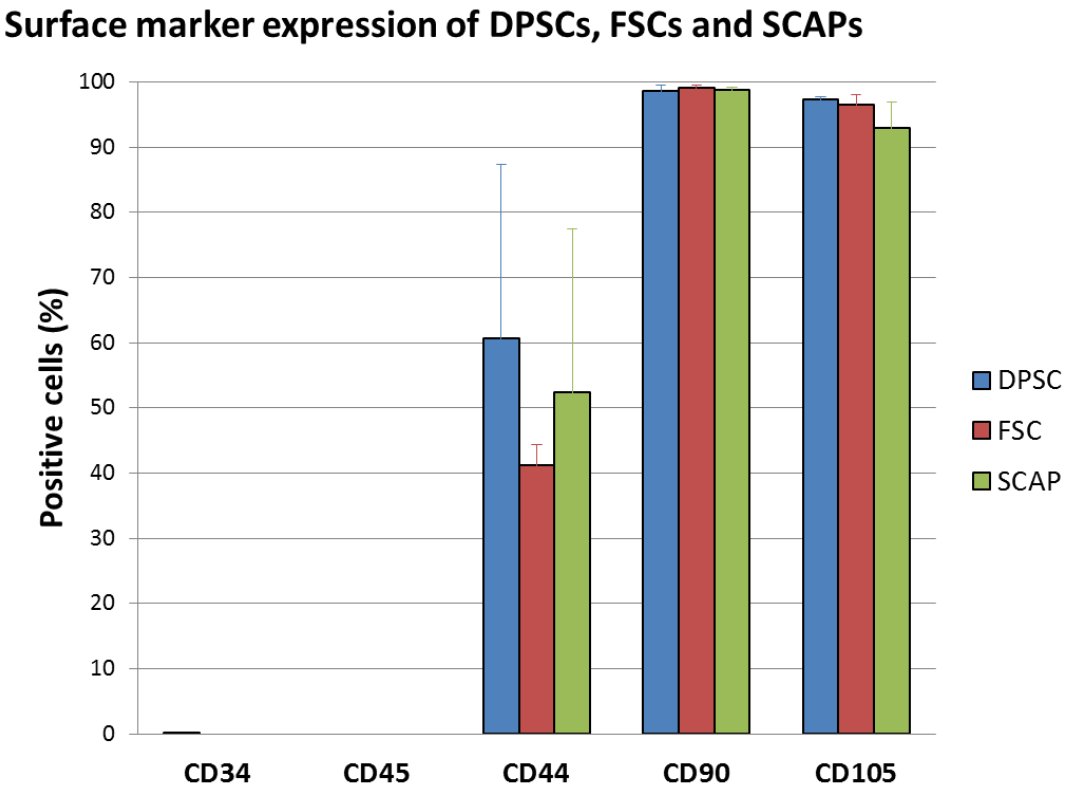

Figure 13: Comparison of surface maker expression profiles of DPSCs, FSCs and SCAPS. The marker expression profiles of DPSCs, FSCs and SCAPs (passage 2,3) were investigated by means of FACS analysis. All DSCs were negative for CD34 and CD45, but strongly positive for CD90 and CD105. Moderate expression levels for CD44 were found for DPSCs, FSCs and SCAPs. A Kruskall-Wallis Test was performed with a Dunn's Post Test to compare marker expression levels. Marker expression was examined on 9 different patients for DPSCs, except for CD105 for which n =2, and on 6 patients for FSCs and SCAPs (n =6) in 10 independent experiments. Data are expressed as mean ± SD.

3.2.4 Immunocytochemical analysis marker expression

Immunocytochemical stainings for CD117 were performed on DPSCs, FSCs and SCAPs. All the populations were positive for CD117, but only subfractions of the DPSCs showed expression with a more perinuclear location (Fig.14 B). A uniform expression was observed for FSCs and SCAPS throughout the entire cell (Fig.14 C, D).

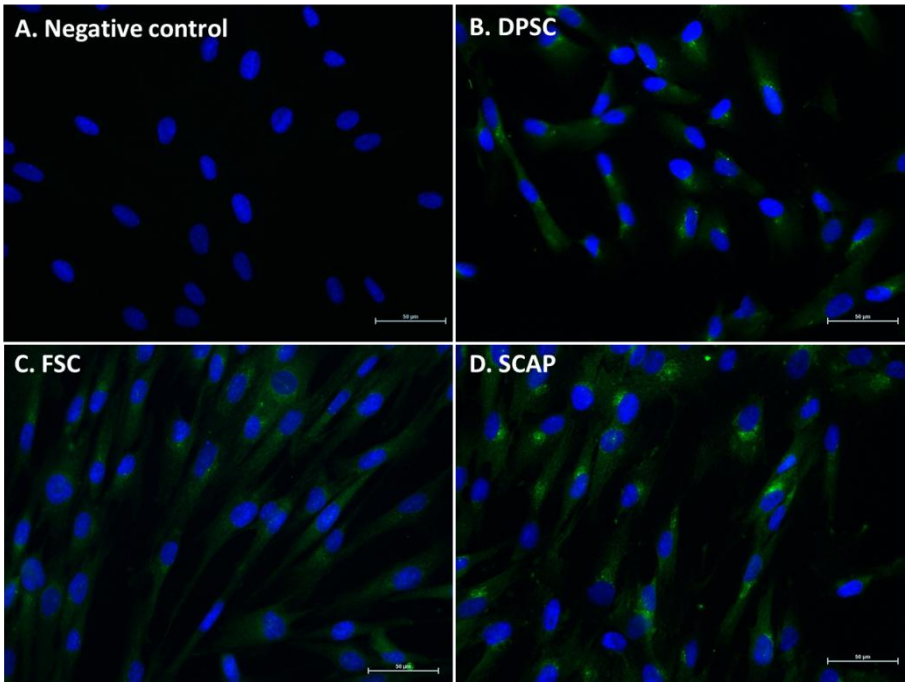

Figure 14: Immunocytochemistry for CD117 on DPSCs, FSCs and SCAPs. Immunocytochemistry was performed for CD117 (green) with a DAPI nucleus staining (blue). All DSCs were positive for CD117, but only subfractions of the DPSCs showed expression with a more perinuclear location (B). A uniform expression was observed for FSCs and SCAPs throughout the entire cell (C,D).The stainings were repeated for 3 different patients (n = 3) in 2 independent experiments. Scale bar = 50µm.

3.2.5 Human Angiogenesis Array

A Human Angiogenesis Array was performed to investigate the relative expression of 55 angiogenesis-related proteins in the CM of DPSCs, DFSCs, SCAPs and HGF-1. DSCM with 0.1% of FBS was included as a control. Expression levels were normalized against positive and negative reference spots. Numerous pro- and anti-angiogenic factors were identified in the CM (Fig.15). The amount of angiogenic factors in the 0.1% DSCM was equal to the negative reference values for most factors or very low, indicating that the identified angiogenic factors are indeed produced by the DSCs and the HGF-1 and thus not already present in the 0.1% DSCM. High levels of the anti-angiogenic factors TIMP-1, PAI-1, TSP-1, PTX-3 and PEDF were observed in the DSC and HGF-1 CM. A higher secretion of the pro-angiogenic factors ANG and IGFBP-2 was found for the HGF-1 compared to the DSCs. DSCs secreted more of the pro-angiogenic factors IGFBP-3, uPA and VEGF compared to the HGF-1. Higher levels of CXCL16, IL-8 and MCP-1 were found for FSCs compared to the other groups. Since the Array was only repeated on 2 different patient samples, RT-PCR and ELISA were performed to study the exact concentrations and expression levels of the identified factors.

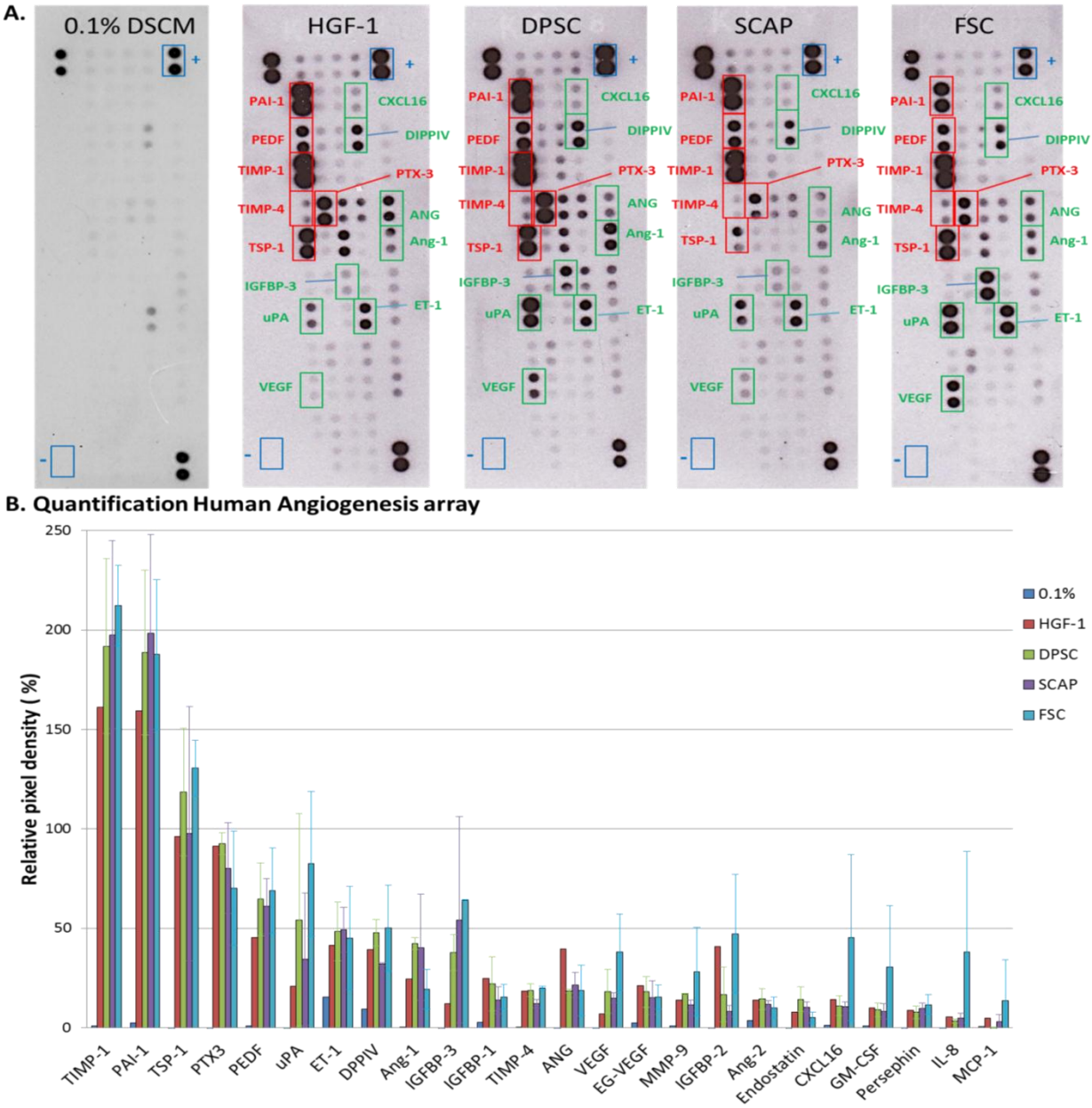

Figure 15: Human Angiogenesis Array on DSC and HGF-1 CM. A Human Proteome Profiler™ Angiogenesis Array was performed to assess the relative expression of 55 angiogenesis-related proteins in the CM of HGF-1s (passage 5), DPSCs, SCAPs and FSCs (passage 2,3). 0.1% DSCM was included as a control. Each blot was normalized against positive (+) and negative (-) reference spots. High levels of the anti-angiogenic factors TIMP-1, PAI-1, TSP-1, PTX-3 and PEDF were observed in the DSC and HGF-1 CM. A higher secretion of the pro-angiogenic factors ANG and IGFBP-2 was found for the HGF-1 compared to the DSCs. DSCs secreted more of the pro-angiogenic factors IGFBP-3, uPA and VEGF compared to the HGF-1. Higher levels of CXCL-16, IL-8 and MCP-1 were found for FSCs compared to the other groups. The array was repeated for 2 different patients (n = 2) in 2 independent experiments with only one repeat for the 0.1% DSCM and HGF-1 CM (n = 1). Data were expressed as mean ± SD.

3.2.6 Reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR was performed on VEGF, to validate this pro-angiogenic factor on mRNA level. β2-microglobuline was used as a housekeeping gene and non-template controls (nt) were included. A moderate expression of VEGF was found for all DSCs populations with a minimal expression by the HGF-1. However, for the DSCs much patient variability was found. A persistent expression of β2-microglobuline was shown for all patient samples.

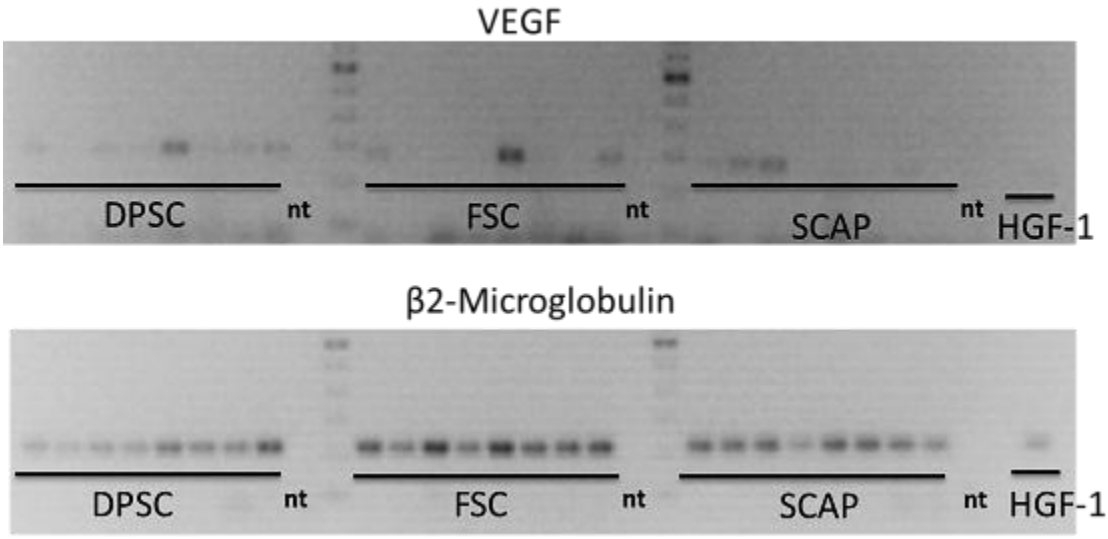

Figure 16. RT-PCR: Expression of VEGF on mRNA level for DSCs and the HGF-1. A moderate expression of VEGF was found for DPSCs, FSCs, SCAPs with a minimal expression for the HGF-1. For the DSCs patient variability was found. A persistent expression of β2-microglobuline was shown for all samples. mRNA expression was examined on 8 different patients samples (n = 8) and the HGF-1 were included once (n = 1).

3.2.7 Enzyme-linked immunosorbent assay (ELISA)

ELISAs were performed on CM of DPSCs, FSCs, SCAPs and the HGF-1 to validate and quantify the factors CXCL-16, IGFBP-3 and VEGF, which were identified in the angiogenesis array. The CXCL-16 levels were lower than the detection limit of the ELISA (data not shown). IGFBP-3 was found in the CM of all DSCs and HGF-1, with a trend towards a higher secretion by the FSCs (Fig.17). High levels of VEGF were shown in the CM of DPSCs, FSCs and SCAPs, with observance of some patient variability. Only low levels of VEGF were detected in the CM of the HGF-1 (Fig.17). A trend towards a higher secretion of VEGF was seen for the DPSCs, however, more repeats are needed to confirm this.

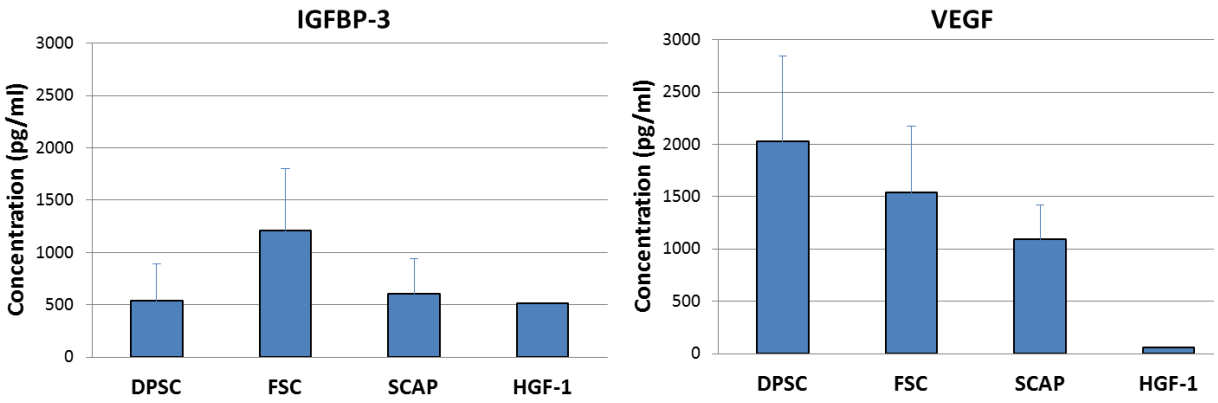

Figure 17: ELISA for IGFBP-3 and VEGF on DSC and HGF-1 CM. The concentration of IGFBP-3 and VEGF in the CM of DPSCs, SCAPs and FSCs (passage 2,3) and the HGF-1 (passage 5) was determined by means of ELISA. IGFBP-3 was detected in the CM of the DSCs and the HGF-1. High levels of VEGF were shown in the CM of the DSCs, with only low concentrations in the CM of HGF-1. No significant differences in IGFBP-3 and VEGF secretion were found between the different DSCs. A Kruskall-Wallis Test was performed with a Dunn's Post Test to compare the groups. ELISAs were performed on 6 different patient samples for the DSCs (n = 6) and the HGF-1 cells were included once (n = 1) in 3 independent experiments for each factor.

3.2.8 *In situ* immunohistochemistry for angiogenic factors

Tissue sections of the different dental tissues were also investigated for expression of VEGF by means of immunohistochemical stainings *in situ*, as an additional validation to test if VEGF secretion by DSCs is an intrinsic property and not induced by *in vitro* culturing conditions. The MSCs and PC marker CD146 was also included. CD146 was mainly found surrounding blood vessel, but subfractions of slightly positive cells were also seen in all dental tissues. The *in situ* stainings revealed the presence of VEGF in all the dental tissues. VEGF levels seemed higher in the pulp and follicle tissues than in the apical pads.

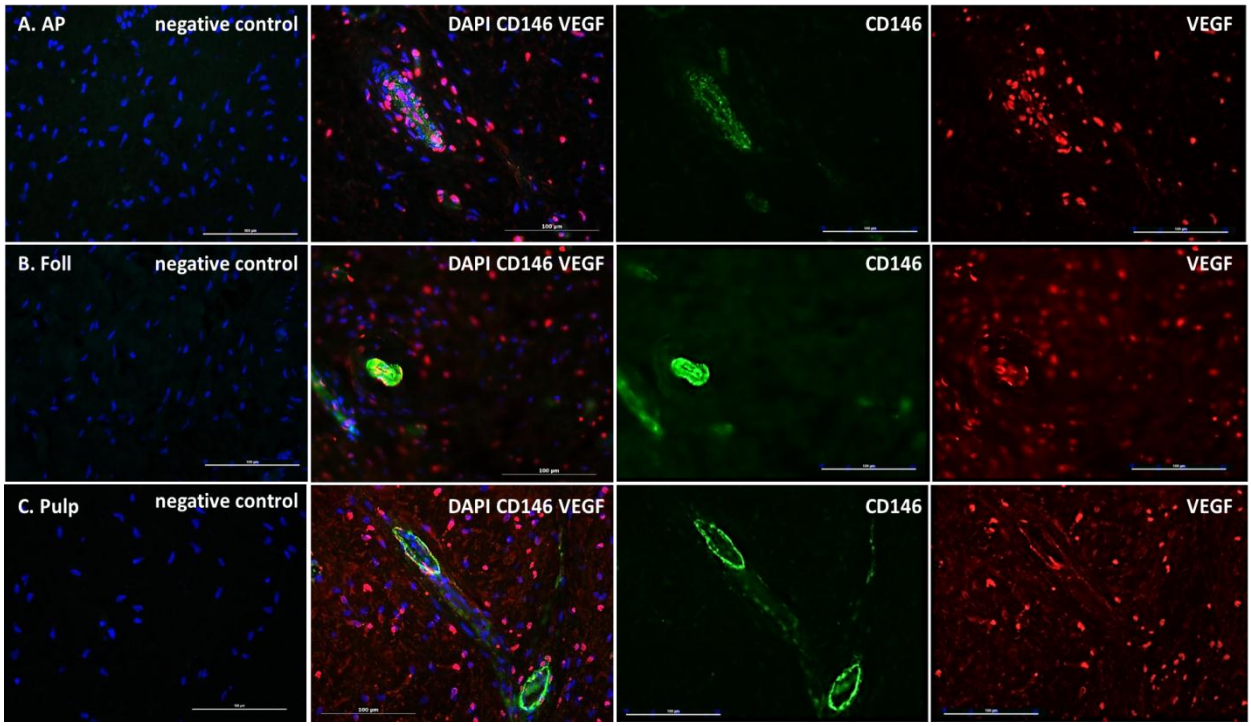

Figure 18: *In situ* immunohistochemical stainings for CD146 and VEGF in the dental tissues. Paraffin sections of the dental tissues were stained against the pro-angiogenic factor VEGF (red) together with the MSCs marker CD146 (green) with a DAPI nucleus staining (blue). CD146 was mainly found surrounding the blood vessels, with slightly positive cells in all dental tissues. *In situ* stainings revealed the presence of VEGF in all the dental tissues. Each staining was repeated for 2 different patients (n = 2) in 2 independent experiments. Scale bar = 100µm

Table 3.1: Immunohistochemistry of the dental tissues

	Apical papilla	Dental follicle	Dental pulp
CD146	+	++	++
VEGF	++	++	+++

+ mild positive, ++ strong positive, +++ very strong positive

3.2.9 MTT proliferation assay

The effect of DSCs on EC proliferation was explored in an MTT assay. HMEC-1 cells were cultured in DSC and HGF-1 CM for 72 hours or in 0.1% and 10% DSCM as a negative and positive control respectively. No significant increases in HMEC-1 proliferation were observed between the conditions with CM of DPSCs, FSCs and SCAPs compared to the negative control of 0.1% DSCM. A trend towards an increase in proliferation was found for the HGF-1 CM. Unfortunately, because of problems in the cultures of these cells insufficient repeats were performed for statistical analysis (n = 2).

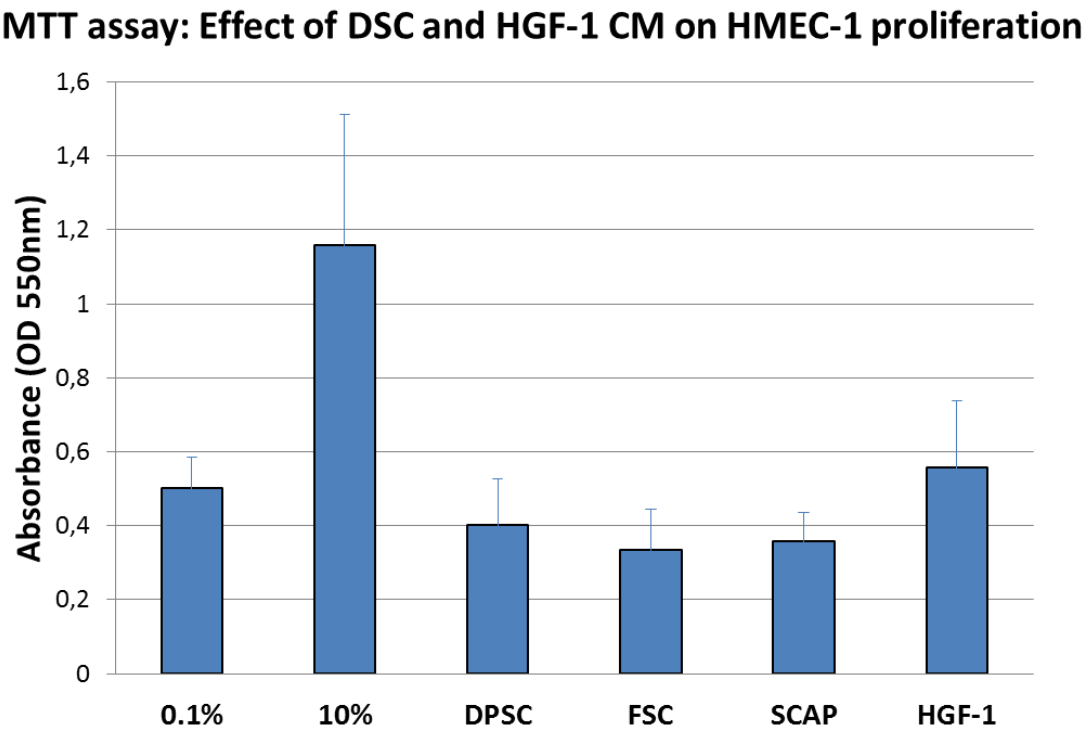

Figure 19: MTT assay: Effect of DSC CM on HMEC-1 proliferation. HMEC-1 cells were seeded at a density of 15625 cells per cm^2. After overnight incubation, the cells were put on DSC of HGF-1 CM for 72 hours and an MTT assay was performed. 0.1% DSCM and standard DSCM were used as negative and positive control respectively. No significant increases in HMEC-1 proliferation were observed between the control conditions and the DSC CM. A trend towards an increase in HMEC-1 proliferation was found for the HGF-1 condition, however, insufficient repeats were performed for statistical analysis (n=2). Data were analyzed by means of a Kruskal-Wallis test with a Dunn's Post Test. The MTT assay was repeated in 4 independent experiments with n = 5 for 0.1%, 10% and DPSC and n= 7 for FSC and SCAP. Data are expressed as mean ± SD.

3.2.10 Transwell migration assay

Since EC migration is an essential step in angiogenesis, it was tested if DSCs induce HMEC-1 migration by means of a Transwell migration system. DPSCs, FSCs and SCAPs were seeded in a lower compartment and were induced to secrete growth factors by serum starvation [84, 87]. HMEC-1 cells were seeded in the upper compartment and migration of the HMEC-1 through an 8µm pore membrane towards the DSCs was examined (Fig.20 A). 0.1% and 10% DSCM were added in the lower compartment as negative and positive control respectively. HMEC-1 migration was induced by all DSC populations. A significant increase in HMEC-1 migration was shown for SCAPs compared to 0.1% DSCM (p-value < 0.05), with a trend towards an increase for the DPSCs and FSCs (Fig.20 D-G).

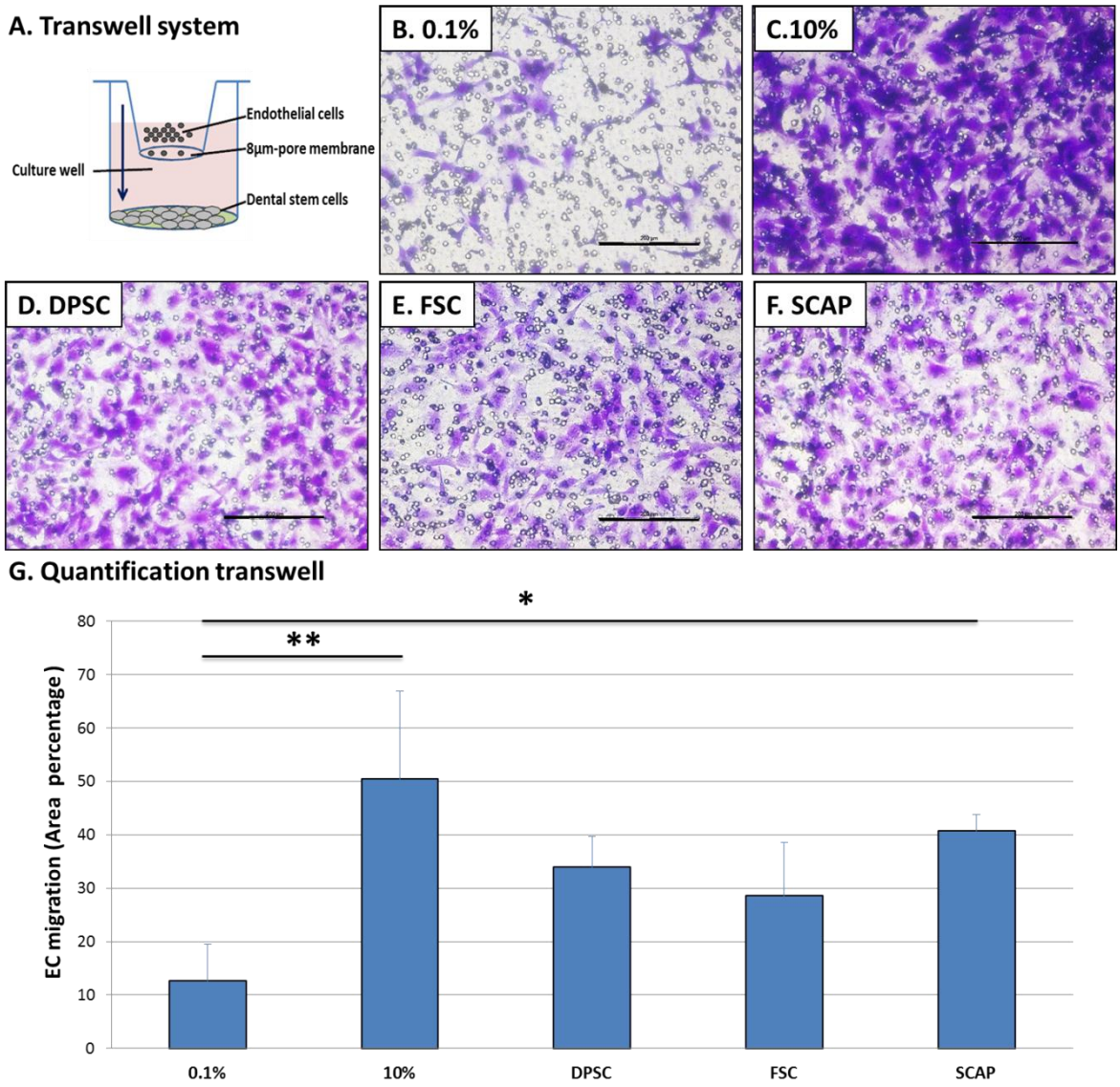

Figure 20: Transwell migration assay. DPSCs, FSCs and SCAPs were seeded in 24-well plates in DSCM at a density of 25000 cell per cm^2 (lower compartment). The cells were put on 0.1% DSCM to induce growth factor secretion. HMEC-1 cells were seeded on Thincert™ tissue culture inserts of pore size 8µm in 0.1% DSCM at a density of 1488 cells per cm2 (upper compartment)(A). After 24 hours the inserts were fixed and migration was quantified. A significant increase in HMEC-1 migration was shown for SCAPs compared to 0.1%, with a trend toward an increase for the DPSCs and FSCs (D-G). The assay was repeated in 5 independent experiments with n = 6 for 0.1%, 10% and the FSCs, n = 4 for the DPSCs and n = 5 for the SCAPs. Data were analyzed by means of a Kruskal-Wallis test with a Dunn's Post Test. * p-value < 0.05; ** p-value < 0.01. Data are expressed as mean ± SD.

3.2.11 Matrigel tube formation assay

To investigate if DSCs induce EC tube formation, a Matrigel assay was performed. HMEC-1 were seeded on growth factor-reduced Matrigel in DSC and HGF-1 CM or in 0.1% and 10% DSCM as negative and positive control respectively. After 24 hours the amount of tube formation was examined. DPSC CM significantly increased tube formation compared to the negative control (Fig.21 C,G) (p-value < 0.05). No significant differences in tube formation were observed between the CM of FSCs, SCAPs and the HGF-1 compared to the negative control (Fig.21 D,E,G).

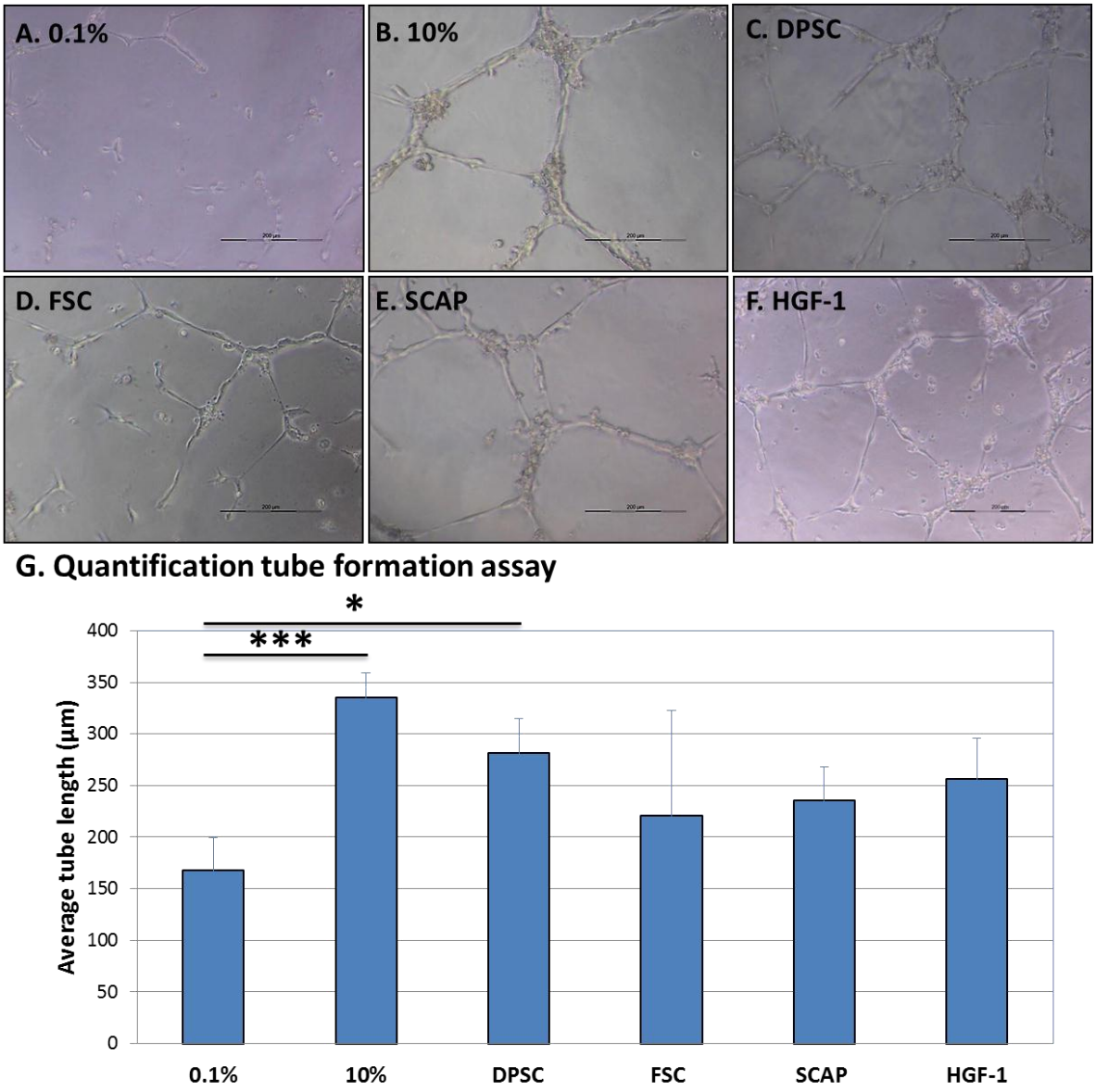

Figure 21: Matrigel tube formation assay. HMEC-1 were seeded on growth factor-reduced Matrigel in 15µ angiogenesis slides at a density of 40 000 cells per cm^2. The HMEC-1 were seeded in DSC or HGF-1 CM with 0.1% DSCM or 10% DSCM as a negative and positive control respectively. After 24 hours, the average length of 5 random tubes was measured. Tube formation was significantly increased by the DPSC CM compared to the negative control (G). No differences in tube formation were observed for FSC (D), SCAP (E) and HGF-1 (F) CM compared to the control conditions. The assay was repeated in 5 independent experiments with n = 5 for 0.1 and 10%, n = 13 for the DPSCs, n = 10 for the FSCs and n = 12 for the SCAPs. Data were analyzed by means of a Kruskal-Wallis Test with a Dunn's Post Test. * p-value < 0.05; *** p-value < 0.001. Data are expressed as mean ± SD.

3.2.12 Chorioallantoic Membrane (CAM) Assay (*in ovo*)

A chorioallantoic membrane (CAM) assay was performed to provide more insights in the angiogenic properties of the DSCs and the HGF-1 *in ovo*. This model resembles the *in vivo* situation, since all blood vessel cell types are involved. A single control and test sponge was placed on the CAM at E9 (Fig.22 A). For the test conditions DPSC CM, DPSC CM concentrate, 10 000 DPSCs and 50 000 DPSCs were used. At E12, the number of blood vessels growing towards the gelatin sponges (*), intersecting a circular grid (Fig. 22 CC), were quantified (Fig.22 B,C,D).

No differences were found for any of the test conditions compared to the controls. Since this was a first pilot experiment, optimization is needed with inclusion of more repeats per condition.

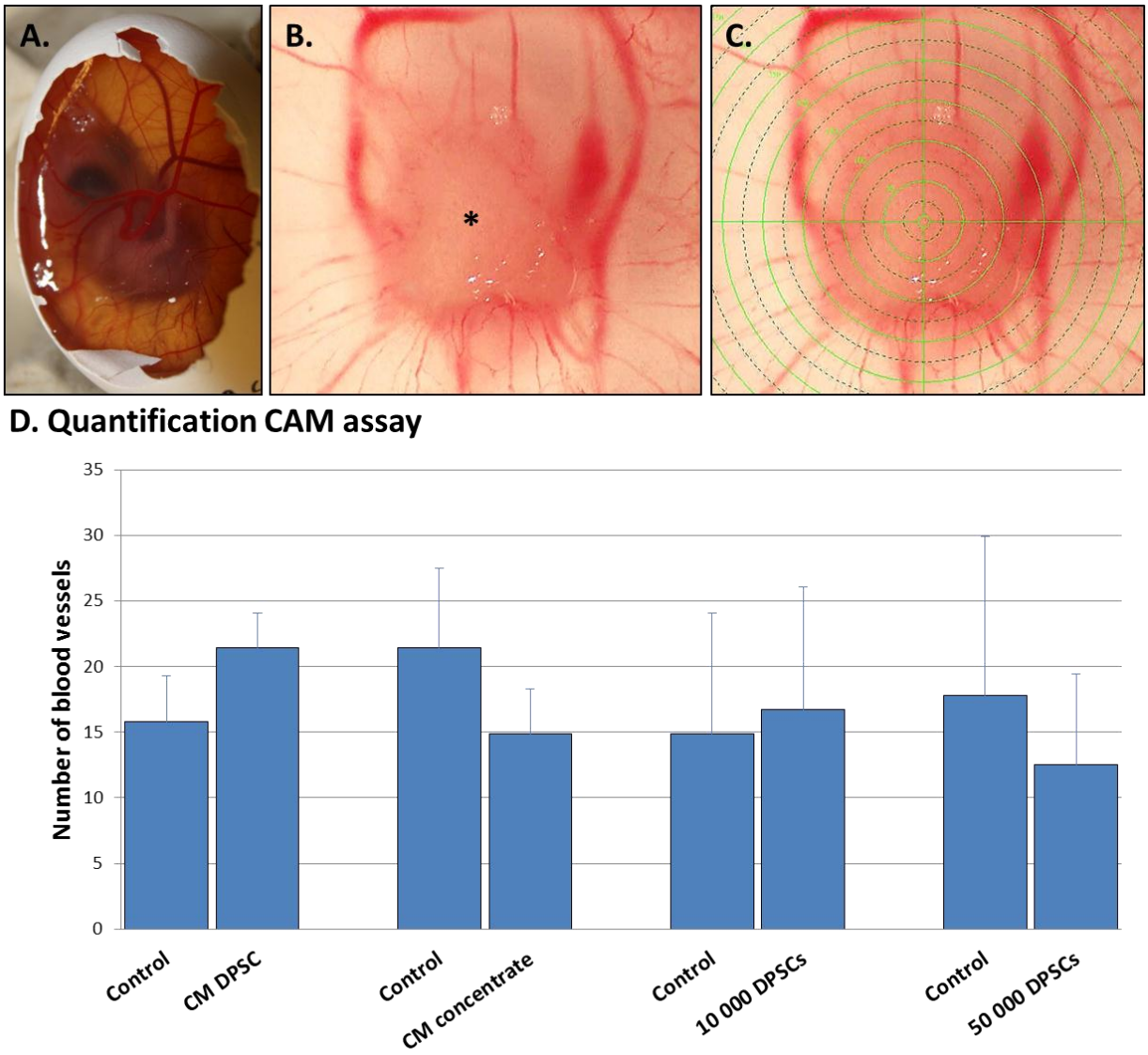

Figure 22: CAM assay: the angiogenic effects of DPSCs and DPSC CM *in ovo*. A control and a test sponge were placed on the CAM of a fertilized chick egg at E9 (A). DPSC CM, DPSC CM concentrate, 10 000 DPSCs and 50 000 DPSCs were used as test conditions together with a 0.1 DSCM control for the CM conditions and a PBS control for the DPSC conditions. At E12, the number of blood vessels growing towards the gelatin sponges (*) were quantified (B,C,D) with the aid of a grid (C). No differences were found for any of the test conditions compared to the controls. Control and test conditions were compared by means of a Mann-Whitney test. Data are expressed as mean ± SD. For DPSC CM n = 5, for CM concentrate n = 7, for 10 000 DPSCs n = 7, for 50 000 DPSCs n = 4 for test and 5 for control.

4. Discussion

4.1 Comparing an EZ and OG method for DPSC isolation.

The main goal of this study was to explore and compare the angiogenic properties of the different DSC populations. However, for use in therapeutic applications, stem cells need to be isolated from their microenvironment and expanded *ex vivo* to obtain sufficient cell numbers [88-91]. Therefore, in addition to considering the therapeutic potential of DSCs in angiogenesis, their behavior *in vitro* needs to be investigated, including the effects of different isolation techniques and the effects of *in vitro* cell expansion. MSCs, including DSCs, can be isolated from different sources, yielding heterogeneous populations; however, no specific marker is available to identify these cells. Consequently, there is a need for validation and standardization of the isolation and expansion protocols for MSCs for experimental and clinical use Moreover, the isolation technique needs to be suited for large scale clinical use, i.e. give rise to a high yield of potent stem cells by means of an efficient procedure [32, 33, 50, 88, 89, 91, 92]. Therefore, the first part this study focused on the effect of the isolation procedures on stem cell properties [77, 78]. Since DPSCs were first identified and are the most studied DSC population, they were chosen for this part of the study [55, 93]. Two widely used isolation techniques were compared here, namely an EZ and an OG technique [77, 78, 89, 90, 93].

For the EZ method, fragments of pulp tissue were digested with a collagenase-dispase solution for one hour, which leads to degradation of the extracellular matrix. After passing the obtained suspension through a strainer, the resulting single cell solution was put into culture. The EZ method already gave rise to adherent cells overnight and confluence was reached after approximately 1 week. Although this is a fast way to start up a culture, this methods is not very specific, since all the cell types of the tissue are included [77, 94]. Indeed, heterogeneous populations of DPSCs were obtained with mostly typical fibroblast-like spindle-shaped cells, but also polygonal cells with multiple processes, which was previously for DPSCs-EZ and SHEDs-EZ [77, 78].

Small fragments of pulp tissue were put into culture without any pretreatment to allow outgrowth of DPSCs in the OG method, since it is based on the migratory and plastic adherence properties of MSCs [40, 77, 90, 91]. The tissue fragments started to adhere after 2 to 4 days and outgrowing cells with an elongated shape were visible from then on. The wells reached confluence after 10 to 14 days. The OG isolation resulted in a more homogeneous population of DPSCs with a spindle-shaped morphology and long cytoplasmic processes. Only the most potent stem cells will be able to migrate out of the explant pieces. After outgrowth of these cells, the explants were removed together with remaining non-migrated cells. In addition, the risks for contamination of other cell types are strongly reduced, in accordance with results for DPSCs of Bakopoulou *et al*., 2011 [77] and Jing *et al*., 2011, for adipose tissue stromal cells [95].

Although fibroblast contamination can still occur, it can be overcome with an additional selection step by culturing the explants on fibronectin-coated surface, as reported by Spath *et al*., 2006 [90]. No clear differences in ultrastructure were observed between the DPSCs-EZ and DPSCs-OG. A normal oval cell shape with a perinuclear organelle-rich zone could be clearly distinguished from of an organelle-poor peripheral zone for both DPSCs-EZ and DPSCs-OG and large euchromatic nuclei with multiple nucleoli were visible. However, extensive vacuolization throughout the entire cytoplasm was found for the DPSCs-EZ, but not for the DPSCs-OG. This could be explained by the fact that the EZ procedure is rather aggressive [91]. Although collagenase specifically degrades collagen, dispase is a non-specific protease, allowing degradation of surface molecules, which could have an effect on stem cell biology and viability [91]. Therefore, analysis of surface marker expression profiles by means of FACS analysis was also performed. Although Bakopoulou *et al*., 2010 have reported differences in expression of the HSC markers CD34 and CD45 on SHEDs isolated by means of EZ and OG methods [77], both DPSCs-OG and DPSCs-EZ were negative for these markers here, indicating a non-hematopoietic origin of both populations. Significant higher expression levels of the MSC markers CD44 and CD90 were observed for DPSCs-OG compared to DPSCs-EZ. A higher level of CD44 positive cells for DPSCs-OG is not surprising, since the hyaluronan receptor CD44 is important for cellular migration and adhesion, in accordance with the isolation procedures of the explant technique (migration and plastic adherence). A higher level of CD90 positive cells could indicate a higher percentage of DPSCs in accordance with the fact that the OG method only selects for outgrowing plastic adherent cells, whereas the EZ method allows the release of all cell types from the pulp explants. Immunocytochemical stainings for the MSC marker CD29 and the HSC marker CD117, on the other hand, did not reveal any differences between DPSCs-OG and DPSCs-EZ, both cell types were positive for these markers. The expression of CD117, however, was dependent on the patient sample, indicating patient variability, in accordance with Gagari *et al*., 2006 [96], which is complemented by the characteristics of teeth used, e.g. stage of root resorption. Inconsistent results for CD117 expression by MSCs have also been reported in the literature, varying from no expression [71, 74, 97], to slightly positive cells [69] and subfractions of $CD117^{+}$ cells reported for BM MSC [98]. These variations can be explained by differences in isolation and culturing procedures, e.g. the stage of culture growth [99]. Although our results for CD117 are in contrast with the literature, CD117 is known as a marker for cells of neuroectodermal or PC origin, indicating a possible subpopulation of neural crest cells [74, 96]. Indeed, our group has reported positive expression of CD146 on both DPSCs-EZ and DPSCs-OG, suggesting their PC or perivascular origin [77, 100]

As already mentioned, for clinical applications large numbers of stem cells need to be transplanted and, therefore, *in vitro* expansion is necessary. The proliferation rates of cells derived from both isolation methods were assessed by means of an MTT assay at different time points after initial seeding. No differences in proliferation between DPSCs-EZ and DPSCs-OG were found here, which is in accordance with previous studies on SHEDs and Wharton's Jelly-derived MSCs [77, 91]. Although Huang *et al*., 2006, have reported faster proliferation rate for DPSCs-EZ compared to DPSCs-OG, the 2 populations were grown different culture media, which could be the cause of differences in proliferation rates [78]. Proliferation or self-renewal was also assessed by means of a CFU-F assay [40, 86]. The assay is based on the principle that a single adherent cell divides to form a colony of daughter cells and, thus, has the capacity to self-renew. Both DPSCs-EZ and DPSCs-OG were capable of forming colonies as previously reported in the literature for DPSCs [93, 101, 102], SHEDs [101, 102], PDLCs [101], Wharton's Jelly MSCs [91] and BM MSCs [101]. The number of colonies observed here was in accordance with the seeding density. No differences in colony frequency were observed between DPSCs-EZ and DPSCs-OG, in accordance with the comparative study of EZ and OG of De Bruyn *et al*., 2011 [91] and the results of the MTT proliferation assay in this study.

Finally, the adipogenic, chondrogenic and osteogenic differentiation potential of DPSCs-EZ and DPSCs-OG were also compared by our research group (data not shown). Both DPSCs-EZ and DPSCs-OG were able to differentiate in the all 3 mesodermal lineages, with no clear differences between both groups. Based on these findings, it can be concluded the both the EZ and OG isolation technique gave rise to DPSC populations which meet the minimal criteria for MSCs defined by the ISCT in 2006 [40]. However, slight differences were observed in marker expression and morphology. Therefore, the OG method was chosen as the preferred isolation method for our the second part of this study on the angiogenic properties of the different DSC populations. Our conclusion is in accordance with the findings for other stem cells types, where the OG method was found to be superior compared to EZ technique for stem cell isolation, e.g. umbilical cord Wharton's Jelly-derived MSCs [91, 103], adipose tissue MSCs [95], skin-derived stem cells [104, 105] and skeletal muscle stem cells [106]. In addition, the use of non-human enzymes limits the use of DPSCs-EZ in clinical applications because of biosafety risks. A final important aspect is that the OG methods is based on the migration properties of potent stem cells, which is important for. migration towards sites of injury in therapeutic applications, such as therapeutic angiogenesis [107].

4.2 Comparing the angiogenic properties of the different DSC populations

Therapeutic angiogenesis is very promising in regenerative medicine and the treatment of pathologies associated with insufficient vascularization. Unfortunately, current revascularization therapies, including stem cell administration, have led to rather disappointing results. Not only does angiogenesis play an important role in the development and repair of dental tissues, but the stem cell populations of the tooth are also located in perivascular niches [55, 66]. Therefore, this study proposes DSCs for vascular regeneration. Angiogenic properties have already been shown for DPSCs, however, little is known about the other DSC types. Therefore, the angiogenic properties of the different DSCs were explored and compared here, with the emphasis on their paracrine actions. A HGF-1 cell line was included, since gingival fibroblasts are known to be involved in pathological angiogenesis.

It was shown here that not only the dental pulp, but also the apical papilla and the dental follicle are rich sources for stem cells isolation. By means of an OG isolation method, considerable numbers of DSCs could be easily isolated from all the dental tissues, which could be expanded *in vitro* to number suited for clinical applications. In general, all DSCs showed a typical fibroblast-like morphology, however, for the FSCs and SCAPS more polygonal cells and cubic cells were also visible in accordance with DPSCs-EZ isolated by Huang *et al*., 2006 [78]. These atypical cells were mostly observed during the isolation or even at early passage for the FSCs. These differences in morphology might indicate that this populations is very heterogeneous, as reported by others [45, 64]. Contamination by non-stem cell types, e.g. epithelial cells or endothelial, can be excluded, since these atypical cells were no longer found at later passages [91]. During the isolation, colonies were visible for all 3 DSC types and, therefore, a CFU-F assay was performed to compare the colony forming properties of the 3 populations. DPSCs, FSCs and SCAPs were able to form colonies as previously reported [54, 61, 63, 64]. No differences in colony numbers were found between DPSCs, FSCs and SCAPs after quantification. However, a trend towards a lower frequency was observed for DPSCs compared to FSCs and SCAPs at a density of 52 cells per cm^2, which was no longer observed at a density of 208 cells per cm^2. This could reflect patient variability since other patient samples were used for the DPSC CFU-F assay than for the FSCs and SCAPs, whereas these latter groups did include the same patient samples. Therefore, the assay should be repeated with DSCs derived from the same patients with inclusion of or more repeats.

The surface marker expression profiles of DPSCs, FSCs and SCAPs were compared by means of FACS analysis to confirm their mesenchymal origin. All 3 DSC populations were negative for the HSC markers CD34 and CD45, indicating their non-hematopoietic origin [50, 51, 64]. Mild to high expression levels were observed for the MSC marker CD44, whereas. all populations were strongly positive for the MSC markers CD105 and CD90.

The observed marker profiles are in accordance with the literature [51, 61, 64] and no significant differences in marker expression were observed between DPSCs, FSCs and SCAPs. Immunocytochemistry revealed expression of the HSC marker CD117 in all DSC populations. However, CD117 was only expressed by subpopulations of DPSCs, while both FSCs and SCAPs showed a uniform expression, which is in contrast with the findings of Luan *et al*., 2011 for FSCs, isolated from Swiss Webster mice dental follicles [108]. Nevertheless, our findings might suggest a higher contribution of cells of neuroectodermal or PC origin for FSCs and SCAPs compared to DPSCs [74, 96, 109] and might indicate a more immature or pluripotent nature of $CD117^+$ cells [109]. Immunostainings for the PC marker CD146 were also performed by our group, with clear expression by all DSCs, confirming a possible PC origin of DSCs [100]. Some of the above mentioned surface markers are not only stem cell markers, but are also known to play a role in angiogenesis, e.g. the marker CD44 is known to be important in EC tube formation [110, 111], CD146 is a PC marker [112], CD105 is involved in tumor angiogenesis by modulating TGF-β signaling [113] and $CD117^+$ stem cells play a key role in angiogenesis induced by bone marrow transplantation via paracrine actions [114, 115]. Since several studies, including results of our research group, have shown that the angiogenic effects of MSCs mainly occur via paracrine actions, we will focus on these properties of the different DSCs and the HGF-1 for the use in therapeutic angiogenesis [19, 69, 87, 114-117]. Since serum starvation is known to promote the angiogenic properties of MSCs by stimulating growth factor secretion, DSCs and HGF-1 cells were cultured in 0.1% DSCM for 48 hours, after which this CM was collected for experimental use. In addition, serum starvation is necessary, since FBS contains numerous angiogenic factors, such as FGF-2 and VEGF which could bias our results [84, 87].

To identify secreted angiogenic factors in the CM of DPSCs, FSCs, SCAPs and HGF-1, a Human Angiogenesis Array was performed. A differential expression of both pro- and anti-angiogenic factors was found for the 4 cell types. High levels of the anti-angiogenic factors TIMP-1, PAI-1, TSP-1, PTX-3 and PEDF were observed in the DSC and HGF-1 CM. A higher secretion of the pro-angiogenic factors ANG and IGFBP-2 was found for the HGF-1 compared to the DSCs. DSCs secreted more of the pro-angiogenic factors IGFBP-3, uPA and VEGF compared to the HGF-1. Higher levels of CXCL16, IL-8 and MCP-1 were found for FSCs compared to the other groups. Some of these identified factors have been previously reported in the literature. For DPSCs, DPPIV, MMP-9, VEGF, TGF-β have been shown on protein or mRNA level [67, 71, 74, 89, 118]. Although FGF-2 was reported in DPSC CM by Tran-Hung *et al.*, 2008 this factor was not detected by the angiogenesis array, nor by *in situ* immunostainings of dental pulp tissue (data not shown). However, it should be mentioned that the CM in their experiments was prepared with 10% FBS, while it was shown that serum increases the concentrations of VEGF and ANG in CM [119]. For FSCs, VEGF was previously shown on mRNA and protein level [120]. For SCAPs, only the relative expression of FGF-2 was reported at gene level [50].

ANG, angiostatin, IL-8, PEDF, TIMP-1, TIMP-2 and VEGF have been shown to be expressed by HGF cells or gingival tissue constructs at mRNA or protein level [121-125]. Therefore, we are the first to identify such an elaborate number of angiogenesis-related proteins for the different DSCs and the HGF-1. For BM MSCs, on the other hand numerous pro-angiogenic factors, such as ANG, Ang-1 and Ang-2, FGF-2, HGF, IGF, IL-6, IL-8, IL-11, MCP-1, uPA, VEGF, IGFBP-3 and anti-angiogenic factors, such as TGF-β, TNF-α TIMP-1, TIMP-2 have been reported [19, 84, 87, 116, 119] However, since the Array only allows relative quantification of angiogenic factors and since only two different patient samples were included, ELISA and RT-PCR were performed to validate and quantify some of the identified factors on protein and mRNA level. ELISA confirmed the presence of the pro-angiogenic factor IGFBP-3 in the CM of all DSCs and HGF-1, with a trend towards higher levels for FSCs, in accordance with the results of the Angio-array. Although IGFBP-3 is known for its pro-angiogenic effects, anti-angiogenic effects have also been shown for this factor in tumor angiogenesis, indicating a possible regulatory function [126]. The pro-angiogenic factor VEGF was validated by means of PCR, ELISA and *in situ* immunohistochemistry. Although at low levels, VEGF was found to be expressed in all DPSC samples. For the FSCs and SCAPs expression was found for some patient samples, but not all. A persistent expression of β2-microglobuline was shown for all patient samples. A very low expression was found for the HGF-1, in accordance with Yoshino *et al.*, 2003 [124]. The ELISA for VEGF, on the other hand, indicated high concentrations of VEGF in the CM of DPSCs, FSCs and SCAPs, in accordance with the literature for DPSCs and [67] FSCs [120]. Very low concentrations were found in the CM of HGF-1, which was in accordance with the results of the Array, the RT-PCR and the observations of others [124]. Although the high levels of VEGF on protein level and the low levels on mRNA level seem contradictory, the results of the RT-PCR only allow comparison between the different DSCs and do not allow quantification. Moreover, the cells for the mRNA samples were cultured in standard DSCM, whereas the CM for the ELISAs was obtained from cells cultured in 0.1% DSCM. Nevertheless, a clear trend towards a higher concentration of VEGF in the CM of DPSCs compared to the FSCs and SCAPs was observed, in accordance with the RT-PCR results. Tissue sections of the different dental tissues were also investigated for expression of VEGF together with the MSC and vascular marker CD146 by means of immunohistochemical staining *in situ.* Expression of CD146 was mainly visible surrounding blood vessels, suggesting perivascular DSCs niches, since the DSCs shown to be $CD146^{+}$ [66, 90, 100]. VEGF was found to be expressed in all the dental tissues, with a seemingly higher expression in pulp and follicle tissues than in apical pads, which is in accordance with the ELISA results. These results indicate the intrinsic expression and secretion of the main pro-agiogenic factor VEGF by DSCs, which is involved in almost all steps of the angiogenesis process [9]. As already mentioned the expression of VEGF on mRNA and protein level was also reported for BM MSCs [19, 116, 127] and adipose MSCs [128], in studies exploring the angiogenic potential of these MSCs.

Functional *in vitro* assays with HMEC-1 were performed to gain more insight in the angiogenic potential of the different DSCs and their effects on the different steps of the angiogenesis process. One of the first steps in the angiogenic process is the proliferation of EC. To test whether DSCs can induce EC proliferation, the HMEC-1 were incubated with CM of DPSCs, FSCs, SCAPs and the HGF-1 for 72 hours and an MTT proliferation assay was performed. No effect on HMEC-1 proliferation was observed for any of the DSCs. This is in accordance with previous results of our research group for DPSCs and CM of BM MSCs reported by Gruber *et al.*, 2005. A trend towards an increase in EC proliferation, on the other hand, was observed for the HGF-1 cells in accordance with the results of Okada *et al.*, 2009 [79]. Unfortunately insufficient repeats of the HGF-1 condition were included for statistical analysis. Although an increase in proliferation has been shown for adipose MSCs and BM MSCs, their CM contained higher levels of FBS [116, 119, 128]. This was also the case for the DPSC side population cells of Iohara *et al*., 2008, who also reported an increase in EC proliferation. However, their CM was had a different constitution and these DPSCs were not only selected for marker expression, but were also derived from porcine tooth germs [71]. Nevertheless, our findings can be explained by the angiogenic secretion profiles of the different DSCs. Although relative high levels of pro-angiogenic factors known to induce EC proliferation, e.g. ANG and VEGF were shown, numerous anti-angiogenic factors with anti-mitotic effects on EC were also found., e.g endostatin and PEDF. Since, hypoxic conditions have been shown to increase EC proliferation by others [128-130], preparation of the CM under hypoxia can be tested, leading to a possible shift in the angiogenic balance [6].

Still the process of angiogenesis is composed of multiple steps, of which the migration of EC towards an angiogenic stimulus is another important phase. To asses if DSCs induce EC migration, a Transwell system was used. The migration of HMEC-1 cells in an upper compartment towards DSCs in a lower compartment was investigated. A significant increase in HMEC-1 migration was shown for SCAPs, with a trend towards an increase in HMEC-1 migration for the DPSCs and FSCs. These results are in accordance with findings for BM MSCs and CM of BM MSCs [119, 131].

Once EC have started to migrate towards the angiogenic stimuli, they need to aggregate into tube-like structures. The ability of DSCs to induce EC tube formation was examined by growing HMEC-1 on growth factor-reduced Matrigel provided with DSC or HGF-1 CM. After 24 hours, tube formation was clearly observed for all conditions, but not for the negative control. However, only DPSCs CM gave rise to a significant increase in HMEC-1 tube formation compared to the negative control. These results are in accordance with previous findings for BM MSCs and co-cultures of human pulp fibroblasts with human umbilical vein ECs [19, 73, 85, 87, 131, 132]. Although Yoshino *et al*., did not report tube formation for their HGF CM, in contrast with the observed trend here, a collagen gel and bovine aortic ECs were used in their experiment. Moreover, their HGF cells were isolated from a fibrotic avascular area of gingiva which could have affected the outcome of their results [124].

These *in vitro* assays showed that DSC and HGF-1 CM induce EC migration and tube formation, but not EC proliferation. However, multiple cell types are involved in the *in vivo* angiogenesis process e.g. EC, SMCs and PCs. Therefore, a CAM assay was performed to gain more insight in the angiogenic potential of the different DSCs. This *in ovo* assay resembles the *in vivo* situation, since it allows integration of the interactions of all blood vessel cell types and includes all steps of the angiogenesis process. A pilot study was performed with DPSCs to test different conditions of DPSCs and DPSC CM. Test conditions were placed on the CAM at E9 on a gelatin sponge, together with a control sponge. At E12, the number of blood vessels migrating towards the gelatin sponges were quantified. Although others have reported an increase in angiogenesis mediated by BM MSCs or BM MSC CM [84, 131], no differences were found for any of the test conditions compared to the controls in our experiment. Optimization, therefore, is needed. Since Oskowitz *et al*., 2009 implanted 0.2 to 2 million cells in their CAM experiment, the used cell number might have been too low [84]. In addition, more repeats need to be included since the CAM is very sensitive to changes in oxygen tension, and inflammatory reactions, e.g. caused by shell dust [133]. Although the gelatin sponge model has been shown to be an efficient vehicle to deliver test substances and cells, different methods of administration have been reported in the literature, e.g. collagen gels, sodium alginate and Matrigel, which can also be adopted [84, 131, 134-136]. In accordance with our findings, difficulties in distinguishing pre-existing vessels from newly formed vessels are known for the CAM gelatin sponge model, since the number of blood vessels is only determined at the end of the experiment. Moreover, the CAM naturally expands within the time frame of experiment [133, 136]. Therefore, by quantifying the blood vessels vertically growing towards the gelatin sponges or inside the sponges, pre-existing vessels can be separated from newly formed ones more properly. Although no differences were found after macroscopic quantification, histological sections of the gelatin sponges can still be quantified for ingrowth of blood vessels [131, 134, 136], since the gelatin sponges and surrounding CAM were fixed after the experiment. Moreover, this would allow investigation of the fate of the stem cells after the implantation of the sponges. For example it can be investigated if the DPSCs survive and if they co-align with the newly formed vessels and interact with the vascular cells by pre-labeling the DPSCs with a cell tracker, such as CellTracker™ CM-DiI, in combination with stainings for Ki-67 or EC markers, such as CD31, respectively [137].

In conclusion, it was shown here that the various DSCs and the HGF-1, differentially express both pro- and anti-angiogenic factors, such as IGFBP-3, IL-8 TIMP-1 and VEGF on mRNA or protein level. Moreover, VEGF was found to be present *in situ* in the dental tissues. Functional *in vitro* assays showed a pre-dominant pro-angiogenic effect of DSCs by induction of EC migration and tube formation, but not EC proliferation. A trend toward induction of EC proliferation and tube formation was found for the HGF-1.

Although both pro- and anti-angiogenic factors were found, these observations can be explained by the fact that the angiogenesis process is orchestrated by a delicate balance of both pro- and anti-angiogenic factors. Pro-angiogenic factors, such as VEGF, are very potent and are involved in multiple steps of the angiogenesis process, therefore, low concentrations are sufficient to induce angiogenesis [9]. Nevertheless, the identification of anti-angiogenic factors is important to prevent pathological angiogenesis e.g. stimulation of tumor formation and hemagioma formation. These side effects have been previously described in clinical trials for therapeutic angiogenesis and limit applications of these therapies [4, 10, 13-15]. Moreover, the identified factor IGFBP-3 is known to have pro-angiogenic effects, however, it has also been shown to induce anti-angiogenic effects in tumor angiogenesis, indicating a possible regulatory function [126].

Although no single population was shown to be superior, the results suggest a stronger angiogenic potential for DPSCs and SCAPs compared to FSCs, but more research is still needed.

5. Conclusion

Insufficient vascularization contributes to numerous pathologies and is a rate limiting step in regenerative medicine. Therapeutic angiogenesis, therefore, is very promising as a solution for these conditions. Unfortunately, current revascularization therapies have led to rather disappointing results. Stem cells therapies are raising new hopes, however, finding a suited stem cell candidate is difficult. Not only does angiogenesis play an important role in the development and repair of dental tissues, but the stem cell populations of the tooth are also located in perivascular niches. Therefore, this study proposes DSCs for vascular regeneration. However, for use in therapeutic applications, stem cells need to be isolated from their microenvironment and expanded *ex vivo* to obtain sufficient cell numbers. Therefore, in addition to considering the therapeutic potential of stem cells, their behavior *in vitro* also needs to be investigated to guarantee the safety of their use in medicine. The effects of 2 main isolation techniques on the stem cell properties of DPSCs were compared in the first part of this study. It was shown here that both an EZ and OG isolation method gave rise to DPSC populations, which could be expanded *in vitro* and were in accordance with the minimal criteria for MSCs defined by the ISCT in 2006. However, the OG method resulted in a more homogeneous population of stem cells, whereas DPSCs-EZ were more heterogeneous with rather atypical polygonal and cuboidal cells. At ultrastructural level DPSCs-EZ showed extensive vacuolization of the cytoplasm, probably caused by the harsh nature of enzymatic digestion. Moreover, higher levels of the MSCs markers CD44 and CD90 were found for DPSCs-OG compared to DPSCs-EZ due to degradation of surface molecules or contamination of other cell types in the EZ method. Furthermore, since the OG method is based on the migration of potent stem cells, this method was preferred for use in therapy, since it might select for stem cells capable of homing and migration to sites of injury.

However, before stem cells, including DSCs, can be put into practice more research is needed to standardize their isolation and culturing. Although the optimal isolation method was investigated here, the effect of *in vitro* culturing needs to be addressed as well. For therapeutic applications, expansion in animal-free serum or media free of animal-derived components is necessary because of risks for the transfer of zoonoses, prions and the danger of inducing immune reactions. Autologous and allogenic human serum or platelate lysate are currently under investigation. Finally, effects of long-term culturing should be assessed to gain insight in the risks of malign transformations, e.g. by looking at telomere length and chromosome stability.

This study was the first to explore and compare the angiogenic properties of the different DSCs with inclusion of a HGF-1. It was found that the various DSC populations and the HGF-1 differentially express both pro- and anti-angiogenic factors, such as IGFBP-3, IL-8, TIMP-1 and VEGF, on mRNA and protein level. In addition, VEGF was shown to be present *in situ* in the dental tissue and therefore is not induced by *in vitro* culturing conditions. Functional *in vitro* assays showed a pre-dominant pro-angiogenic effect of DSCs by induction of EC migration and tube formation, but not EC proliferation. A trend towards induction of EC proliferation and tube formation was found for the HGF-1. Although both pro- and anti-angiogenic factors were found, these observations can be explained by the fact that the angiogenesis process is orchestrated by a delicate balance of both pro- and anti-angiogenic factors. The identification of anti-angiogenic factors here is important to prevent pathological angiogenesis e.g. stimulation of tumor formation and hemagioma formation. Angiogenic properties have already been reported for DPSCs, however, this study was the first to report angiogenic properties for FSCs and SCAPs. Therefore, it was shown here that the human third molar serves as a potential source for 3 different stem cell populations with angiogenic properties Although no single population was shown to be superior, the results suggest a stronger angiogenic potential for DPSCs and SCAPs compared to FSCs, but more research is still needed to confirm this. Since differential expression of angiogenic factors were observed, complementary effects of the different DSCs are also an aspect that needs further investigation. Only a few of the factors identified in the Angiogenesis Array were validated on mRNA and protein level and therefore this this validation still needs to be performed. Additional information would be provided by investigating which of the identified factors are responsible for the reported angiogenic effects. Therefore, blocking antibodies or siRNAs against the identified angiogenic factors can be applied in the *in vitro* assays to test this. Moreover, not all steps of the angiogenesis process have been investigated here. The effect of DSCs on ECM and EC survival still needs further investigation. The *in ovo* CAM assay should be optimized and an *in vivo* study will also give additional insights in the angiogenic properties of the different DSCs, e.g. with a subcutaneous sponge or polymer mouse model or a Zebrafish model.

Application of DSCs in therapeutic angiogenesis will not only require angiogenic potential, but also survival of the DSCs in an hypoxic and inflammatory environment. Therefore, the angiogenic properties of the different DSCs should also be examined in these conditions. Immunomodulatory effects have been shown for DSCs, which would create additional clinical benefit. Moreover, it would allow allogenic use, creating the possibility for an off-the-shelf therapy and expanding the administration of cells derived from a single donor to more individuals. The valorisation possibilities of DSC therapy are even more increased by the fact that wisdom tooth extractions are routinely performed, in which third molars are discarded as medical waste. Moreover, DSC banking is already being performed, indicating valorization opportunities for DSC therapies in the near future.

Therapeutic angiogenesis with DSCs would have a wide range of clinical applications, since insufficient vascularization contributes to numerous pathological conditions, e.g. ischemia and chronic wounds. Moreover, vascularization of tissue grafts is a rate limiting step in the field of tissue engineering. In dentistry, DSCs would enable pulp revascularization and regeneration, or even whole tooth engineering. Because of their multipotent characteristics DSCs can even be applied as a therapy for many degenerative conditions, in which revascularization is an additional benefit, e.g. after a myocardial infarct and stroke. Our results indicate that therapeutic angiogenesis with DSCs could provide opportunities to treat many patients, simply with cells gathered from their own or other individuals' teeth, in a safe and responsible way.

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Supplementary files

S.1.1 Angiogenic factors and their functions

Pro-angiogenic factors	
Angiogenin	EC proliferation
Angiopoietin 1 and 2	EC migration, survival Vessel stabilisation, EC sprouting (only in the presence of VEGF)
Angiotropin	EC migration
CCL chemokines e.g. monocyte chemotactic protein 1 (MCP-1/CCL2), macrophage inflammatory protein-1 (MIP-1α/CCL-3)	EC migration and monocyte recruitment
Coagulation factor III/tissue factor	Induction signalling and gene transcription angiogenesis (via HIF1α)
Colony stimulating factor (CSF)	EC proliferation, migration
Connective tissue growth factor (CTGF)	EC adhesion, migration Mediator of TGF-β, growth factor-induced DNA synthesis
CXC chemokines with ELR motif e.g. AP-3/CXCL1, IL-8/CXCL-8, CXCL16	EC proliferation, migration
Cyclo-oxygenase2 (COX-2)	EC migration, differentiation
Dipeptidyl peptidase IV (DPPIV) /CD26	Activation neuropeptide Y (EC migration, proliferation and tube formation)
Endothelin 1 (ET-1)	EC proliferation, migration, VEGF induction
Epithelial growth factor (EGF) family e.g. Amphiregulin, transforming growth factor α, neuregulin (NRG1-β)	EC proliferation, survival
Erythropoietin (EPO)	EC proliferation
Fibroblastic growth factor (FGF)-family e.g. FGF-1/aFGF, FGF-2/bFGF	EC proliferation, migration Increase Plasminogen activators, upregulation adhesion molecules
Granulocyte [monocyte] colony stimulating factor (G[M]-CSF)	EC proliferation, migration
Hepatocyte growth factor (HGF)	EC and SMC proliferation, migration
Hypoxia inducible factor 1 (HIF-1)	Transcriptional activator of pro-angiogenic factors
Insulin-like growth factor (IGF)	EC proliferation, survival Induction VEGF and plasminogen activators
Insulin-like Growth Factor Binding Protein 1, 2, 3 (IGFBP-1, -2, -3)	EC migration, tube formation Induction HIF-1
Integrins	EC migration, attachment, survival and differentiation
Interleukins (IL-1α, IL-3, IL-8)	Activation VEGF-VEGFR2 signaling EC proliferation, migration, differentiation
Matrix metalloproteinases (MMPs)	ECM degradation EC migration, invasion
Nitric oxide (NO)	Increase vessel permeability EC proliferation, migration FGF release, Suppression angiostatin
Placental growth factor (PLGF)	Binds VEGF-R1 and VEGF-R2 and stimulates vessel formation
Platelet-derived growth factor (PDGF-BB)	SMC, PC proliferation, vessel stabilisation
Platelet endothelial cell adhesion molecule 1 (PECAM-1/CD31)	EC migration, aggregation Tube formation, vessel stabilisation
Prostaglandin E1, E2	EC differentiation
Retinoids	Tube formation Induction HGF and angiopoietin 2
Transforming growth factor β (TGF-β) family e.g. artemin, endoglin/CD105 (part TGF-β1 receptor complex)	Tube formation ECM synthesis, vessel stabilisation Angiogenesis in inflammation
Urokinase plasminogen activator (uPA)	EC migration via ECM degradation
Vascular endothelial-cadherin	EC survival, vessel stabilisation
Vascular endothelial growth factor (VEGF) family	Increase vessel permeability, ECM degradation EC proliferation, migration, survival, tube formation

EC=endothelial cell; SMC=smooth muscle cell; MC=mesenchymal cell; PC=pericyte; ECM=extracellular matrix; Adapted from: Distler JH, Hirth A, Kurowska-Stolarska M, Gay RE, Gay S, Distler O. *Q J Nucl Med*. 2003, 47(3):149-61. Liekens S, De Clercq E, Neyts J. *Biochem Pharmacol*. 2001, 61(3):253-70. Bhadada SV, Goyal BR, Patel MM. *Fundam Clin Pharmacol*. 2011, 25(1):29-47.

Anti-angiogenic factors	
Angiostatin/plasminogen	Inhibition EC proliferation, migration, tube formation EC apoptosis
Angiopoietin 2	EC apoptosis Vessel destabilisation
Canstatin	EC apoptosis Inhibition EC proliferation, migration Inhibition tube formation
CXC chemokines without ELR motif e.g.IP-10/CXCL-10	Inhibition FGF and $VEGF_{165}$ binding to their receptors
Endostatin//Collagen XVII	Inhibition EC proliferation, migration EC apoptosis Inhibition of MMPs
Insulin-like growth factor binding protein 3 (IGFBP-3)	Inhibition EC proliferation, EC apoptosis Down regulation gene transcription pro-angiogenic factors
Interferon γ (IFN-γ)	Inhibition EC proliferation
Interleukin 4 (IL-4)	Inhibition EC migration
2-Methoxyoestradiol	EC apoptosis Inhibition EC proliferation, migration
Pentraxin-related protein 3 (PTX-3)	Inhibition FGF-2 angiogenesis (FGF-2 antagonist)
Pigment epithelium-derived factor (PEDF)/serpin F1	Inhibition EC proliferation, migration
Plasminogen activator inhibitor 1 (PAI-1)	Inhibition uPA
Retinoic acid	Inhibition EC migration
Thrombospondin 1 and 2 (TSP-1 and -2)	Inhibition EC migration, EC apoptosis Prevents VEGF-induced angiogenesis by directly binding to it
Tissue inhibitor of metalloproteinases 1, 2, 4 (TIMP-1, -2, -4)	Inhibition MMPs
Transforming growth factor β (TGF-β) family e.g. activin A (ligand TGF-β receptor)	Inhibition EC proliferation, migration Decrease Plasminogen activators EC apoptosis Increase TIMPs
Troponin I	Inhibition EC proliferation
Tumor necrosis factor α (TNF-α)	Inhibition FGF-induced EC proliferation EC apoptosis

EC=endothelial cell; SMC=smooth muscle cell; MC=mesenchymal cell; PC=pericyte; ECM=extracellular matrix; Adapted from: Distler JH, Hirth A, Kurowska-Stolarska M, Gay RE, Gay S, Distler O. *Q J Nucl Med*. 2003, 47(3):149-61. Liekens S, De Clercq E, Neyts J. *Biochem Pharmacol*. 2001, 61(3):253-70. Bhadada SV, Goyal BR, Patel MM. *Fundam Clin Pharmacol*. 2011, 25(1):29-47.

S.1.2 Pathologies associated with a disturbed angiogenic balance

Pathologies insufficient angiogenesis		**Pathologies excessive angiogenesis**	
Pathology	***Angiogenic mechanism***	***Pathology***	***Angiogenic mechanism***
Myocardial Ischemia	Imbalance in capillary-to-cardiomyocyte fiber ratio	Hemangiomas	Vascular anomaly
Myocardial hypertrophy	Mismatch between the number of capillaries and cardiomyocytes	Psoriasis	Inflammatory response Abnormal vasculature and angiogenic balance
Hypertension	Impaired vasodilatation or angiogenesis	Age-related macular degeneration	Vision loss because of choroidal angiogenesis
Atherosclerosis	Impaired collateral vessel development	Kidney Disease	Loss peritubular capillaries interstitial cells Aneurysms responsible for renal bleeding
Peripheral Arterial Disease and Critical Lower Limb Ischemia	Impaired growth of collateral vessels in response to obstructive arterial disease	Inflammatory Bowel Disease	Inflammation is favoured and maintained by pathological angiogenesis
Cerebral Ischemic Stroke	Correlation of capillary density (angiogenesis) and survival	Ulcerative colitis and Crohn's disease	Inflammation is favoured and maintained by pathological angiogenesis
Wound Healing	Insufficient invasion angiogenic sprouts wound clot	Cancer	Tumor growth, progression and metastasis
Ulcer Healing	Delayed healing because of insufficient angiogenesis	Arthritis	Angiogenesis is key in formation and maintenance of the pannus in rheumatoid arthritis
Menorrhagia	Inadequate vascular remodeling/maturation and excessive blood loss		
Diabetes Mellitus	Nefast effects of hyperglycemia on EC proliferation, ECM and metalloproteases EC dysfunction		
Bone Disease	Impaired bone formation Osteoporosis Impaired healing of fractures		
Emphysema	Alveolar EC apoptosis upon VEGF inhibition		
Pre-eclampsia	EC dysfunction, resulting in organ failure, thrombosis and hypertension because of deprivation of VEGF by soluble Flt1		

Adapted from: Bhadada SV, Goyal BR, Patel MM. *Fundam Clin Pharmacol*. 2011, 25(1):29-47.

S.1.3 Characteristics of the different DSC populations and the HGF-1

Properties	DPSC	SHED	SCAP	FSC	PDLSC	HGF-1
Location	Dental pulp	Dental pulp	Apical papilla	Dental follicle	Periodontal ligament	Gingiva
Proliferation rate	Moderate	High	High	High	High	High
Colony formation	Yes	Yes		Yes		Yes
Heterogeneity	Yes	Yes	Yes	Yes	Yes	Yes
Differentiation potential						
Odontogenic	+	+	+	+	+	
Cementogenic		+			+	
Adipogenic	+	+	+	+	+	
Chondrogenic	+	+		+	+	
Osteogenic	+	+	+	+	+	
Myogenic	+	+		+		
Neurogenic	+	+	+	+	+	
Tissue formation						
Dentin-pulp tissue	+	+	+			
Bone-like tissue	+	+				
PDL				+	+	
Root				+	+	
Marker expression						
STRO-1	+	+	+	+	+	
CD13	+		+	+	+	
CD24	-	-	+	-	-	
CD29	+		+	+	+	
CD44	+		+	+	+	
CD59	+			+	+	
CD73	+		+	+		
CD90	+		+	+	+	
CD105	+		+	+	+	
CD106			+			
CD117	+/-					+
CD146	+	+	+	+	+	
Nanog		+				
OCT4	+	+				
Notch-1				+		
Nestin	+			+		
	+					
CD14						
CD34	-		-			
CD45	-	-	-	-		
	-					

Adapted from Rodriguez-Lozano FJ, Bueno C, Insausti CL, Meseguer L, Ramirez MC, Blanquer M, et al. *Int Endod J.* 2011, 44(9):800-6.

S 2.1 Fluorescence activated cell sorting (FACS)

- Seed cells provided with culture medium into a V-bottom FACS-plate (Becton Dickinson) at a density of 50 000 cells per well
- Wash 3 times in 100 to 150 µl FACS buffer (2% FBS in PBS)
- Recovery step: leave at room temperature for 30 minutes
- Centrifuge 6 minutes at 300g at 4°C
- Remove supernatant and dissolve in 100µl of primary antibody solution
- Incubate for 45 minutes in the dark at 4°C
- Wash 3 times in 100 to 150 µl FACS buffer
- In case of secondary antibody → dissolve in 100ml secondary antibody solution
  In case of labeled primary without secondary antibody → dissolve in FACS buffer
- Incubate for 30 minutes in the dark at room temperature
- Wash 3 times in 100 to 150 µl FACS buffer
- Remove supernatant and dissolve in 150µl FACS buffer
- Transfer to FACS tubes
- Analyze samples with Becton Dickinson FACS Calibur

S 2.2 Immunohistochemical stainings (Fluorescence)

- Deparaffinization and rehydration:
  - Xylene (VWR, Fontenary, France) twice for 5 minutes
  - Series of descending Ethanol (VWR, Fontenary, France) concentrations
  - Aquadest
- Antigen retrieval:
  - 3 alternating boiling (5 minutes) and cooling steps (2 minutes) in 1X Citrate buffer solution (Dako, Carpinteria, USA)
  - Final cooling step of 30 minutes
- Wash 3 times in PBS 1X (0.01M, pH 7.2) for 3 to 5 minutes
- Block in 10% serum in PBS at room temperature for 20 minutes
- Wash 3 times in PBS 1X for 3 to 5 minutes
- Primary antibody at 4°C overnight in the dark
- Wash 3 times in PBS 1X for 3 to 5 minutes
- Secondary antibody at 4°C for 30 minutes in the dark
- Wash 3 times in PBS 1X for 3 to 5 minutes
- Nucleus counterstaining with 4',6-diamidino-2-phenylindole (DAPI) for 10 minutes in the dark at room temperature
- Wash 3 times in PBS 1X for 3 to 5 minutes
- Mount and coverslip with Dako Immunofluorescence mounting medium (Carpinteria, USA)

S 2.3 Immunocytochemical staining (Fluorescence)

- Wash 3 times in PBS 1X (0.01M, pH 7.2) for 3 to 5 minutes
- In case of intracellular antigen→ Permeabilisation in 0.05% Triton X in PBS at 4°C
- Block in 10% serum in PBS at room temperature for 20 minutes
- Wash 3 times in PBS 1X for 3 to 5 minutes
- Primary antibody at 4°C overnight in the dark
- Wash 3 times in PBS 1X for 3 to 5 minutes
- Secondary antibody at 4°C for 30 minutes in the dark
- Wash 3 times in PBS 1X for 3 to 5 minutes
- Nucleus counterstaining with 4',6-diamidino-2-phenylindole (DAPI) for 10 minutes in the dark at room temperature
- Wash 3 times in PBS 1X for 3 to 5 minutes
- Mount and coverslip with Dako Immunofluorescence mounting medium (Carpinteria, USA)

S 2.4 Immunocytochemical stainings (DAB)

- Wash 3 times in PBS 1X (0.01M, pH 7.2) for 3 to 5 minutes
- Peroxidase blocking in 0.03% hydrogen peroxide containing sodium azide (EnVision®+ System-HRP Kit, Dako, Carpinteria, USA).
- Block in 10% normal goat serum in 1X PBS at room temperature for 20 minutes
- Wash 3 times in PBS 1X for 3 to 5 minutes
- Primary antibody 1 hour at room temperature
- Wash 3 times in PBS 1X for 3 to 5 minutes
- Secondary goat anti-mouse or anti-rabbit antibody conjugated with HRP-labelled polymer at room temperature for 30 minutes in the dark
- Wash 3 times in PBS 1X for 3 to 5 minutes
- DAB-chromogen in substrate buffer (20µl DAB per ml substrate buffer) at room temperature 1 to 10 minutes until brown-colored precipitate is visible
- Rinse with distilled water
- Counterstaining with haematoxylin for 8 minutes at room temperature
- Wash 3 times in tap water for 3 to 5 minutes
- Mount and coverslip with Aquatex (Merck, Darmstadt, Germany)

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Richting: **master in de biomedische wetenschappen-klinische moleculaire wetenschappen**
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Fanton, Yanick

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