2011
2012

GENEESKUNDE

master in de biomedische wetenschappen: klinische moleculaire wetenschappen

Masterproef

Dental stem cells in angiogenesis and tissue engineering

Promotor :
Prof. dr. Ivo LAMBRICHTS
dr. Annelies BRONCKAERS

Jessica Vanormelingen

Masterproef voorgedragen tot het bekomen van de graad van master in de biomedische wetenschappen , afstudeerrichting klinische moleculaire wetenschappen

Maastricht University

universiteit hasselt
KNOWLEDGE IN ACTION

De transnationale Universiteit Limburg is een uniek samenwerkingsverband van twee universiteiten in twee landen: de Universiteit Hasselt en Maastricht University

universiteit hasselt
KNOWLEDGE IN ACTION

Maastricht University

Universiteit Hasselt | Campus Diepenbeek | Agoralaan Gebouw D | BE-3590 Diepenbeek
Universiteit Hasselt | Campus Hasselt | Martelarenlaan 42 | BE-3500 Hasselt

2011
2012

GENEESKUNDE

master in de biomedische wetenschappen: klinische moleculaire wetenschappen

Masterproef

Dental stem cells in angiogenesis and tissue engineering

Promotor :
Prof. dr. Ivo LAMBRICHTS
dr. Annelies BRONCKAERS

Jessica Vanormelingen

Masterproef voorgedragen tot het bekomen van de graad van master in de biomedische wetenschappen , afstudeerrichting klinische moleculaire wetenschappen

Maastricht University

universiteit hasselt
KNOWLEDGE IN ACTION

Table of contents

Preface

During the last 8 months, I had the chance to complete my internship at the Department of Morphology at Hasselt University. This was a great experience as I have learned much more than I had ever expected. Moreover, I had the opportunity to work in a great team environment in which everybody was always prepared to help wherever needed. Therefore, I would like to thank every person that made it possible for me to complete all my experiments successfully.

First of all, I would like to thank Prof. Dr. Ivo Lambrichts for giving me the opportunity to work in his research group. Moreover, a big thank you for your great help with the EM, your guidance, your kindness and most of all for your enthusiasm and the creation of the feeling of always being welcome.

Furthermore, I would like to express my gratitude to my co-promotor and daily supervisor Dr. Annelies Bronckaers. Thank you for all the guidance, patience, help and advice whenever needed. Most of all, I would like to thank you for all your understanding, your comments on the right time and for you always being enthusiastic. I will keep a lot of your words in mind.

I would also like to thank Petra Hilkens for her help in the laboratory and for my thesis, Pascal Gervois for his good guidance on our trips to the hospital and the rest of the research group for all the little helps which made it possible to bring this internship to a great end.

My gratitude also goes out to Prof. Dr. Constantinus Politis of Ziekenhuis Maas en Kempen, for providing us with patient material and to Dr. Bertie Souvereyns for providing me with the Ti-scaffolds

A word of appreciation goes out to Marc Jans, Jeanine Santermans and Igna Rutten. Marc, without your hard work, I wouldn't have finished my EM-research in time. Jeanine, thank you for your guidance with the stainings. Igna, without your help with the computer for picturing the gels and for reading the absorbance, a lot of my experiments were done for nothing. Thank you!

Much gratitude goes also to my fellow students Yanick, Stephanie and Sarah for always being there when again something went wrong, for listening whenever needed and for the pleasant moments in the basement. Yanick, thank you for your help, your patience, your jokes and your brains ;) during this internship. Our collaboration made this last part of my school career unforgettable.

Moreover, I would like to thank my boyfriend Patrick for his support during my internship and studies, for helping me and asking difficult and 'pain in the ass' questions and understanding that I couldn't be present more. Especially a great thanks for all the efforts and pains you put in our house and doing almost everything alone.

Als laatste, maar zeker niet als minste zou ik graag mijn ouders danken om mij altijd de kans te geven om te studeren wat ik graag deed, mij te steunen in mijn keuzes en mij te helpen waar mogelijk. Om te luisteren naar het geklaag als het eens teveel was, om mij te verwennen en alles te doen wat mogelijk was om mij door de studeer-en schrijfperiodes heen te loodsen, en mij altijd net dat tikkeltje meer moed te geven om alles tot een goed einde te brengen.

Jessica Vanormelingen; June 11th, 2012

List of abbreviations

BMC	bone marrow derived cell
CAM	chick embryo chorioallantoic membrane
CM	conditioned medium
DSC	dental stem cell
DPSC	dental pulp stem cell
EC	endothelial cell
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
EZ	enzymatic digestion
FBS	fetal bovine serum
FSC	follicle stem cell
HGF	human gingival fibroblast
HMEC-1	human microvascular endothelial cell line 1
IL8	interleukin 8
MCP1	monocyte chemotactic protein 1
MSC	mesenchymal stem cell
OG	outgrowth method
PDLSC	periodontal ligament stem cell
RT-PCR	reverse transcriptase polymerase chain reaction
SCAP	stem cell of the apical papilla
StDev	standard deviation
TEM	transmission electron microscopy
Ti	titanium
uPa	urogen plasminogen activator
VEGF	vascular endothelial growth factor

Abstract

Introduction: Stem cells are an ideal cell source to use in tissue engineering as they are able to differentiate into multiple cell lineages and tissues. Besides cells, a scaffold and blood vessels are required in this promising technique for treating the loss or failure of tissues or organs. The scaffold provides a three-dimensional support desirable for the adhesion, proliferation, migration and differentiation of the responding cells. In this study, titanium scaffolds will be used as they are biocompatible, have a high strength to weight ratio and are highly corrosion resistant. Blood vessels are necessary for the delivery of oxygen, nutrients and inductive signals to the implanted cells. These blood vessels can be formed by means of angiogenesis. Stem cells can promote this by either differentiating into endothelial cells or by excreting pro-angiogenic factors. In this study, three different dental stem cell (DSC) populations are explored and compared to investigate their angiogenic properties, as recent studies indicate that dental stem cells might possess these properties. Moreover, they are relative easy to isolate, they can be safely cryopreserved and possess immunological and anti-inflammatory properties. Furthermore, these stem cells are mesenchymal cells which implies they are able to differentiate into chondrocytes, osteocytes and adipocytes.

Materials and methods: By means of an angiogenesis antibody array, we elucidated which angiogenic factors are secreted by the three DSC populations. This was validated by PCR and ELISA.
Moreover, immunostainings against IL8 and MCP1 were implemented to investigate if the tissues and stem cells express these angiogenic markers.
Next, as migration is one of the key events in angiogenesis, we evaluated the effects of the dental pulp stem cells (DPSCs) on the migration potential of endothelial cells *in vitro* by means of a transwell migration assay. As preliminary data have shown that DPSCs secrete the pro-angiogenic factors IL8, VEGF and MCP1, we examined if inhibition of these factors also results in the suppression of the chemotactic activities of the DPSCs.
In the last part of this thesis, we investigated if the DPSCs could grow and differentiate into bone on a titanium scaffold. This was examined by means of transmission electron microscopy.

Results and discussion: Based on the angiogenesis antibody array, PCR and ELISA can be concluded that the three different DSC populations excrete pro-angiogenic as well as anti-angiogenic factors, such as uPa, angiogenin, IL8, VEGF, TIMP1, TIMP4 and Pai-1. DPSCs have moreover a positive effect on the migration of endothelial cells. Inhibition of the pro-angiogenic factor VEGF leads to a significant decrease of migration towards the DPSCs. Blocking of IL8 and MCP1 also slightly suppressed endothelial cell migration, but this inhibition was not statistically significant. DPSCs are furthermore capable of growing and differentiating into osteocytes on titanium scaffolds.

Conclusion: Dental pulp stem cells can grow on a titanium scaffold and can moreover differentiate into osteocytes. These are promising results for usage in tissue engineering to treat the loss or failure of tissues or organs. Moreover, dental stem cells are able to induce blood vessel formation which is necessary for the survival of new body parts and new tissue inserts.

Samenvatting

Inleiding: Stamcellen zijn een ideale bron om te gebruiken in 'tissue engineering' omdat ze kunnen differentiëren in meerdere cellijnen en weefsels. Naast cellen zijn een drager en bloedvaten nodig om met deze veelbelovende techniek, beschadigde of verloren weefsels of organen te regenereren. De drager voorziet een driedimensionale structuur waarop de gebruikte cellen zich kunnen hechten, prolifereren, migreren en differentiëren. In deze studie zullen titanium dragers gebruikt worden aangezien ze biocompatibel, sterk en zeer corrosie resistent zijn. Bloedvaten zijn daarnaast nodig voor de overdracht van zuurstof, voedingsstoffen en inductieve signalen naar de getransplanteerde cellen. Deze bloedvaten kunnen gevormd worden door angiogenese. Stamcellen kunnen dit bevorderen door enerzijds te differentiëren naar endotheelcellen of door anderzijds pro-angiogene factoren uit te scheiden. In deze studie worden de angiogene eigenschappen van drie verschillende dentale stamcelpopulaties onderzocht en vergeleken, aangezien recente studies aantonen dat deze stamcellen zulke eigenschappen bezitten. Daarnaast zijn ze vrij makkelijk te isoleren, kunnen ze veilig gecryopreserveerd worden en bevatten ze immunologische en ontstekingsremmende eigenschappen. Daarbovenop zijn het mesenchymale stamcellen waardoor ze gedifferentieerd kunnen worden naar chondrocyten, osteocyten en adipocyten.

Materiaal en methode: We hebben door middel van een angiogenese antilichaam test bepaald welke angiogene factoren uitgescheiden worden door de drie verschillende dentale stamcelpopulaties. Dit werd achteraf gevalideerd met PCR en ELISA. Daarnaast werden er immunokleuringen tegen IL8 en MCP1 uitgevoerd om te kijken of de weefsels en stamcellen deze angiogene markers tot expressie brengen. Daarbovenop hebben we de effecten van dentale pulp stamcellen (DPSCs) op de migratie van endotheelcellen bepaald aangezien migratie een zeer belangrijk proces is in de vorming van bloedvaten. Dit hebben we *in vitro* gedaan met behulp van een transwell migratie test. Aangezien preliminaire data ook aangetoond hebben dat DPSCs de pro-angiogene factoren IL8, VEGF en MCP1 uitscheiden, hebben we onderzocht of inhibitie van deze factoren ook leidt tot onderdrukking van de aantrekkingskracht van de DPSCs. In het laatste stuk van deze thesis hebben we geanalyseerd of de DPSCs konden groeien en differentiëren in bot op een titanium drager. Dit werd nagegaan door middel van transmissie elektronen microscopie.

Resultaten en discussie: Gebaseerd op de angiogenese antilichaam test, PCR en ELISA kunnen we besluiten dat de 3 verschillende dentale stamcelpopulaties zowel pro- als anti-angiogene factoren uitscheiden zoals uPa, angiogenine, IL8, VEGF, TIMP1, TIMP4 en Pai-1. DPSCs hebben daarbovenop een positief effect op de migratie van endotheelcellen. Inhibitie van de pro-angiogene factor VEGF leidt tot een significante migratieafname naar de DPSCs. Het blokkeren van IL8 en MCP1 leidt ook tot een lichte afname van migratie, maar dit was statistisch niet significant. DPSCs kunnen bovendien ook groeien en differentiëren in osteocyten op een titanium drager.

Conclusie: Dentale pulp stamcellen kunnen groeien en differentiëren in osteocyten op een titanium drager. Dit zijn veelbelovende resultaten voor het gebruik in 'tissue engineering' om het verlies of falen van weefsels of organen op te lossen. Daarnaast kunnen dentale stamcellen bloedvatvorming induceren, nodig voor de overleving van nieuwe lichaamsdelen.

1. Introduction

1.1. Tissue engineering

Tissue engineering is a promising technique in science and medicine as it holds a great potential to treat the loss or failure of tissues or organs, which is one of the most frequent and expensive problems in human health care today [1]. It is a treatment in which laboratory-grown molecules, cells, tissues or organs are developed and manipulated to repair or replace the function of defective or injured body parts [2]. The three key ingredients for tissue engineering are an extracellular matrix (scaffold), responding cells and inductive signals (regulatory biomolecules, morphogens)[2].

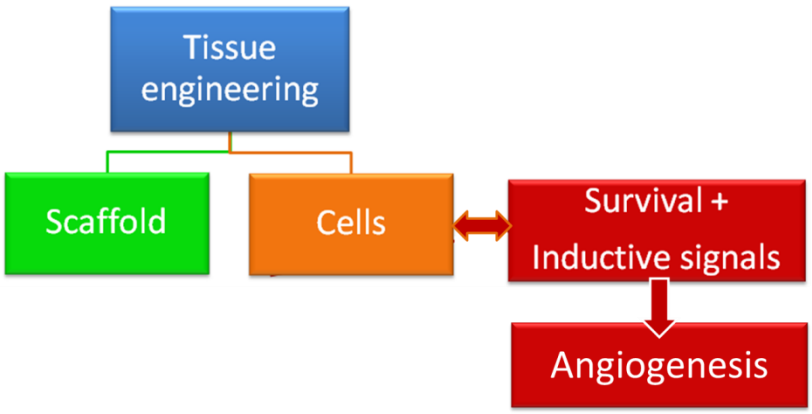


Figure 1: For accomplishing tissue engineering, scaffolds are needed on which cells can grow and differentiate. In order to promote survival, growth and differentiation of these cells, inductive signals, delivered by blood vessels, are required.

The **scaffold** is needed to provide support to the damaged region in order to form a healthy bone or tissue at the site of damage. It provides a three-dimensional environment that is desirable for the adhesion, proliferation, migration and differentiation of the responding cells. This scaffold provides mechanical support while the cells multiply and eventually differentiate into functional tissue-specific cells [3]. Metals, polymers and ceramics have been widely used as tissue scaffolds. Polymers and some bioceramics are able to be resorbed, forming a healthy tissue. However, these polymer bioscaffolds lack the strength and therefore cannot be applied for load bearing structures, e.g. bones. On the other hand, metallic and ceramic implants have the disadvantage of stress shielding. Porous titanium has properties that are intermediate between soft polymer scaffolds and solid metallic or ceramic implants. Moreover, titanium is biocompatible, possesses a high strength to weight ratio and is highly corrosion resistant [4].

Besides a scaffold, **cells** are required which can differentiate into the cells of the damaged region. Stem cells are an excellent source of cells for this application because they still can differentiate into multiple lineages of cells and tissues.

Finally, **inductive signals** are necessary. These regulatory molecules (e.g. growth factors) are capable of stimulating cellular growth, proliferation and cellular differentiation. Moreover, they also can govern the pattern of tissue development and the positions of various specialized cell types within a tissue.

In order to stimulate the growth, differentiation and long-term survival of these cells and to accomplish the delivery of the regulatory molecules, **blood vessel formation (i.e. angiogenesis)** is necessary. These blood vessels can provide oxygen, nutrients and regulatory molecules to the tissue implant in order to avoid necrosis and for promotion of the differentiation to the wanted tissue.

In order to investigate if dental pulp stem cells are able to be used in tissue engineering, this study examines if they are able to grow and attach onto titanium (Ti) and moreover, if they are able to differentiate into osteocytes. This will be investigated by means of transfer electron microscopy (TEM).

1.2. Angiogenesis

Blood vessels are important for the adequate supply of oxygen needed for normal tissue function and for delivery of inductive signals to the cells. These vessels arise in the embryo through vasculogenesis, which is the *de novo* formation of a poorly functional vasculature through differentiation of angioblasts, pluripotent precursor cells, to endothelial cells. This primitive network becomes remodeled and grows into a functional adult circulatory system via angiogenesis. Angiogenesis is a normal and vital process in growth and development, wound healing, the female reproductive organs and in the development of collateral circulations at sites of ischemia [5].

Angiogenesis is a multistep process and can be divided into several distinct, often overlapping phases. In general, angiogenesis involves vasodilatation, pericyte detachment and degradation of the extracellular matrix (ECM) and basement membrane. Subsequently, endothelial cells begin to proliferate and migrate towards the mitogenic stimulus. During the final maturation stages, there is a lumen and basement membrane formation and peri-endothelial cells (pericytes for small capillaries and smooth muscle cells for larger vessels) are recruited for further support of the new vessel. (see figure 2) [5].

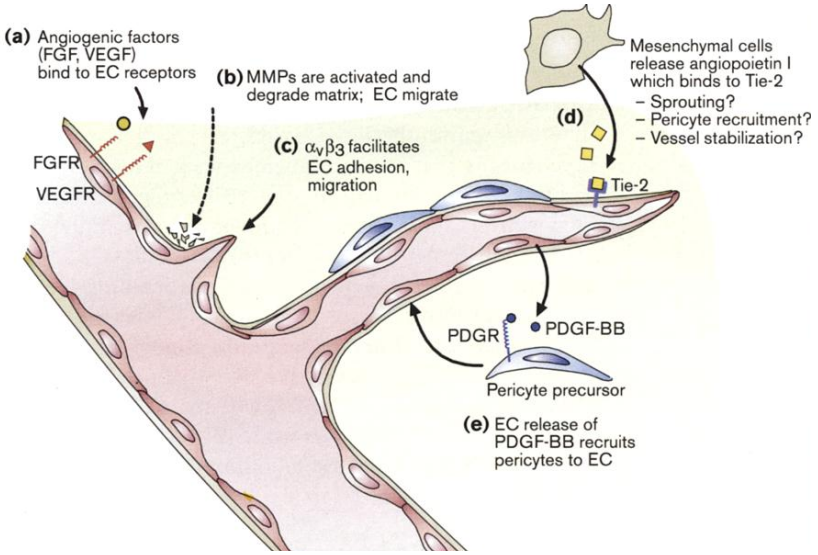


Figure 2: Process of angiogenesis (Klagsbrun M, Moses MA. Molecular angiogenesis. Chem Biol. 1999 Aug; 6(8):R217-24.)

This multistep process is controlled by a balance between pro- and anti-angiogenic factors. These can arrive in the form of cytokines and proteases, growth factors and inhibitors as well as extracellular matrix proteins and adhesion molecules (see table a, supplement). These extracellular cues are then transduced to the cytoplasm by various different classes of cell surface receptors [5].

In normal conditions, these pro-and anti-angiogenic factors are in perfect harmony. However, in case of disease, the balance between stimulators and inhibitors is tilted, resulting in an angiogenic switch. If the stimulators dominate, cancer metastasis, multiple sclerosis or rheumatoid arthritis can occur. On the other hand, if there is a majority of anti-angiogenic factors, this can result in preeclampsia, diabetes, stroke or ischaemic heart disease. Moreover, blood vessel formation is also necessary in tissue engineering in order to prevent necrosis of the tissue implant and for delivery of the inductive signals to the implanted cells. In these cases, (re)vascularization is necessary. However, currently used methods of revascularization are no longer effective for a significant number of patients. For this reason, stem cells are under investigation as an alternative method for the stimulation of angiogenesis [5, 6]. Moreover, scientists currently hypothesize that stem cells can be a continuous source of angiogenic factors.

1.3. Stem cells

Stem cells are unspecialized cells capable of dividing and renewing themselves for long periods and able to give rise to specialized cell types. They have the remarkable potential to develop into many different cell types in the body during early life and growth and serve as a kind of internal repair system in many tissues, able to divide without limit to replenish other cells [7].

Stem cells are able to divide in two different ways. They can divide symmetrically, required to expand in number during development or after injury. On the other hand, asymmetric division enables them to simultaneously renew themselves as stem cells and generate progeny that can differentiate into mature cells (see figure 3) [8].

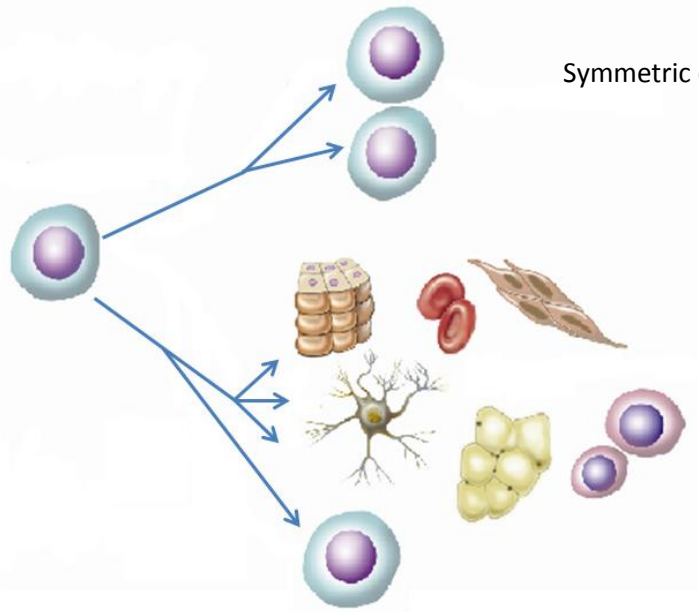


Figure 3: Symmetric and asymmetric division of stem cells (Barlow, C. and K. Treuner, *DNA instability in the brain: survival of the 'fittest'.* Nat Med, 2005. 11(5): p.474-5.)

There are multiple sources of stem cells. **Totipotent stem cells**, are derived from a fertilized egg and are able to give rise to the, mesoderm, endoderm, ectoderm, the germ cells and to extra-embryonic tissues, needed for the survival of the developing embryo. **Pluripotent stem cells** on the other hand, such as embryonic stem cells, are derived from the inner cell mass of an early blastocyst (7-14 days after fertilization) and are able to give rise to all derivatives of the three primary germ layers and germ cells. This means they can generate all cell types of the body, but are not able to develop into a fetus, because they lack the potential to contribute to extra-embryonic tissue. The use of embryonic stem cells however is controversial because of the creation, manipulation and destruction of (human) embryos

Adult stem cells can be categorized based on their origin into two main groups: germline and somatic stem cells. Adult stem cells are multipotent and can be found in different adult organs. Multipotent stem cells are able to give rise to multiple, but not all celltypes. On the other hand, **unipotent stem cells** are cells that have the capacity to differentiate in only one cell type [9].

1.3.1. Mesenchymal stem cells

Mesenchymal stem cells are a subdivision of somatic stem cells. They originate from the mesoderm, which is the middle layer of the three primary germ layers in the early embryo. Mesenchymal stem cells can be characterized by means of three criteria. (i) Firstly they are multipotent with the ability to differentiate into chondrocytes, the cells of cartilage, osteocytes, the cells of bone and adipocytes, the cells of fatty tissue. (ii) Moreover they must express the mesenchymal stem cell markers CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19. (iii) As a last feature, mesenchymal stem cells must be able to adhere to plastic when cultured under standard conditions [10]. Besides their trilineage differentiation potential, mesenchymal stem cells are also able to transdifferentiate into neurons and endothelial cells [11].

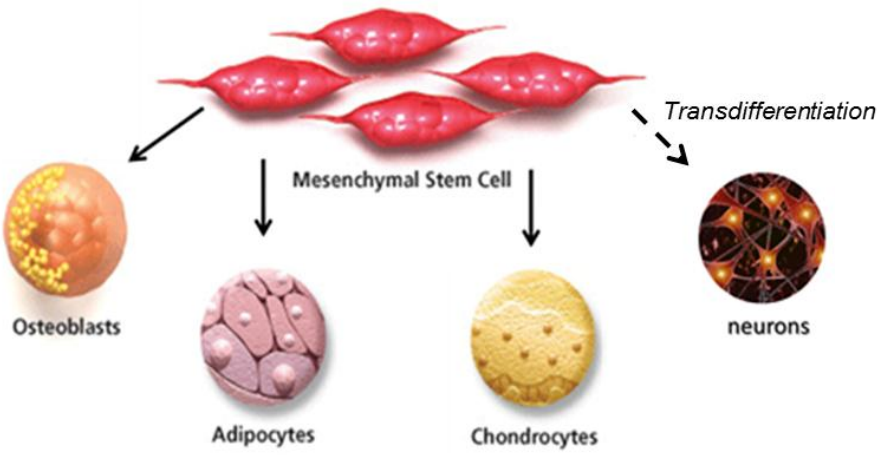


Figure 4:Differentiation potential of mesenchymal stem cells. Besides their characteristic trilineage differentiation potential, they are also able to transdifferentiate into neurons.

Mesenchymal stem cells can be found in different tissues of the body including the stroma of adult bone marrow and in soft tissues of the tooth. However, extracting stem cells from the bone marrow is an invasive operation with the possibility to causing adverse effects [12]. Dental stem cells (DSCs) on the other hand can be easily retained from discarded teeth after orthodontics operation with very

low morbidity and few ethical issues. Moreover, DSCs can be safely cryopreserved, making them ideal for bio-banking. Additionally, they possess immunological and anti-inflammatory abilities favorable for allotransplantation [12-14].

1.3.2. Dental stem cells

The idea of teeth containing stem cells was based on the well-known repairing ability of dentin after injury. This idea was confirmed by *Gronthos et al*. in 2000 by discovering the dental pulp stem cells (DPSCs). Afterwards, a lot of research has been conducted to investigate the location and properties of these stem cells. Besides this characterization, other stem cell niches have been found to exist in the human permanent tooth, namely dental follicle stem cells (FSCs), stem cells of the apical papilla (SCAPs) and periodontal ligament stem cells (PDLSCs). They are all present in different niches of the developing tooth (see figure 5) [15]. It has been shown that DSCs are an adult mesenchymal stem cell population originating from the neural crest, expressing the stem cell surface markers CD29, CD44, CD73, CD105, CD106, CD146, CD166 and STRO-1. Besides this, they are negative for CD14, CD34 and CD45. Moreover, DSCs are also able to differentiate into adipocytes, osteocytes and chondrocytes [16-18].

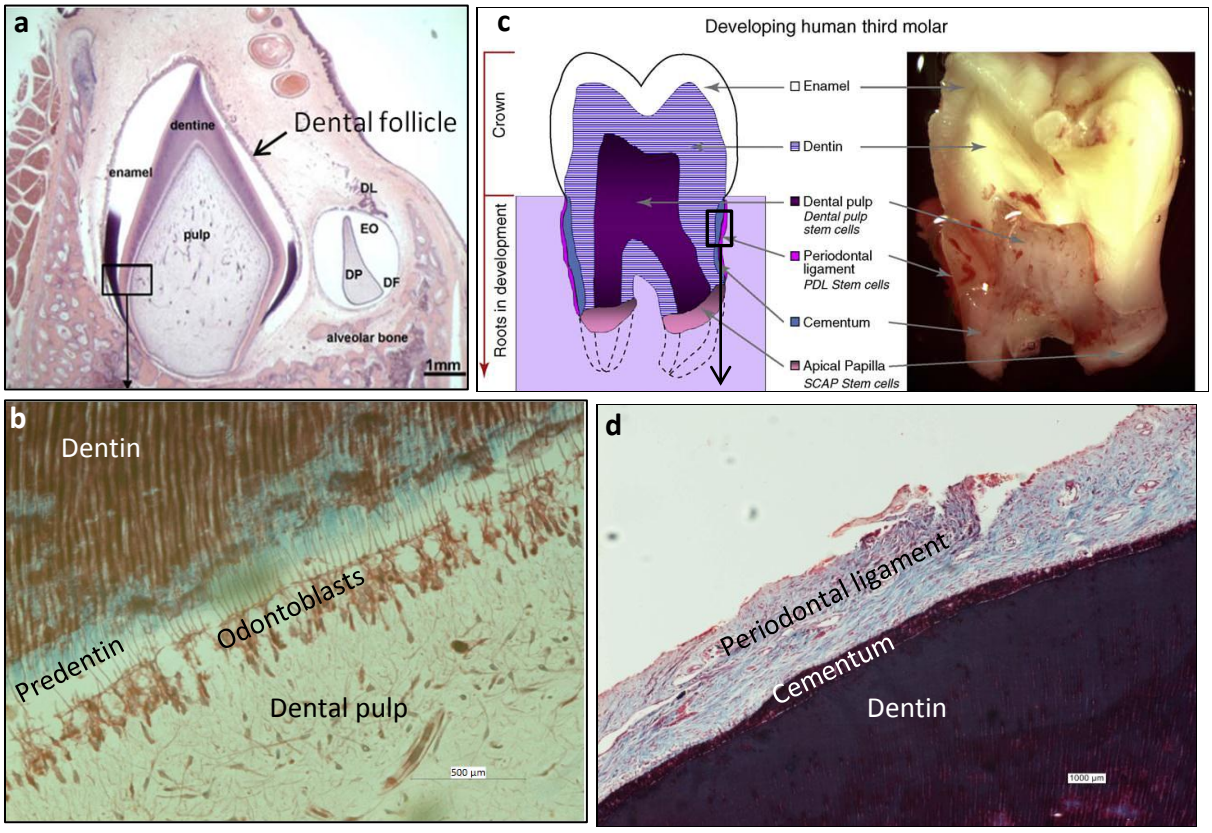


Figure 5: The four subpopulations of DSCs present in human permanent teeth are (1) stem cells from the dental pulp, (2) the periodontal ligament, (3) the dental follicle and (4) the apical papilla. A: An unerupted tooth showing the dental follicle (Yen AH, Sharpe PT. Stem cells and tooth tissue engineering. Cell Tissue Res. 2008 Jan;331 (1):359-72).; B: Magnification of A showing the dentin, predentin, the odontoblasts and the dental pulp.; C: A developing third molar showing the dental pulp, the periodontal ligament and the apical papilla (Volponi AA, Pang Y, Sharpe PT. Stem cell-based biological tooth repair and regeneration. Trends Cell Biol. 2010 Dec; 20 (12):715-22).; D: Magnification showing the periodontal ligament, cementum and dentin.

FSCs are derived from the **dental follicle** which is surrounding the developing tooth germ prior to eruption. It contains progenitor cells that form the periodontium, including the cementum, the periodontal ligament and the alveolar bone. Moreover, the dental follicle is involved in root formation and tooth eruption. The developmental cascade occurring in this tissue confirms the existence of stem cells in the dental follicle [19, 20]. *In vivo*, transplantation of FSCs in a critical-size defect created in calvaria of immunodefficient rats led to bone-formation [20].
The **DPSCs** are stem cells residing within the perivascular niche of the **dental pulp**, situated in the central region of the tooth (figure 5c). This tissue is characterized by the presence of odontoblasts and is surrounded by a rigid mineralized texture formed by dentin and enamel. After injury to the tooth, there is an induction of a regeneration process in the dental pulp by the DPSCs, producing new odontoblasts and the secretion of reparative dentin. Moreover, the dental pulp is infiltrated by a network of vessels and nerve bundles emanating from the apical region [18]. Besides the characteristic trilineage differentiation potential, DPSCs are also able to differentiate into neural lineages [12, 21, 22]. *In vivo*, DPSC transplantation in immunocompromised mice generated functional dental tissue in the form of dentine/pulp-like complexes [18].
SCAPs are derived from the **apical papilla**, which is located at the tips of growing tooth roots and is derived from the same precursor tissue as the dental pulp, namely the dental papilla. As these roots still develop postnatally, the apical papilla is accessible in dental clinical practice after the extraction of wisdom teeth. Therefore it is an active source of stem cells with embryonic-like properties (i.e. in the process of development).[12, 18]. *In vivo*, SCAPs were able to form a functional bio-root in a minipig model [23].
The **PDLSCs** are derived from the **periodontal ligament** (PDL), which is a ligament between the tooth and the alveolar bone and is essential for many functions that support the tooth. The periodontal ligament is derived out of the dental follicle and consists out of collagen fibers, blood vessels and nerve fibers. It has a nutritive, protective and sensory function for the tooth. As this ligament is constantly under tension while chewing, the PDLSCs are likely to play an endogenous role in the maintenance of PDL cell numbers [18].
In this project, we will focus on FSCs, DPSCs and SCAPs.

1.3.3. Isolation of dental stem cells

The isolation of DSCs out of the tissue pieces can be performed by means of two methods. The first isolation method is the explant method in which the tissue is mechanically fractionated into little pieces and the cells are allowed to grow out of the tissue fragment. The second technique used to isolate DSCs is enzymatic digestion. Here, the tissue pieces are digested in a collagenase/dispase solution, after which the obtained suspension is put into culture.

Both techniques are used in the dental stem cell field, but there is a lot of debate about which method is the best way of isolation. Therefore, this thesis provides a comparison between the two methods by means of ultrastructural analysis and investigation of mesenchymal stem cell properties (see chapter 3.1).

1.4. Angiogenesis and stem cells

Stem cells can promote angiogenesis either (i) by differentiating into endothelial cells themselves, (ii) by excreting pro-angiogenic factors or (iii) by a combination of both. Multiple sources of stem cells have already been proposed as potential pro-angiogenic cell therapies, including bone marrow-derived cells (BMCs), endothelial precursor cells (EPCs), embryonic stem cells, cardiac stem cells and mesenchymal stem cells [6, 24-26].

1.4.1. Angiogenesis and mesenchymal stem cells

The differentiation capacity of mesenchymal stem cells to endothelial cells after addition of endothelial culture medium containing VEGF or after injection in an ischemic region has already been shown *in vitro* and *in vivo* in BMCs, kidney-derived MSCs and umbilical cord-derived MSCs [25, 27-29].

Moreover, MSCs such as adipose-derived stem cells (ASCs), kidney-derived MSCs and BMCs are able to promote angiogenesis by the secretion of an array of different pro-angiogenic peptides including vascular endothelial growth factor (VEGF), Interleukin 6 and 8 (IL6 and IL8), hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor (bFGF), angiopoeitin-1 (Ang-1), urokinase plasminogen activator (uPA) and tumor necrosis factor alpha (TNFα) [30, 31]. BMCs are furthermore able to form tube-like structures *in vitro*, can enhance wound healing and promote therapeutic angiogenesis in hindlimb ischaemia through VEGF production [11, 24, 25, 27, 32, 33].

In contrast to these pro-angiogenic properties, mesenchymal stem cells are also able to inhibit angiogenesis when administered in large numbers by the production of reactive oxygen species [34].

Due to these controversies, the lack of unambiguous results and good results in easy obtainable stem cells, this thesis will investigate DSCs as angiogenic promoters.

1.4.2. Angiogenesis and dental stem cells

Because harvesting stem cells from the bone marrow is a painful and invasive operation and DSCs are easily retainable after orthodontics operation, this project will investigate if different dental stem cell populations have angiogenic opportunities. This is highly suggested as angiogenesis is important in both tooth development and healing and DSCs are furthermore located in perivascular niches [35].
It has already been shown that dental pulp stem cells, derived from the highly vascular pulp, secrete factors which interact with endothelial cells and affect the process of angiogenesis, VEGF and fibroblast growth factor 2 (FGF-2) [36]. Additionally, they are able to differentiate into endothelial cells *in vitro* in the presence of VEGF [17]. Moreover, intracardiac injection of DPSCs can reduce the area of myocardial infarction, improve ventricular function, and induce revascularization around the injection region [37]. However, the differentiation potential of DPSCs into endothelial cells *in vivo* has not been shown yet.
As the apical papilla is derived from the apical part of the precursor tissue of the radicular pulp and the blood vessels firstly invade the apex of the developing tooth, SCAPs are believed to be good

angiogenic promoters. They are moreover derived from a developing tissue that may represent a population of early stem cells [20]. The same holds true for the FSCs. These stem cells are derived from the follicle, which is the first tissue surrounding the developing tooth, which gets infiltrated with a vascular supply and contains many blood vessels [12].

In this thesis, we will investigate the array of angiogenic factors secreted by the different dental stem cell populations by means of an angiogenesis antibody array, the ability of attraction of endothelial cells by means of a transwell assay and the induction of blood vessel formation *in vivo* using a CAM assay.

2. Materials and methods

2.1. Isolation and culture of cells

Dental stem cells (DSCs) are collected from normal human third molars (patients 16-18 years old) after orthodontics operation with informed consent of the patients at Ziekenhuis Maas en Kempen, Bree. After extraction, the DSCs are isolated by means of two methods which will be compared in this thesis. (see chapter 3.1)

The first method is the **outgrowth method** in which the tissue is mechanically fractionated into pieces of 1-2 mm^3. Afterwards these fragments are seeded into 6-well plates in α-modification of Eagle's medium (α-MEM, Invitrogen, Merelbeke, Belgium), supplemented with 100 Units/ml Penicilin, 100 µg/ml Streptomycin (penstrep, Invitrogen) and 2 mM L-Glutamin (L-Glut, Invitrogen) (further referred to as standard culture medium) and 10% Fetal Bovine Serum (FBS, Invitrogen). This is placed in an incubator at 37°C with 5% CO_2 to allow the stem cells to grow out of the tissue fragment.

The second method is **enzymatic digestion** in which the mechanically fractionated tissue is digested in a solution of 3mg/ml collagenase type I and 4mg/ml dispase type II (Roche Diagnostics, Vilvoorde, Belgium) for 1h at 37°C. After centrifugating this suspension for 5 minutes, single cell suspensions are obtained by passing the cells through a 100 µm cell strainer (BD, Erembodegem, Belgium). This cell suspension is seeded in culture dishes and is maintained in standard culture medium supplemented with 10% FCS under standard conditions. Subconfluent cultures are harvested with 0.05% trypsine/EDTA (Invitrogen) and seeded in a density of 4000 cells/cm^2.

Cells of passage 1 to 4 are used for all experiments.

Moreover, we use an immortalized human dermal microvascular endothelial celline (HMEC) transfected with a PBR-322-based plasmid containing the coding region for the simian virus 40 A gene product and a large T antigen. These HMEC-1 are cultured in MCDB-131 medium (Gibco, Paisley, UK), supplemented with 100 Units/ml Penicilin, 100 µg/ml Streptomycin (penstrep, Invitrogen), 1mM L-Glutamin, 1µg/ml hydrocortisone (Sigma, Bornem, Belgium) and 10ng/ml human recombinant endothelial growth factor (hEGF, Gibco).

2.2. Conditioned medium

Cells from passage 1-4 were seeded in a density of 20,000 cells/cm^2 in standard culture medium. After 24 hours, the cells are rinsed with PBS and the medium is replaced by α-MEM, supplemented with **0.1%** FBS (Invitrogen). After 48 hours medium is harvested, centrifugated for 6 minutes and stored at -80°C.

2.3. Angiogenesis antibody array

An angiogenesis antibody array was performed to simultaneously elucidate the factors secreted by the DSCs. This was performed on cell culture supernates using the Human Angiogenesis Array kit (R&D systems, Mineapolis, USA) by following manufacturer's instructions. This encompasses blocking the membranes for one hour with blocking buffer. In the meantime samples are incubated with reconstituted detection antibody cocktail for one hour at room temperature. Hereafter, sample/antibody mixtures were added to the membranes and incubated overnight at 2-8°C. After this incubation, membranes were washed with 1X wash buffer for 3 times 10 minutes. After this, streptavidin-HRP was added to the membranes for 30 minutes at room temperature. After a second wash, chemiluminescent detection reagents (Amersham ECL plus™ Western Blotting Detection System, GE Healthcare, Diegem, Belgium) was spread onto each membrane for 5 minutes, after which the membranes were exposed to X-ray film for 1-10 minutes.

2.4. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

DSCs were seeded at 4000 cells/cm^2 and are trypsinised when confluent. After washing the cells with PBS, total RNA was isolated by means of the RNeasy Plus Mini Kit (Qiagen, Venlo, the Netherlands). From this total RNA, 700 ng was transcribed reversely into cDNA according to manufacturer's instructions of the Reverse Transcription System (Promega, Leiden, the Netherlands). Next, the Polymerase Chain Reaction (PCR) was performed using Taq DNA Polymerase (1 U/µl) (Roche Diagnostics) according to the protocol in table 2.1. Primers were designed using primer-blast on the NCBI website. Primers are developed in such a way that they span exon-exon junctions to avoid amplification of undesired genomic sequences. All primers are delivered by Eurogentec S.A. (Seraing, Belgium). All forward and reverse sequences, their product size and melting temperature (Tm) are presented in table 2.2.

Table 2.1: PCR protocol

PCR mix per sample (µl)		PCR program		
10x PCR-buffer	2.5	1x	5 minutes	94°C
dNTP mix	0.25	35x	1 minute	95°C
Forward primer (25µM)	1		1 minute	60°C
Reversed primer (25µM)	1		45 seconds	72°C
Taq polymerase	0.75	1x	10 minutes	72°C
MiliQ	18.5			

Table 2.2: Primer sequences for RT-PCR

Gene	Primer	Sequence	Product size (bp)	Tm (°C)
TIMP-1	Forward	GCT-TCT-GGC-ATC-CTG-TTG-TT	462	60
	Reverse	TTT-GCA-GGG-GAT-GGA-TAA-AC		58
Housekeeping gene	**Primer**	**Sequence**	**Product size (bp)**	**Tm (°C)**
β2-microglobulin	Forward	CTC-ACG-TCA-TCC-AGC-AGA-GA	213	62
	Reverse	CGG-CAG-GCA-TAC-TCA-TCT-TT		60

2.5. Enzyme Linkend Immunosorbent Assay (ELISA)

ELISAs were performed on conditioned medium of DPSCs, SCAPs, FSCs and HGF-1 cells to determine the concentrations of the pro-angiogenic factors IL8 (Biolegend, Antwerp, Belgium) and angiogenin (RayBiotech, Inc., Boechout, Belgium) and the anti-angiogenic factor TIMP4 (RayBiotech, Inc.). This was done following manufacturer's instructions.

2.6. Immunostaining on tissues and cells

For (immuno)histochemic analysis on tissues, tissues (pulpa, apical pad, follicle) were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 hour, dehydrated in graded ethanol, embedded in paraffin and serially sectioned at 7 µm. For immunohistochemistry on cells, cells were fixed with Unifix (Klinipath, Geel, Belgium).

Tissue sections were deparafinated and antigen was retrieved by use of 10% target retrieval solution (DAKO, Heverlee, Belgium) in PBS heated 3 times 5 minutes, with pauses of 2 minutes. Afterwards tissues were blocked by 10% serum for 20 minutes and primary antibody (see table 2.3) was incubated overnight. Afterwards secundary antibodies (donkey anti-mouse or donkey anti-rabbit) were incubated for 30 minutes and DAPI for 10 minutes.

Table 2.3: primary antibodies

Antibody	Dilution	Type *	Supplier
CD 29	1/200	rPab	Abcam (Cambridge, USA)
CD 146	1/100	rMab	Abcam
IL-8	1/50	mMab	R&D systems (Mineapolis, USA)
MCP-1	1/25	mMab	Abcam

*mMab: Mouse monoclonal antibody rMab: Rabbit monoclonal antibody
rPab: Rabbit polyclonal antibody

2.7. Transwell migration assay

The influence of the DPSCs on endothelial cell migration was investigated in a transwell migration assay using HMEC-1 cells. hDPSCs were seeded in a density of 50,000 cells/cm^2 and were allowed to attach for 24 hours. Afterwards, standard culture medium supplemented with 10% FCS was replaced by culture medium with 0.1% FCS. After 24 hours, translucent ThinCert™ tissue culture inserts (pore size 8 µm; Greiner Bio-One, Wemmel, Belgium) were placed, containing 25,000 cells/cm^2 HMEC-1 cells in standard culture medium with 0.1% FCS. These were allowed to migrate for 24 hours, after which the inserts were washed in PBS, fixed with 4% PFA for 20 minutes and stained with chrystal violet for 10 minutes. Pictures were taken with an inverted microscope (Nikon Eclipse TS100),

equipped with a Progres C3 digital microscope camera (Jenoptik, Germany) after which analysis was performed with the AxioVision software, edition 4.6.3 (Carl Zeiss NV-SA, Zaventem, Belgium).
In order to investigate the impact of the angiogenic factors secreted by the DPSCs, antibodies against anti-VEGF (1µg/ml), anti-IL8 (1µg/ml) and anti-MCP1 (1µg/ml) were added to the DPSCs.

2.8. Chick embryo chorioallantoic membrane (CAM) gelatin sponge assay

To test the angiogenic properties of DPSCs *in vivo*, a CAM gelatin sponge assay was performed. Fertilized chicken eggs were incubated for 3 days at 37°C at constant humidity, after which 3 ml of albumen is removed with a syringe in order to detach the developing CAM from the shell. A square window is opened in the egg shell exposing the CAM and for validation of the fertilization. This window is sealed with cellophane tape and the eggs are returned to the incubator.
At day 9 of embryonic development, 2 mm^3 steril gelatin sponges (Gelfoam Upjohn, Kalamaoo, MI) were placed on top of the CAM. Test compounds were placed onto these sponges, after which the window is closed again with cellophane tape and the eggs are returned to the incubator. In one experiment, the conditioned medium was concentrated (in order to obtain a higher concentration of the secreted growth factors for achieving a stronger angiogenic effect). This was done by means of a protein concentrator column with a molecular weight cut-off of 9 kDa (Pierce protein concentrators, 9K MWCO, Pierce Thermo Scientific, Erembodegem, Belgium).
After 3 days, the CAMs are removed and are photographed with the Wilo M3Z microscope (Nikon).

2.9. Osteogenic induction (on titanium)

To investigate if DPSCs can be used in tissue engineering and are able to differentiate into osteocytes on titanium, DPSCs were seeded at a density of approximately 4.2x10^3 cells/cm^2 on different titanium scaffolds with a variety of surface structures (see figure 6), provided by Dr B. Souvereyns and Prof. M. Van Bael (IMO, Uhasselt). The different surface structures are Ti spread across the whole scaffold (TiO_2 dens), Ti orientated in lines across the scaffold (TiO_2 lines), in squares (TiO_2 squares) and porous divided titanium (TiO_2 porous).

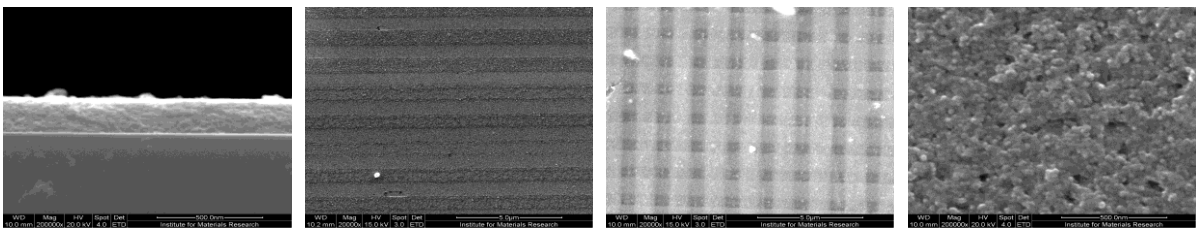


Figure 6: TiO_2 dens | TiO_2 lines | TiO_2 squares | TiO_2 porous

Osteogenic differentiation was induced by culturing the DPSCs in standard culture medium, supplemented with 10% FBS and 5% osteogenic supplement (R&D systems, Mineapolis, USA) containing dexamethasone, ascorbate-phosphate and β-glycerolphosphate. Osteogenic medium was replaced every 3-4 days and the Ti-scaffolds were fixed with glutaraldeyde after 3 days, 1 week and 3 weeks to investigate them with TEM.
Control DPSCs were cultured in the same medium without the osteogenic supplement.

3. Results and discussion

3.1. Comparison of DPSCs isolated by means of 2 different methods (explant vs enzymatic)

3.1.1. Morphology

DPSCs were isolated by means of two different isolation techniques: namely the outgrowth method by which the cells were allowed to grow out of a tissue piece (DPSC-OG), and enzymatic digestion by which the tissue is dissolved in a collagenase/dispase solution and single cell suspension is seeded in culture dishes (DPSC-EZ).

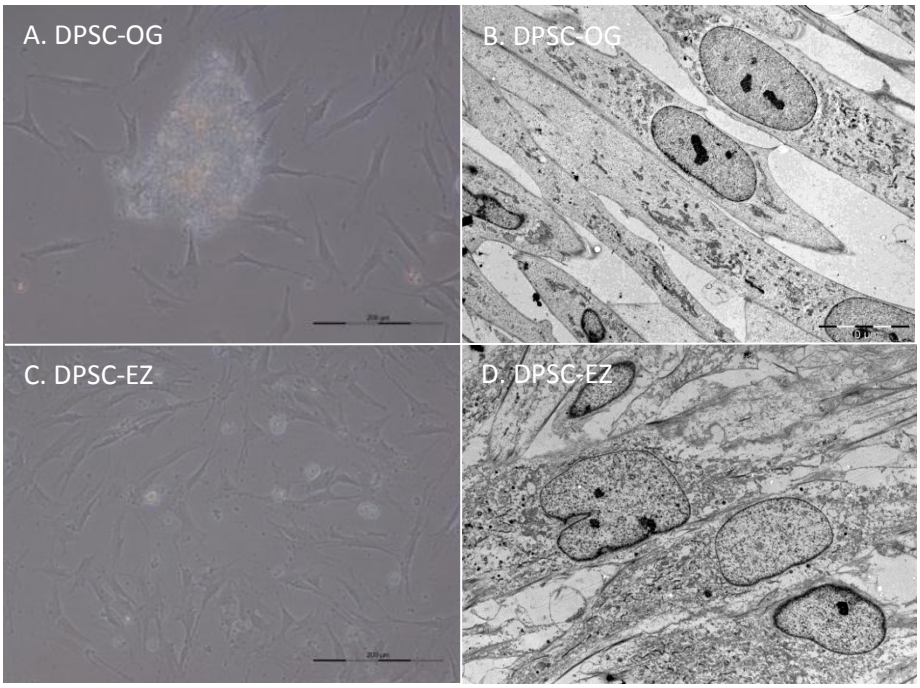


Figure 7: DPSCs isolated by means of the outgrowth method (OG) or by enzymatic digestion (EZ)

Cells isolated by means of the outgrowth method show a homogeneous shape with a fibroblast-like morphology (see figure 7a). On the other hand, enzymatic digestion leaded to a more heterogeneous cell distribution, including fibroblast-like and round cells. There is moreover more cell debris present after enzymatic digestion (see figure 7b). This finding is not peculiar as the EZ-method allows different types of cells to be released during tissue dissociation. A more uniform migration of fibroblast-like cells is allowed by outgrowth as non-migrating cells disintegrate within the tissue explants. This is in line with the findings of Bakopoulou A. *et al*. who compared two isolation methods of DSCs from human exfoliated deciduous teeth [38] and with Salehinejad P. *et al*. who investigated the best isolation method with human umbilical cord Wharton's jelly [39].
Electron microscopic analysis also reveals much less vesicles in the DPSCs isolated by means of the outgrowth method, compared to those isolated by means of enzymatic digestion. The cell organelles of the DPSCS-OGs are moreover particularly perinuclear located with a rather electron lucent peripheral region, in contrast to the DPSC-EZs, where organelles are spread throughout the entire cytoplasm. Large euchromatic nuclei with one or more nucleoli were visible in both DPSC-EZ as in DPSC-OG.

3.1.2. Stem cell markers

In order to elucidate and compare the mesenchymal stem cell properties of the dental stem cell populations isolated by two different isolation methods, immunohistochemic analysis with the mesenchymal stem cell marker CD146 was performed.

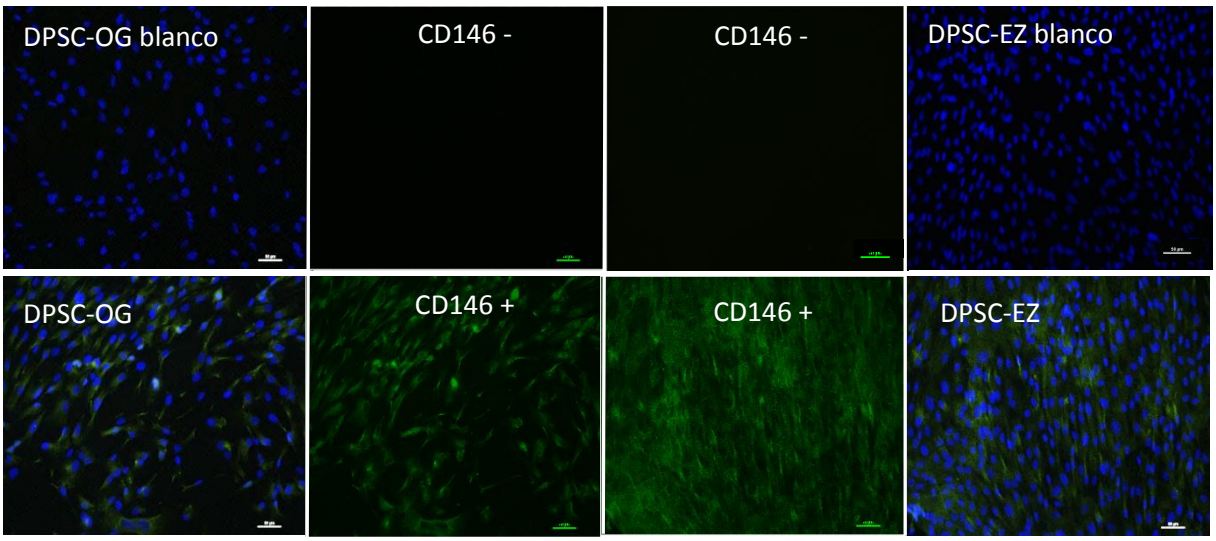


Figure 8: Comparison of the immunohistochemical staining against the mesenchymal stem cell marker CD146 between DPSCs isolated by means of the outgrowth method and through enzymatic digestion. This is a representative staining for 6 different patients (n=6).

Both DPSCs isolated by the outgrowth method as DPSCs isolated through enzymatic digestion express the mesenchymal stem cell marker CD146. This is in line with the findings of Fanton Y., who demonstrated the expression of another mesenchymal stem cell marker CD29 in both DPSC-OG and DPSC-EZ. This is moreover in agreement with the findings of Struys T. *et al.* who also showed the expression of the mesenchymal markers CD29 and CD146 by DPSC-OG [40] and with Shi *et al*. who showed the expression of CD146 in DPSC-EZ [35].

In order to definite decide which isolation method is the best, the expression of more mesenchymal stem cell markers, such as CD90, CD105 and Stro-1 needs to be investigated. Moreover, this expression has to be validated quantitatively by means of for example FACS. Furthermore, the effect of the isolation method on the differentiation potential of the DSCs remains to be elucidated.

3.2. Angiogenic properties of dental stem cells

3.2.1. Morphology of different dental stem cell populations

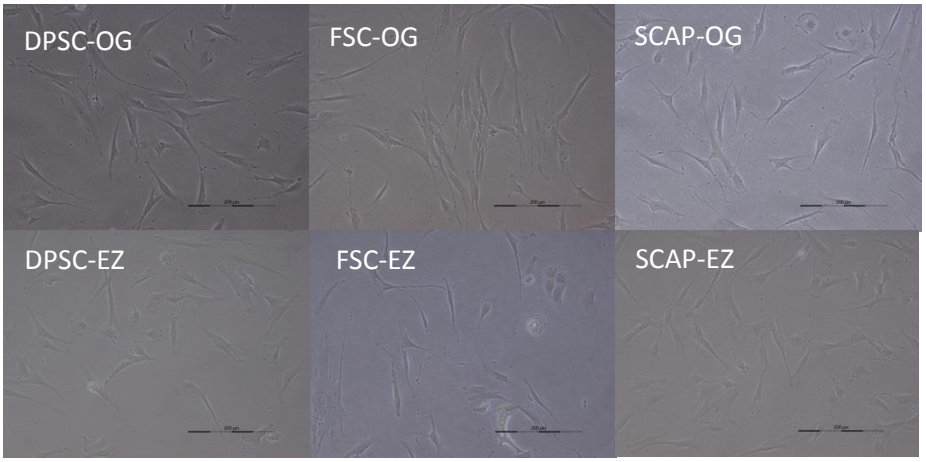


Figure 9: Dental stem cells were isolated by means of two different isolation methods, namely the outgrowth method (OG) and the enzymatic digestion method (EZ).

Both DSCs isolated by means of the outgrowth method as through enzymatic digestion show a fibroblast-like morphology characteristic to mesenchymal stem cells [41]. However, as the literature and our preliminary data show that preferentially the outgrowth method should be applied, it was decided that for our further experiments only DSCs isolated by the outgrowth method will be used.

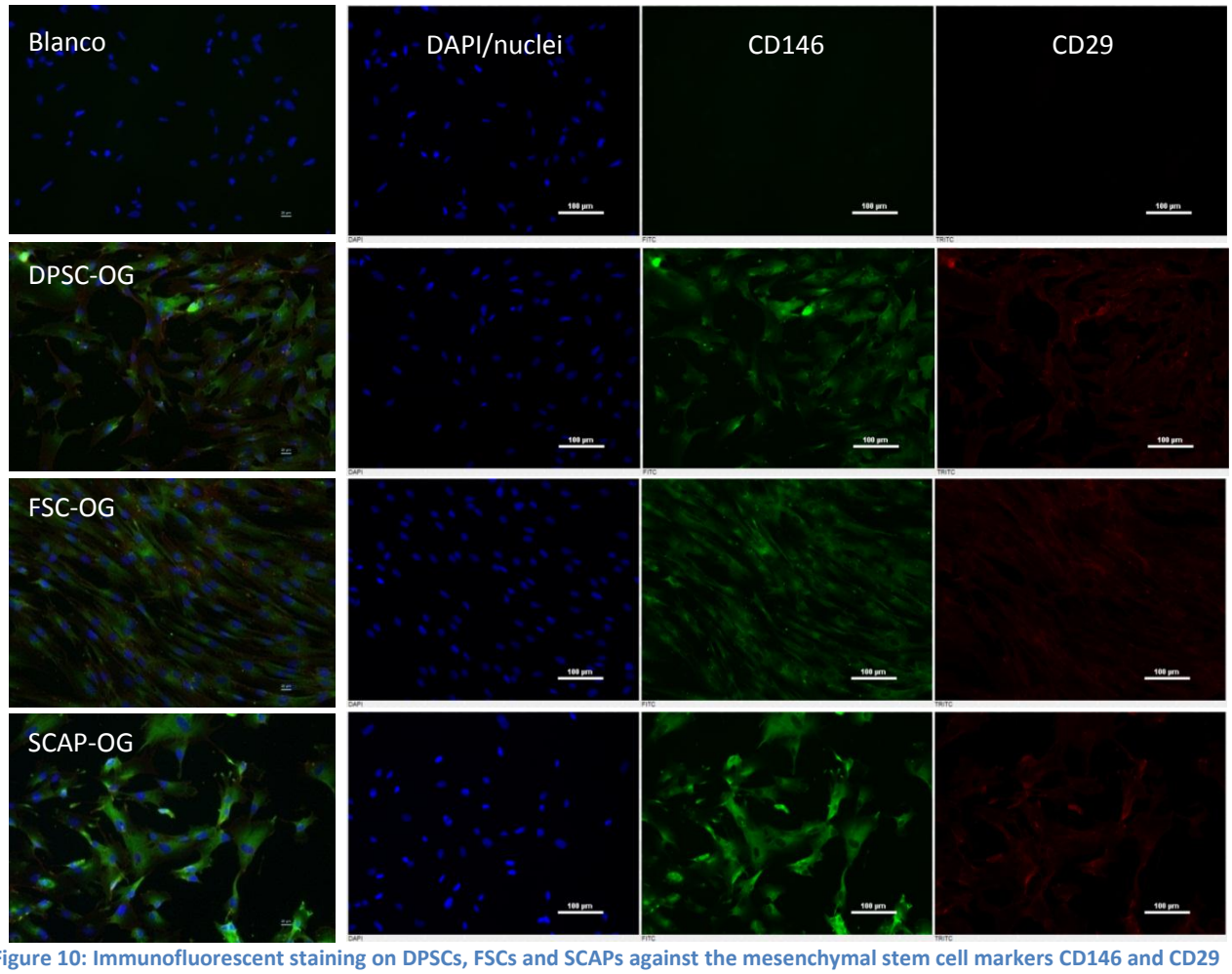


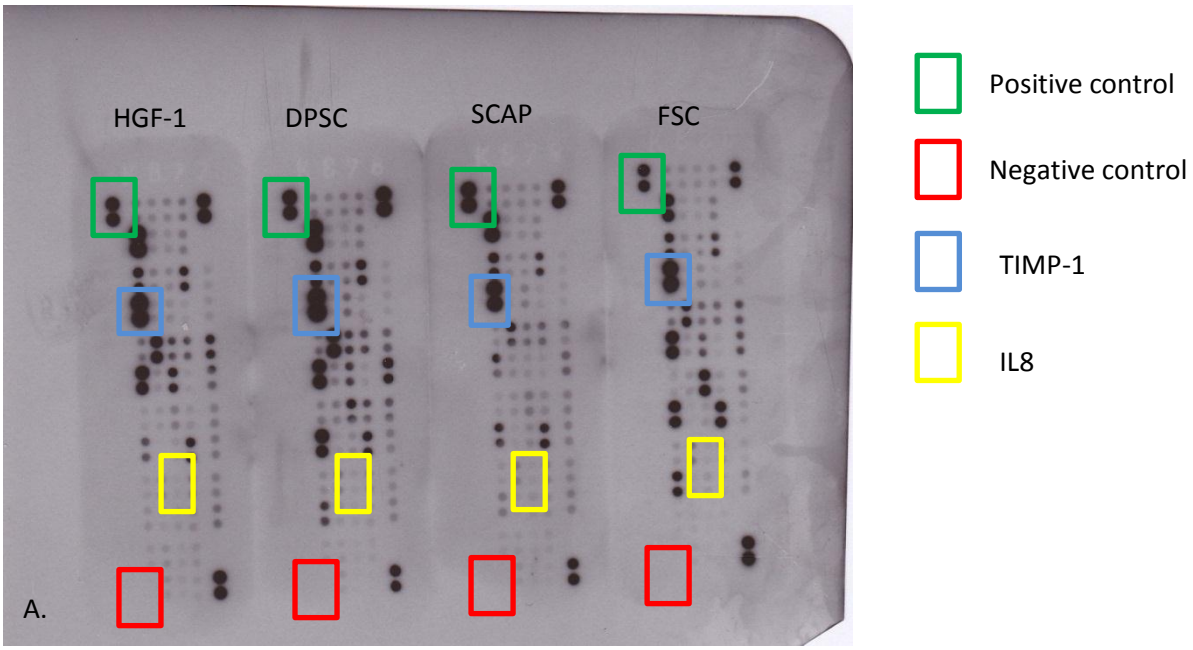
Figure 10: Immunofluorescent staining on DPSCs, FSCs and SCAPs against the mesenchymal stem cell markers CD146 and CD29

All three stem cell populations express the mesenchymal stem cell markers CD146 and CD29. This is in agreement with the findings of Jamal M. *et al*. [42] who showed the expression of CD146 and CD29 in all dental stem cell populations.
In order to confirm these mesenchymal stem cell properties of the DSC populations, further experiments need to be conducted, such as the investigation of the expression of more MSC markers, such as CD90, CD105 and Stro-1.

3.2.2. Expression pattern of angiogenic factors

3.2.2.1. Angiogenesis antibody array

In order to investigate which angiogenic factors are secreted by the DSCs and the HGF-1 cells, an angiogenesis antibody array was performed. To ensure there are no influencing/angiogenic factors in the culture medium, an array with 0.1% culture medium was performed as a control. This revealed that there were no angiogenic factors present in the medium (see supplemental information figure b). Each blot was moreover normalized against a positive and negative control.



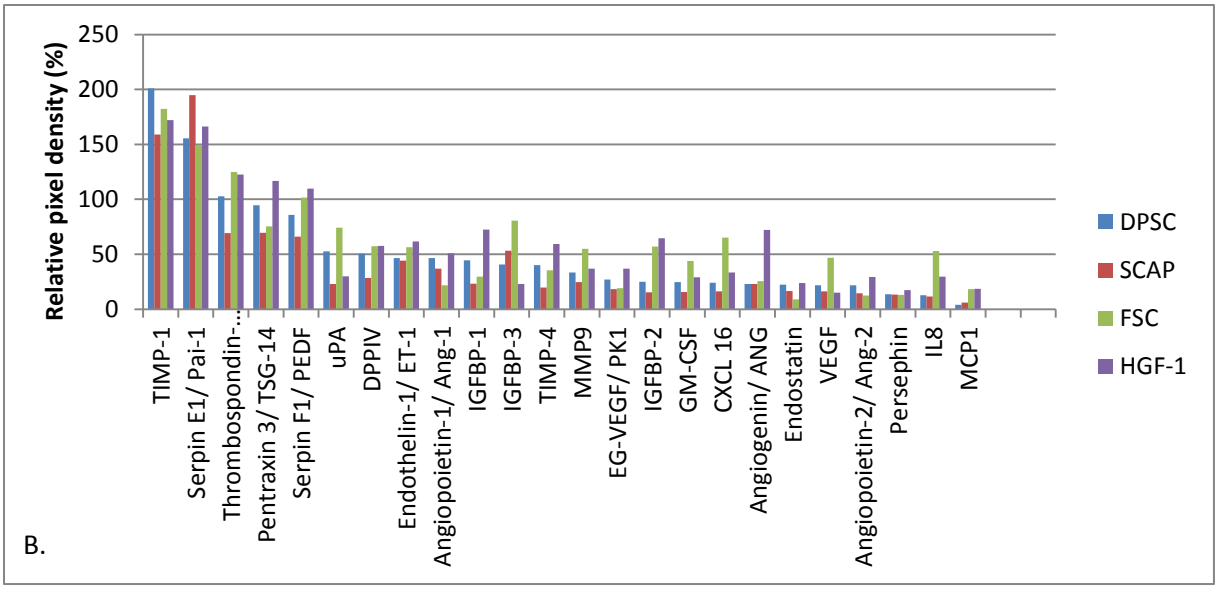


Figure11: An angiogenesis antibody array was performed on the CM of DPSCs, SCAPs, FSCs and HGF-1 cells to determine the relative expression of 55 angiogenesis related proteins. A. The blot on which the angiogenic factors expressed in the CM of the dental stem cell populations and the HGF-1 cells are presented. B. Analysis of the angiogenic factors excreted by the DPSCS, SCAPs, FSCs and HGF-1 cells. (n=2)

Various pro-and anti-angiogenic factors were expressed in the conditioned medium (CM) of the DSCs and the HGF-1 cells. TIMP1, PAI-1 and Thrombospondin were identified in high levels in the CM of the DSCs and the HGF-1 cells. Moreover, TIMP4 and angiogenin were excreted more by the HGF-1 cells compared to the DPSCs, SCAPs and FSCs. IL8 was furthermore present in higher conditions in FSCs than in DPSCs, SCAPs and HGF-1 cells. These factors were selected to be validated further by means of RT-PCR and ELISA. On the other hand, IGFBP-3 and VEGF were more present in the CM of the DSCs. These factors were validated by Fanton Y.

This secretion profile accords to the secretion profile of MSCs isolated from human bone marrow (BM-MSCs) which also show expression of TIMP1, angiogenin, VEGF, IL8 and MCP1 [31]. Moreover, MSCs from cord blood expressed a lot of cytokines including GM-CSF, IL8, VEGF, MCP1, IGFBP-1, IGFBP-3 and TIMP1 [43].

Table 3.1: Factors secreted by the dental stem cells and the HGF-1 cells and their function

Factors	Pro-or anti-angiogenic	Function
Factors expressed by all studied cells		
TIMP-1	Anti	Inhibitor of MMPs (which promote extracellular matrix (ECM) degradation)[44] [45]
Serpin E1/ PAI-1	Anti	Inhibits uPA
Thrombospondin	Anti	Inhibits proliferation and migration of endothelial cells (ECs) [46]
Pentraxin 3	Anti	Binds to FGF-2 and so inhibits FGF-2 induced EC proliferation [47]
Serpin F1/ PEDF	Anti	Inhibits EC migration and proliferation [48] Negative regulator of VEGF [49]

Urokinase plasminogen activator (uPA)	Pro	Promotes ECM degradation[50] Necessary for VEGF action [50]
DPP IV/ CD26	Pro	Role in the mobilization of hematopoietic progenitor cells [51]
Endothelin 1	Pro	Stimulates migration, proliferation and invasion of EC [52]
Angiopoietin 1	Pro	EC sprouting Vessel stabilization [44]
CXCL16	Pro	Stimulates proliferation and chemotaxis of ECs [53]
IL8	Pro	Stimulates EC migration, proliferation and survival [44] [54] Enhances MMP expression [54]
Factors secreted more by HGF-1 than by the dental stem cells		
TIMP-4	Anti	Inhibitor of MMPs (which promote ECM degradation)[44] [45]
Angiogenin	Pro	EC proliferation [44]
Factors secreted more by the dental stem cells than by HGF-1		
IGFBP-3	Pro	Stimulation of EC motility Stimulation of network formation[44]
VEGF	Pro	EC proliferation and migration EC apoptosis ↓ Permeability ↑ [44]

The angiogenesis antibody array reveals that both pro- as anti-angiogenic factors are secreted by the DSCs (see table 3.1). The transwell migration assay (chapter 3.2.3) will elucidate whether the pro-angiogenic factors prevail over the actions of the anti-angiogenic factors.

3.2.2.2. Validation with RT-PCR

For validation of the expression of the anti-angiogenic factor TIMP1 at mRNA level, RT-PCR was performed on mRNA samples of 6 different patients from all three DSC populations. This was performed together with the housekeeping gene β-microglobulin. The expression of TIMP1 (462 bp) and β-microglobulin (213 bp) is shown in figure 12.

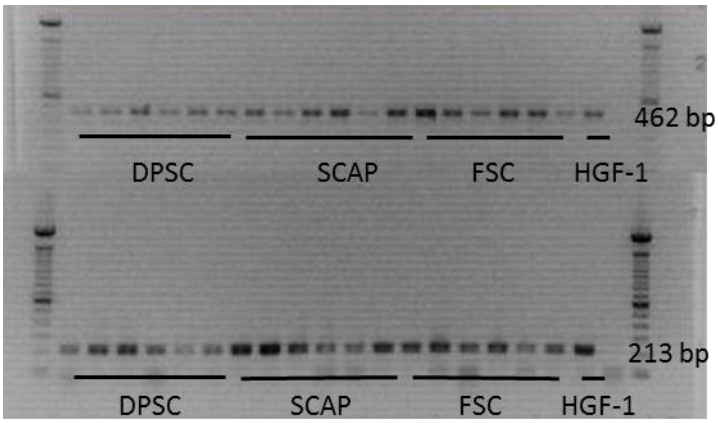


Figure 12: RT-PCR: Expression of the angiogenic factor TIMP1 at mRNA level by the three different dental stem cell populations and HGF-1 cells. RT-PCR was performed on cDNA of 6 different patients. Samples were loaded on a 1.2% agarose gel together with a 100 base pair ladder. Empty lanes at the end are non-template controls.

TIMP1 is expressed by all 6 patients of the three different DSC populations. TIMP1 seems to be more expressed by FSCs but in order to validate this quantitative difference, a q-PCR or FACS has to be performed. Moreover, HGF-1 cells do also express TIMP1. This is in line of the findings of the angiogenesis antibody array (see 3.2.2.1), where the protein expression of TIMP1 was found in all examined cells. Moreover, Kagiwada H. *et al*. showed expression of TIMP1 by BM-MSCs and primary human fibroblast cells (2F0-C75) [31]. TIMP1 is an anti-angiogenic factor which inhibits the action of matrix metalloproteinases (MMPs) which normally induce angiogenesis by degrading the ECM. The presence of anti-angiogenic factors, such as TIMP1 in the DSCs holds the angiogenic balance in equilibrium in order to prevent for example cancer metastasis.

3.2.2.3. Validation with ELISA

The secretion at protein level of the pro-angiogenic factors IL8 and Angiogenin (Ang) and the anti-angiogenic factor TIMP4 was validated by means of ELISA.

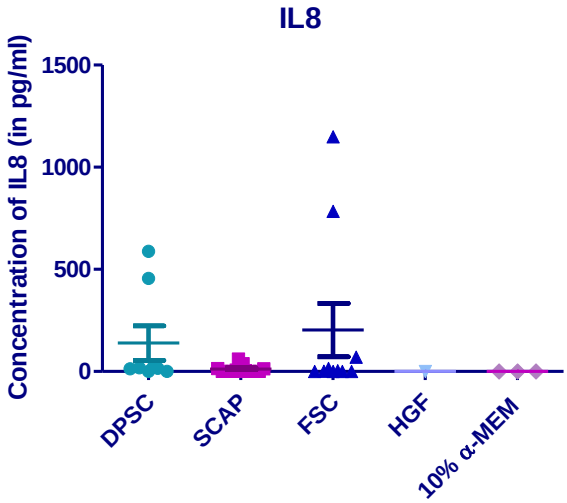


Table 3.2: IL8 concentrations secreted by DPSCs, SCAPs and FSCs. The concentration of IL8 is very variable between different patients.

DPSC (n=8)	SCAP (n=11)	FSC (n=10)
15.548390	12.645160	**784.471600**
20.387100	61.032260	0.000000
0.000000	0.000000	0.000000
0.000000	0.000000	4.280000
588.480000	0.000000	70.880000
455.680000	6.347826	0.000000
12.880000	0.000000	0.000000
17.652170	38.086960	**1149.448000**
	4.391304	2.483871
	0.000000	15.526320
	14.826090	

Figure 13: Distribution of IL8 concentrations was investigated on the CM of different patients on the 3 DSC populations. IL8 was not secreted by HGF-1 cells or was not present in the standard medium (10% α-MEM). Data were analysed by means of a Kruskal-Wallis test followed by a Dunns post test. Data are presented as means ± StDev. Number of repetitions is presented in table 3.2.

Based on the angiogenesis antibody array, it could be concluded that IL8 concentration was highest in the follicle. However, the ELISA showed no significant difference between the secretion of IL8 in the FSCs compared to the DPSCs and SCAPs (see figure 13). This can be due to too less repetitions of the angiogenesis antibody array, as it was just performed on two patients. These two patients show also a high expression of IL8 in the ELISA (see aberrant triangles on the graph and presented bold in table 3.2). Moreover, as is shown in table 3.2, there is a great patient variability in expression of IL8. As IL8 is a pro-inflammatory cytokine [55], an augmentation can be due to an infection, such as a mound infection or pulpitis (an inflamed pulp). Furthermore, capping materials, pulpotomy agents or stress can induce IL8 expression [56, 57].
Furthermore, HGF-1 cells showed a greater secretion of IL8 in the angiogenesis antibody array compared to the DPSCs and SCAPs. However, this is again not confirmed in the ELISA where there is no expression of IL8 from the HGF-1 cells. Moreover, Kagiwada H. *et al*. showed the expression of IL8 in both BM-MSCs and primary human fibroblast cells (2F0-C75) [31]. In order to correct or confirm our low IL8 expression by HGF-1 cells, the ELISA needs to be repeated more on HGF-1 cells. Besides this, IL8 is also secreted by adipose tissue-derived MSCs and MSCs from cord blood [43, 58]. IL8 is a pro-angiogenic factor which enhances MMP expression and stimulates endothelial cell migration, proliferation and survival. This is a positive feature when transplanting or using the DSCs for promotion of angiogenesis as these characteristics are all stimulating the formation of blood vessels.

Furthermore, the expression of angiogenin at protein level was investigated at 4 different patients (see figure 14). However, there was no significant difference in the secretion of this angiogenesis related factors between the 3 DSC populations. This is in accordance with the angiogenesis antibody array where the DPSCs, SCAPs and FSCs showed the same secretion profile. However, the HGF-1 cells had a higher expression of angiogenin in the angiogenesis antibody array compared to the DPSCs, SCAPs and FSCs. This is not confirmed by the ELISA, which shows a lower expression of Ang by the HGF-1 cells. This can be explained by too less repetitions of the angiogenesis antibody array and the ELISA on HGF-1 cells. In line with these findings, Kagiwada H. *et al*. showed a clear expression of angiogenin by BM-MSCs and human primary fibroblast cells. Angiogenin is a pro-angiogenic factor stimulating endothelial cell proliferation which is beneficial for the formation of blood vessels.

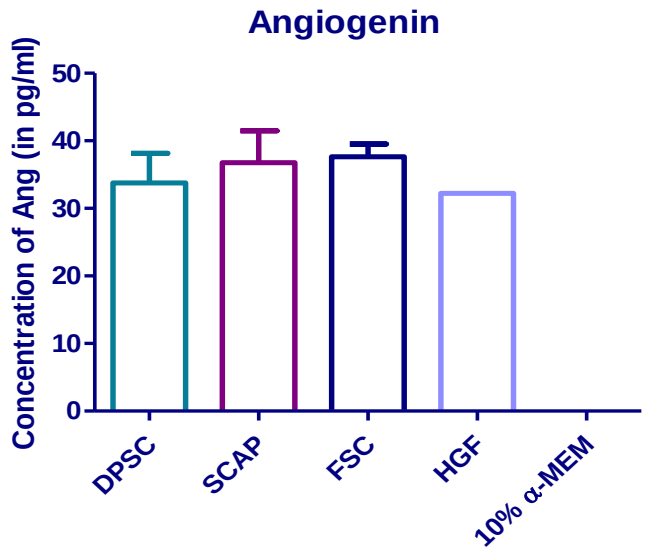


Figure 14: Distribution of TIMP4 concentrations investigated by means of ELISA on CM of 4 different patients on the 3 DSC populations. Data were analysed by means of a Kruskal-Wallis test followed by a Dunns post test. Data are presented as mean ± StDev.

The angiogenesis antibody array revealed a low TIMP4 expression in SCAPs and a high expression in the HGF-1 cells. However, examination of protein expression of TIMP4 by means of ELISA did not confirm these findings, as in the ELISA no secretion of TIMP4 by the HGF-1 cells was shown. Furthermore, the CM of DPSCs had less TIMP4 than the CM of SCAPs and FSCs, but this trend was not statistically significant leading to the conclusion that more patient material needs to be examined. TIMP4 is just as TIMP1, an anti-angiogenic factor inhibiting MMPs and thereby preventing the ECM degradation, necessary for the equilibrium of the angiogenic balance.

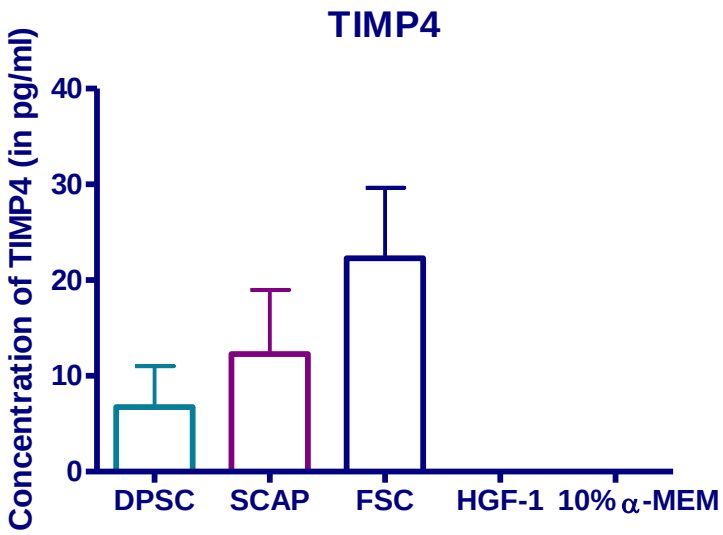


Figure15: Distribution of TIMP4 concentrations investigated on the CM of 6 different patients on the 3 DSC populations. Data were analysed by means of a Kruskal-Wallis test followed by a Dunns post test. Data are presented as mean ± StDev and correspond to n=6 assays.

3.2.2.4. Immunological analysis

In situ immunological analysis was performed on tissue sections of the different dental tissues out of which the DSCs are isolated in order to investigate the expression of angiogenic factors. This gives an indication that the expression of these molecules is not induced by the isolation and culturing conditions, but are already intrinsic present in the dental tissues.
We stained for the angiogenic markerprotein IL8 (see figure 16) as it shows expression differences in the angiogenesis antibody array and ELISA. Moreover, MCP1 (see supplemental information figure a) was stained based on the findings of Bronckaers A. *et al.* who showed a high expression of MCP-1 in DPSCs. Besides this, the tissues were stained for CD146 and CD29, MSC markers which are expressed in all examined dental tissues.

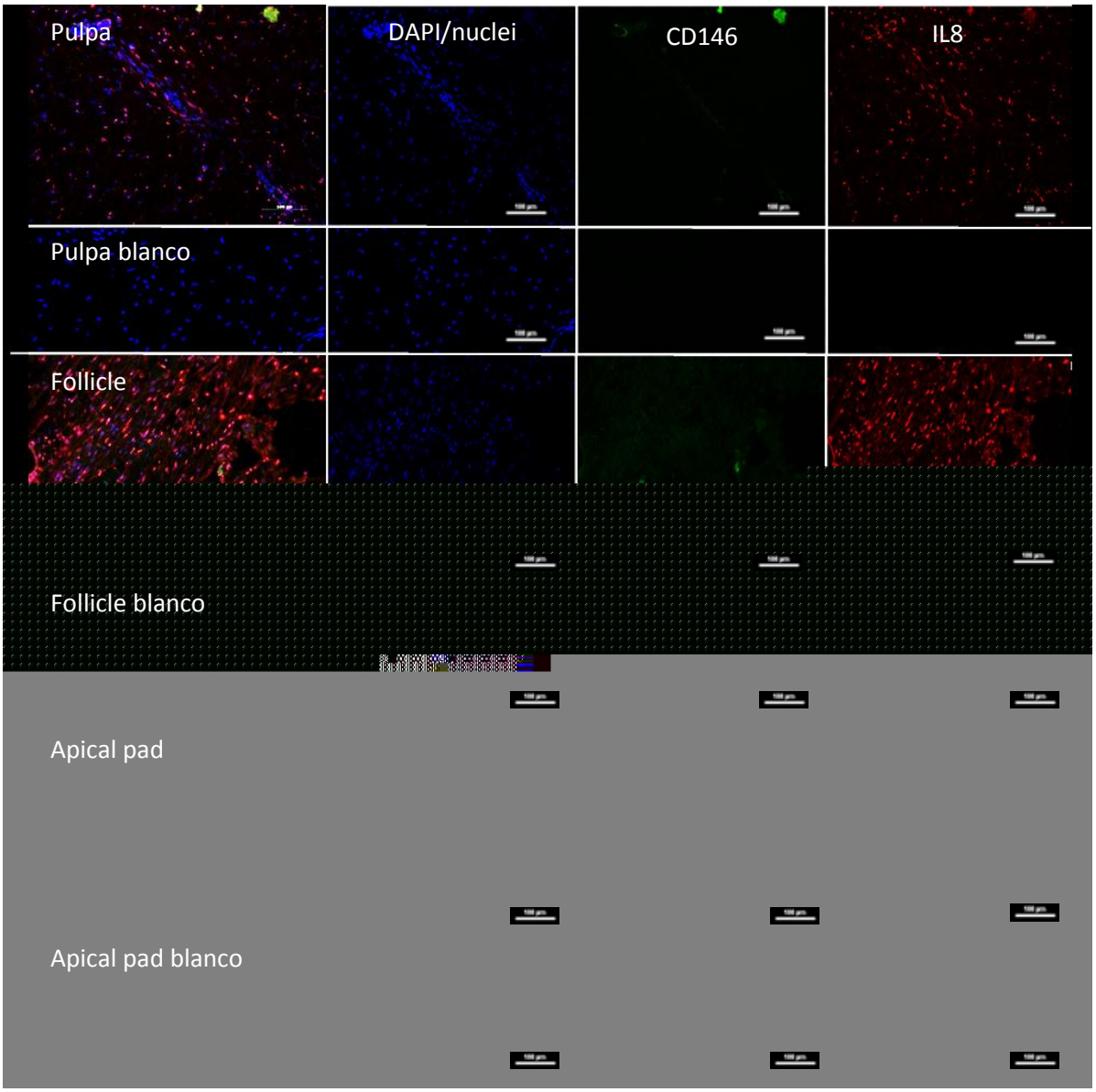


Figure 16: Immunofluorescent staining on tissues against CD146 and IL8. This staining is a representative staining for 6 different patients (n=6). IL8 was evenly distributed throughout the 3 dental tissues, but was more profound in the dental follicle. Scalebar= 100 µm

All three dental tissues express the mesenchymal stem cell markers CD146 and CD29 in the entire tissue, but they were more prominent in the linings of blood vessels. Moreover, the expression of the angiogenic markerprotein MCP1 (see supplemental information figure a) is evenly distributed throughout the entire tissue of all 3 stem cell populations. However, the IL8 expression is higher in the dental follicle compared to the apical and pulp tissue. This is in agreement with the angiogenesis antibody array which showed a higher IL8-expression in the FSCs. Though, the ELISA contradicts this as there is no significant difference between the expression profile of IL8 between the different DSC populations. Furthermore, the ELISA is performed on the conditioned medium of cultured cells while this immunological analyses is performed on tissue samples. Thus, an ELISA investigates the secretion of IL8 while immunohistochemistry shows the intracellular IL8 distribution. It can be possible that IL8 is present in the tissue but that there are no excretion mechanisms through which IL8 can be secreted in equal amounts as is present in the tissue.

Moreover, Fanton Y. demonstrated the presence of VEGF in all three dental tissues, with a higher expression in the dental pulp. Therefore, we can conclude that also *in situ* the dental tissues express angiogenic factors like VEGF, IL8 and MCP1.
However, in order to affirm the quantitative differences of the various angiogenic markers between the different dental tissues, a quantitative analysis should be performed. This can be accomplished by means of a Western blot.

3.2.2.5. Conclusions

Table 3.3: Summary of the conclusions from the angiogenesis antibody array, RT-PCR, ELISA and the immunohistochemistry.

	Angiogenesis antibody array	RT-PCR	ELISA	Immuno-histochemistry	Conclusion
Angiogenin	Equal distribution in all 3 DSC populations Trend to be more in HGF-1 cells	Not determined (ND)	Equal distribution in all 3 DSC populations	ND	Angiogenin is present in all 3 DSC populations
IL-8	Trend to be more in the FSCs	ND	High patient variability	Present in all tissues, more in the follicle	Trend to be more present in the FSCs
TIMP1	High expression in all DSCs and HGF-1 cells	Present in all 3 DSC populations and HGF-1 cells	ND	ND	Present in all 3 DSC populations
TIMP4	Trend to be more in the HGF-1 cells	ND	Trend to be more in the FSCs	ND	Present in all 3 DSC populations

3.2.3. Transwell migration assay

Migration of endothelial cells is one of the key events in angiogenesis. For this reason, we investigate the potential effects of DPSCs on the migration capacity of endothelial cells (HMEC-1) *in vitro* by means of a transwell migration assay. In this assay, an inducer of angiogenesis is placed in the lower well, in our case the DPSCs. Afterwards, endothelial cells (ECs), e.g. HMEC-1 cells, are placed in the upper well with a pore size of 8 µm (see figure 17).

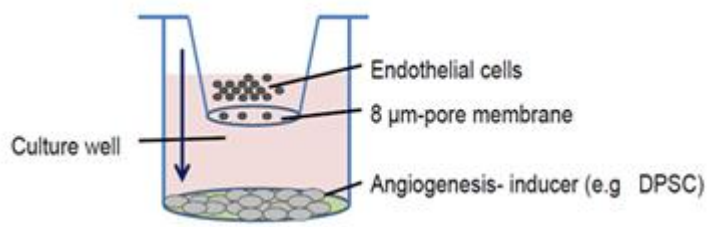


Figure 17: Upset transwell migration assay. In the lower well, DPSCs are placed in order to investigate if they are able to attract endothelial cells, which are placed in the upper well.

As a negative and positive control situation, standard culture medium (0.1% α-MEM) and medium supplemented with 10% FCS (10% α-MEM) is used respectively. DPSCs are cultured one day in 0.1% α-MEM before starting the transwell migration experiment in order to exclude the factors present in the 10% culture medium and in order to allow the DPSCs to secrete their angiogenic factors in this medium.

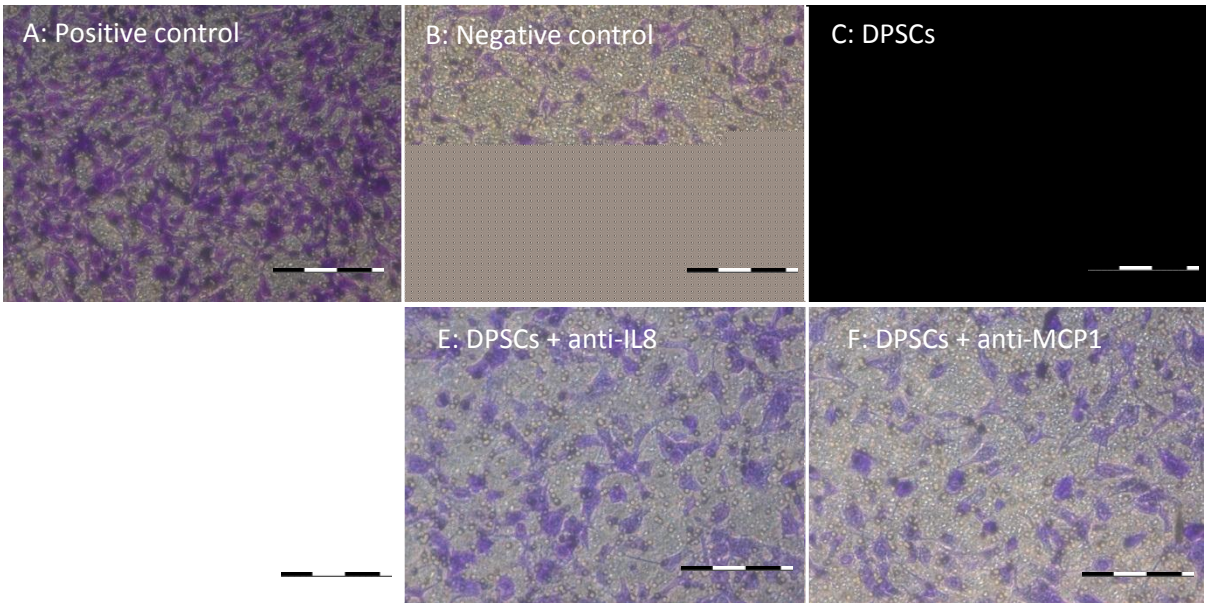
As is presented in figure 17, HMEC-1 cells migrate most to the standard culture medium supplemented with 10% FCS, as this FCS contains a lot of attracting factors. The negative control situation does almost not induce migration. The DPSCs do attract the endothelial cells, which means they excrete pro-angiogenic factors in the culture medium.

The angiogenesis antibody array revealed the expression of both pro-as anti-angiogenic factors. Based on the EC migration assay, it could be concluded that the angiogenic balance is shifted towards the pro-angiogenic factors.

In order to evaluate the effects of the secreted factors, neutralizing antibodies against some pro-angiogenic proteins were added. As preliminary data and the angiogenesis antibody array have shown that DPSCs secrete IL8, VEGF and MCP-1, we added anti-VEGF, anti-IL8 and anti-MCP1 antibodies to the culture medium of the DPSCs before starting the experiment (see figure 18 D-F).

Addition of anti-VEGF leads to a significant decrease of migration of ECs (see figure 18). Furthermore, inhibition of IL8 and MCP-1 also leaded to a decrease in migration, but this inhibition was not statistically significant. This can be due to too less repetitions of the transwell assay in addition with anti-IL8 (n=4) or anti-MCP1 (n=5). Addition of both anti-IL8 and anti-VEGF did not lead to more inhibition of EC migration compared to the conditions where only one of these antibodies was used (as we expected that application of two antibodies would further reduce the amount of migrated ECs) (see figure 18). This might be explained by the fact that anti-VEGF blocks the effects of anti-IL8 or vice versa. Another possible explanation can be found in compensation mechanisms, in which the cell upregulates other pro-angiogenic factors when adding both anti-VEGF and anti-IL8. The inhibition of both VEGF and MCP1 or IL8 and MCP1 can lead to the same working mechanisms.

A third possibility for the reason why the migration is not completely stopped by adding both anti-IL8 and anti-VEGF, is that maybe other angiogenic factors play an additional role in the attraction of ECs towards the DPSCs. For example, as we showed that angiogenin is also expressed by the DPSCs, the effect of anti-angiogenin on the DPSC-induced EC migration should be studied.



G.

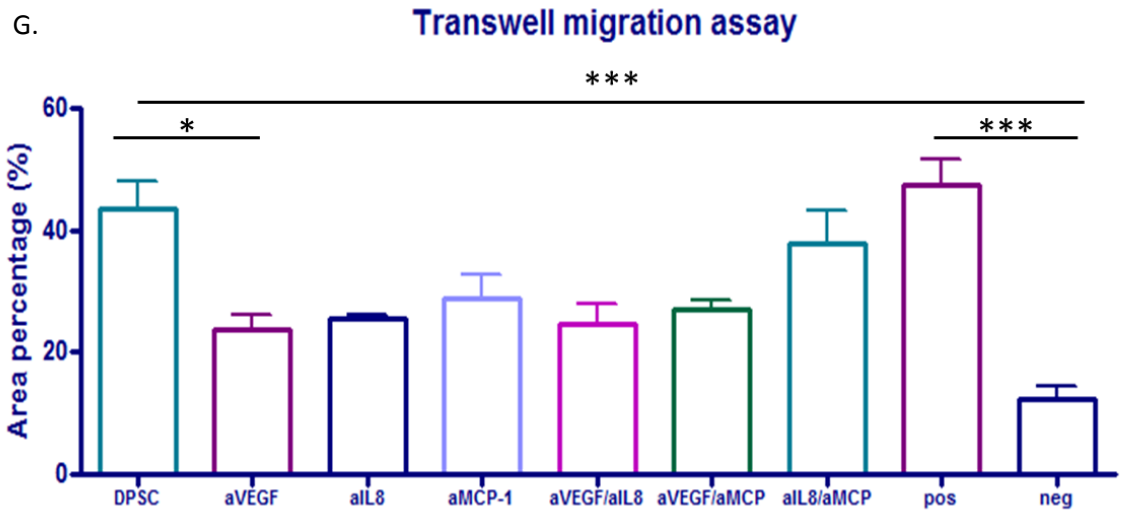


Figure 18: The transwell migration assay is performed with positive (A) and negative (B) control situations and DPSCs (C). EC migration is induced by 10% FCS (positive control, A) and DPSCs (C) compared to the negative control (B). D-F: The transwell migration assay was moreover performed with antibodies against VEGF, IL8 and MCP1 or combinations of different antibodies. (scalebar= 2µm); G Statistical analysis of the transwell migration assay. There is a significant increase of migration by DPSCs compared to the negative control situation. Moreover, addition of anti-VEGF leads to a significant decrease of migration. However, adding antibodies against IL8 or MCP1 does not give a significant migration diminution, as is also the case when administering combinations of antibodies against pro-angiogenic factors. Data were analysed using a Kruskal-Wallis test followed by a Dunn's multiple comparison test. Data are represented as mean ± StDev and corresponds to n= 8 assays. *= p-value< 0.05 and ***= p-value< 0.001

In conclusion, we showed for the first time that DPSCs are able to induce EC migration *in vitro*. However, more studies should be performed to verify whether DPSCs are also involved in other stages of the multistep process of angiogenesis. In this multistep process, the attraction of ECs is without doubt not enough to form new blood vessels. Several other *in vitro* models need to be performed to fully simulate the process of blood vessel formation. First of all, the effect of DSCs on EC proliferation needs to be investigated. This can be done by for example a MTT assay. Next, migration of ECs alone is not sufficient, but a directional migration is needed. This can be simulated by a wound healing assay. But besides the paracrine actions of DSCs, they are furthermore able to differentiate into ECs themselves. This can be investigated by addition of endothelial induction media.

3.2.4. Chick embryo chorioallantoic membrane assay (CAM assay)

Besides investigation of angiogenesis *in vitro*, the chick embryo chorioallantoic membrane (CAM) assay provides a method to evaluate angiogenesis *in vivo*. This technique is based on the implantation of gelatin sponges on top of the growing CAM on day 9 of embryonic development. After implantation, the sponges were treated with several test and control compounds, namely DPSCs (10,000 or 50,000 cells), CM or concentrated CM, in order to investigate the effects on blood vessel formation. An angiogenic response needs to occur 72–96 hours after stimulation in the form of increased vessel density around the implant, with the vessels radially converging toward the center like spokes in a wheel. Here, blood vessels growing towards the sponges were counted (see figure 19 b) on day 12.

There were no significant differences found between the test and control compounds placed on the sponges on top of the CAM (see figure 19). This can be due to the usage of a too low amount of cells as Lee M. *et al.* administered 100,000 cells [59] and Oskowitz *et al*. administered 200,000 to 2,000,000 cells [60] in order to elicit an angiogenic response. However Ribatti D. *et al*. applied 18,000 cells/sponge (mm^3) in order to provoke an angiogenic respons [61]. A second explanation could be that the applied DPSCs did not survive and could not perform their intended action. This can be investigated by incorporation of the GFP^+-gene into the genome of the DPSCs in order to test viability by staining proliferative cells with a KI67 double staining. Another way to optimize the CAM experiment is to apply the DPSCs onto the CAM by bringing a solution of DPSCs in straw disks [59], by usage of small volumes of Matrigel mixtures who polymerize rapidly onto the CAM or by means of pre-gelated defined aliquots of matrigel supplemented with test compounds on nylon meshes and afterwards placed onto the CAM [62]. Kidney MSCs have already shown to induce angiogenesis and vasculogenesis using matrigel-filled implantable angioreactors as Chen J. *et al*. showed [28].

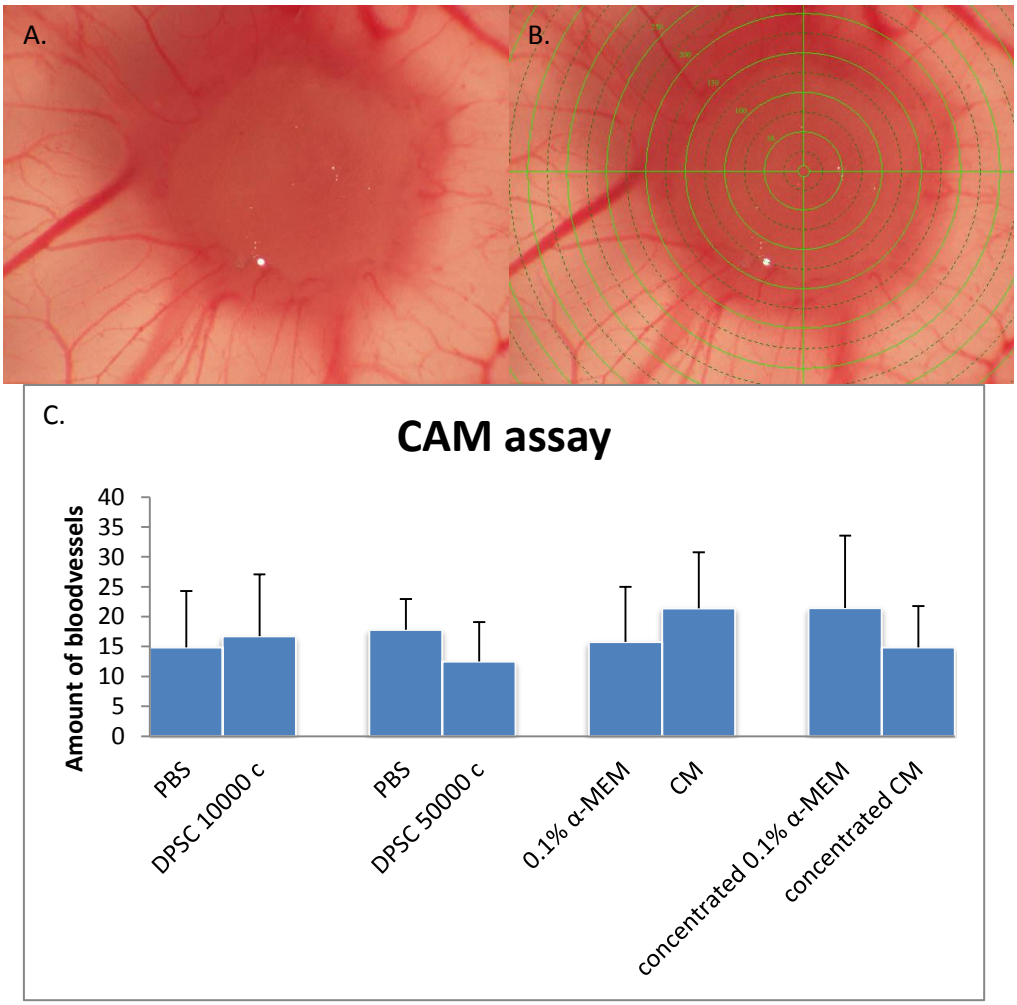


Figure 19: CAM assay: A: A gelatin sponge with test compounds placed on top of the CAM induces blood vessel formation. B: In order to quantify the blood vessels, a grid was placed on top of the figure and the blood vessels intersecting these circles were counted; C: Graph representing the amount of blood vessels leading towards the gelatin sponges placed on top of the CAM containing test and control compounds. There was no significant difference found between control and test compounds. Data were analysed using a Mann-Whitney test.

Also other *in vivo* models to assess the angiogenic properties of the DSCs could be applied. The matrigel plug assay is a valuable technique and is based on subcutaneously injecting a matrigel containing test cells or substances. Once injected, the matrigel forms a plug which can be recovered after 7-21 days and can be examined histologically to determine the amount of new blood vessels that have been grown into the plug [63]. Another *in vivo* assay is the corneal angiogenesis assay which uses the avascular cornea of a rabbit or mouse as test environment. A pocket is made in the cornea and test substances applied in this pocket elicit the ingrowth of new vessels from the peripheral limbal vasculature. Any vessel seen in the cornea after stimulation by angiogenesis-inducing factors, is a new vessel (as the cornea is avascular)[63].

3.3. Tissue engineering

As dental stem cells could be a good source of cells for regenerating injured or defective body parts, and are able to induce angiogenesis, we here investigate the possibility of DPSCs to grow on a titanium scaffold. Moreover, we investigate the possibility of differentiating towards an osteogenic lineage when growing on the Ti-scaffold. This is done by seeding the cells onto different Ti-scaffolds with a variety of surface structures (see figure 6 materials and methods). Furthermore, the effect of the different Ti-scaffolds on the morphology of the DPSCs will be compared.
For examination of growth and differentiation of DPSCs onto the Ti scaffold, electron microscopic analysis was performed.

3.3.1. Electron microscopic analysis

Normal undifferentiated DPSCs have a typical MSC morphology: they are spindle-shaped, have a nucleus with prominent nucleoli and the cellular organelles are only found in the perinuclear area (figure 20 a). When grown on the different Ti-scaffolds for several days, Ti particles were observed in cellular vesicles meaning that the cells were able to incorporate Ti as is shown in figure 20 c and d.

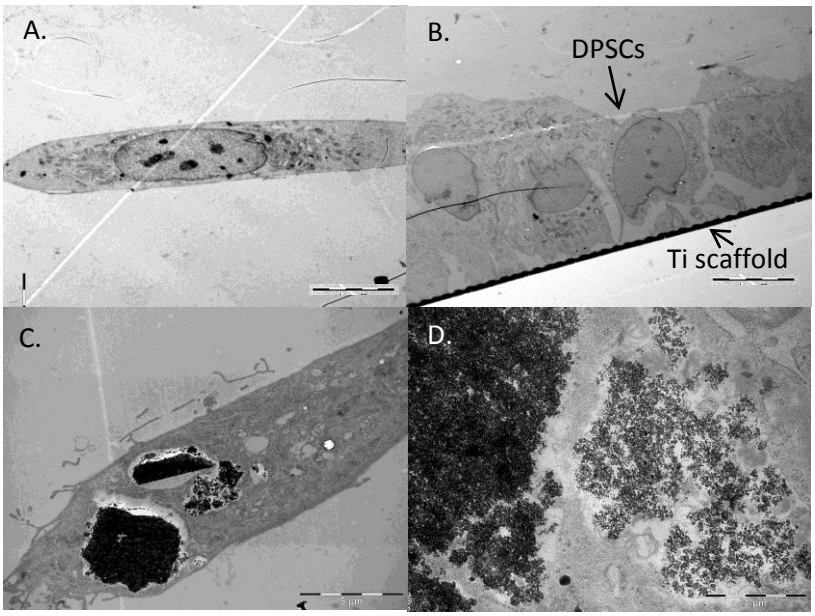


Figure 20: Electron microscopic analyses of DPSCs grown on titanium. A: Control cell with a typical spindle-shaped MSC morphology (scale bar=10 µm); B: DPSCs grown on a Ti-scaffold (scale bar=1 µm); C: Ti particles incorporated by the DPSCs (scale bar=5 µm); D: magnification of the Ti particles taken up by the DPSCs (scale bar= 1 µm).

After 7 days of incubation in osteogenic medium, the deposition of extracellular collagen fibres could be observed in all conditions. However, the osteogenic differentiation was incomplete as no extracellular vesicles and hydroxyapatite needles were found (see figure 21 b). After 3 weeks of osteogenic induction, the morphology of the DPSCs was completely altered. The cells had a more rounded morphology and had a lot of cellular vesicles (see figure 21 c). Furthermore, there were numerous amounts of collagen fibres and extracellular matrix vesicles present (figure 21 d and e). The formation of hydroxyapatite needles could also be observed (figure f). However, there was no difference between the cells incubated on thermanox or on the different Ti-scaffolds.

In order to determine whether a difference in Ti- scaffolds also results in a quantitative difference in bone quality, more quantitative studies need to be performed in the future. For example, the amount of Ca^{2+}-deposits can be determined by a quantitative alizarine red staining. Moreover, in order to clarify which scaffold provides the best grow environment, the cells could be counted after 3 weeks of differentiation on the different scaffolds.

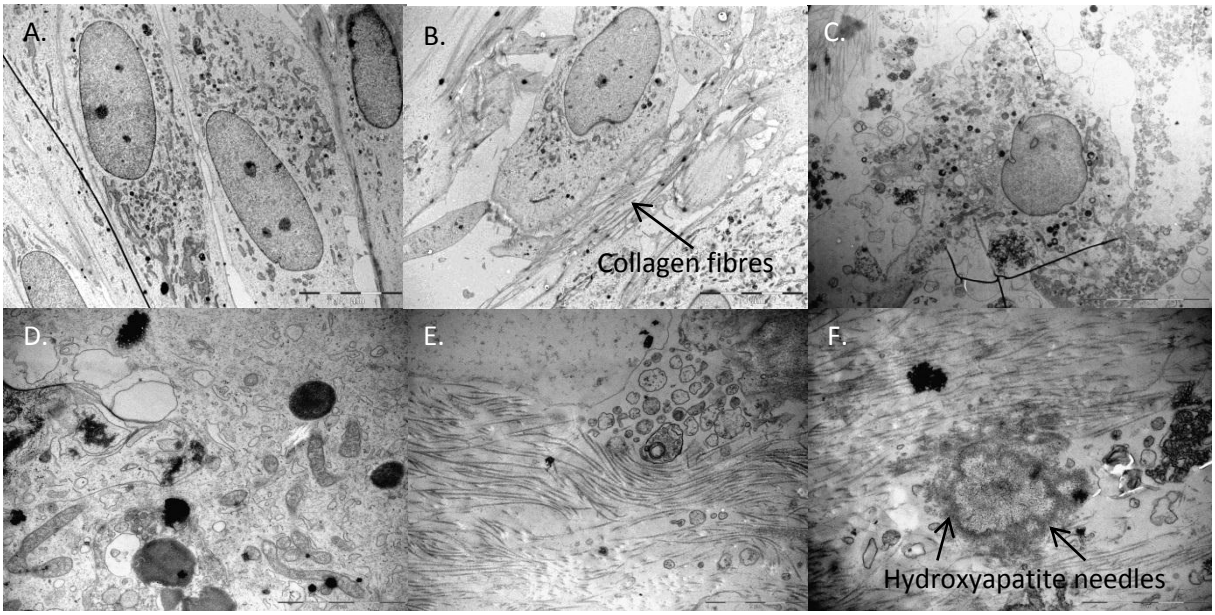


Figure 21: Electron microscopic analyses of DPSCs grown onto Ti after osteogenic induction. A: Control cells (7d) with a typical MSC morphology with the organelles situated around the nucleus (scale bar= 10 µm); B: Deposition of extracellular collagen fibres after 7 days of osteogenic differentiation (scale bar= 10 µm); C: DPSCs after 3 weeks of differentiation show a more rounded shape and a lot of cellular vesicles (scale bar=10 µm); D and E: Numerous amounts of extracellular matrix vesicles and collagen depositions are present after 3 weeks of differentiation (scale bar= 2 µm); F: Hydroxyapatite needles were also present after 3 weeks of osteogenic induction (scale bar= 2 µm).

4. Conclusion and synthesis

In this study, we first investigated the optimal way of isolating dental pulp stem cells from wisdom teeth. We compared the outgrowth method in which the cells were allowed to grow out a tissue piece, and enzymatic digestion, in which the tissue piece was mechanically fractionated and digested with a mixture of collagenase-dispase. In order to decide which method leads to the best results, microscopic, electron microscopic and immunohistochemic analyses were performed. Isolation by means of the outgrowth method leaded to a more homogeneous cell population and a more mesenchymal stem cell like morphology was observed after electron microscopic analyses, e.a. a spindle-shaped fibroblast-like cell with a prominent nucleus and peri-nuclear organised organelles. Immunohistochemic analyses did not show a difference between DPSCs isolated by means of the outgrowth method or by means of enzymatic digestion. In the next studies, where the angiogenic properties of three different dental stem cell populations are compared, it was chosen to isolate all dental stem cells by means of the outgrowth method. However, more experiments need to be performed to really validate these conclusions. This encompasses the investigation of more mesenchymal stem cell markers and the effect of the isolation method on the differentiation potential of the DPSCs.

Besides the optimization of the isolation method, this study further explores the angiogenic properties of different dental stem cell populations. Adequate angiogenesis with a well-balanced equilibrium between pro-and anti-angiogenic factors is important for normal body function. However, if this equilibrium is disrupted, several diseases can occur. If the stimulators dominate, cancer metastasis, multiple sclerosis or rheumatoid arthritis can arise. On the other hand, if there is insufficient angiogenesis, this can result in diabetes, stroke or ischaemic heart diseases. Moreover, blood vessel formation is also necessary in tissue engineering in order to prevent necrosis of the tissue implant and for delivery of the inductive signals to the implanted cells. In these cases, (re)vascularization is necessary. However, currently used methods of revascularization are no longer effective for a significant number of patients. For this reason, we investigated the effects of DSCs as an alternative method for the stimulation of angiogenesis. By means of the angiogenesis antibody array, we found that the DSCs secrete a wide variety of pro-angiogenic factors, such as uPA, DPP4, Endothelin 1, Angiogenin, VEGF and IL8. Besides the secretion of pro-angiogenic factors, also various anti-angiogenic factors such as TIMP1, TIMP4, Serpin E1, Thrombospondin and Pentraxin 3 are secreted. RT-PCR analysis revealed the presence of TIMP1 in all DSCs. In order to quantify which population secretes the most, a q-PCR or FACS has to be performed. Moreover, the protein expression of the anti-angiogenic factor TIMP4 and the pro-angiogenic factors angiogenin and IL8 were investigated by performing an ELISA. The secretion of TIMP4 was highest in FSCs, however this does not confirm the findings of the angiogenesis antibody array. In order to further confirm the secretion profile of TIMP4 by DSCs, more patient material should be studied as now only the TIMP4 levels of only 4 patients were included in this study. ELISA showed that all DSCs expressed the same amount of angiogenin. Protein expression of IL8 was also shown in all 3 stem cell populations but showed a great patient variability. Moreover IL8 was *in situ* demonstrated in all three dental tissues with a greater expression in the follicle. Our group is the first to show expression of the pro-angiogenic factor angiogenin and the anti-angiogenic factors TIMP1 and TIMP4 by dental stem cells.

As both pro-and anti-angiogenic factors are secreted by the dental stem cells, and endothelial cells are known to migrate towards angiogenesis-inducing factors, a transwell migration assay was performed. In this way, we could elucidate which factors prevail. First of all, DPSCs were able to induce migration of endothelial cells significantly compared to the negative control situation. This means DPSCs can attract endothelial cells and they could lead to blood vessel formation. Next, in order to elucidate which factors had a role in this endothelial cell migration, neutralizing antibodies against some pro-angiogenic factors were added. Inhibition of VEGF leaded to a significant decrease of migration towards the DPSCs. Consequently, VEGF plays a major role in the attraction of endothelial cells. However, counteraction of IL8 or MCP1 did not lead to a significant migration decrease, but a trend towards less migration can be seen. Hence, we can conclude that VEGF has a predominant effect in the attraction of endothelial cells towards the DPSCs. However, as migration is not completely stopped, other angiogenic factors play an additional role in the attraction of endothelial cells towards the DPSCs. Furthermore, angiogenesis was tested *in vivo* by means of the CAM assay. However, there was no difference present between DPSCs and control situations. This can be explained by a too low amount of cells used or the dying of the cells, meaning that the CAM assay needs further optimization.

Nonetheless, the secretion of an array of angiogenic factors and the stimulation of migration of endothelial cells, provides a hopeful future for the treatment of diseases in which angiogenesis is insufficient. DSCs could be used in the future as a potential remedy for curing such disorders, such as diabetes, stroke and ischaemic heart disease. By injecting them into the patient, they can provide a favourable environment for the formation of blood vessels. They will secrete their angiogenic factors and elicit the attraction of endothelial cells. However, before DSCs can be used in the clinic, more investigations are required, like for example the application of these cells in various mouse angiogenesis models such as the mouse matrigel assay.

As a last subject, this study has investigated the possibility of using DPSCs in tissue engineering. As they are able to induce angiogenesis and as stem cells maybe can regenerate lost or damaged tissue parts, this would give hopeful results. We studied the possibility of DPSCs to grow on different titanium scaffolds and explored their osteogenic induction potential. We found that DPSCs attach and grow on all titanium scaffolds and moreover can be differentiated into osteocytes. This implies great future possibilities. They can be used to regenerate lost or damaged bones by just placing a titanium scaffold containing DPSCs. These will proliferate towards osteocytes and create new bone. Moreover, DPSCs are able to induce angiogenesis, so inductive signals can be delivered at the place of action. These regulatory molecules are necessary to stimulate cellular growth, proliferation and differentiation of the new grown cells. Moreover, blood vessels are necessary to provide oxygen and nutrients to accomplish long-term survival of the new tissue.

References

1. Laschke, M.W., et al., *Angiogenesis in tissue engineering: breathing life into constructed tissue substitutes.* Tissue Eng, 2006. **12**(8): p. 2093-104.
2. Nahmias, Y. and M.L. Yarmush, *Tissue engineering application in general surgery*, in *Fundamentals of tissue engineering and regenerative medicine*2009. p. 855-869.
3. Djouad, F. and R.S. Tuan, *Mesenchymal Stem Cells: New Insights Into Tissue Engineering and Regenerative Medicine*, in *Fundamentals of Tissue Engineering and Regenerative Medicine* p. 177-188.
4. Yeong, W.Y., et al., *Development of scaffolds for tissue engineering using a 3D inkjet model maker.* Virtual Modeling and Rapid Manufacturing, 2005: p. 115-118.
5. Carmeliet, P. and R.K. Jain, *Molecular mechanisms and clinical applications of angiogenesis.* Nature, 2011. **473**(7347): p. 298-307.
6. Sieveking, D.P. and M.K. Ng, *Cell therapies for therapeutic angiogenesis: back to the bench.* Vasc Med, 2009. **14**(2): p. 153-66.
7. Loeffler, M. and I. Roeder, *Tissue stem cells: Definition, plasticity, heterogeneity, self-organization and models - A conceptual approach.* Cells Tissues Organs, 2002. **171**(1): p. 8-26.
8. Morrison, S.J. and J. Kimble, *Asymmetric and symmetric stem-cell divisions in development and cancer.* Nature, 2006. **441**(7097): p. 1068-74.
9. Lakshmipathy, U. and C. Verfaillie, *Stem cell plasticity.* Blood Rev, 2005. **19**(1): p. 29-38.
10. Dominici, M., et al., *Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement.* Cytotherapy, 2006. **8**(4): p. 315-7.
11. Liu, J.W., et al., *Characterization of endothelial-like cells derived from human mesenchymal stem cells.* J Thromb Haemost, 2007. **5**(4): p. 826-34.
12. Huang, G.T., S. Gronthos, and S. Shi, *Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine.* Journal of Dental Research, 2009. **88**(9): p. 792-806.
13. Papaccio, G., et al., *Long-term cryopreservation of dental pulp stem cells (SBP-DPSCs) and their differentiated osteoblasts: a cell source for tissue repair.* J Cell Physiol, 2006. **208**(2): p. 319-25.
14. Ding, G., et al., *Suppression of T cell proliferation by root apical papilla stem cells in vitro.* Cells Tissues Organs, 2010. **191**(5): p. 357-64.
15. Maurer, M.H., *Proteomic definitions of mesenchymal stem cells.* Stem Cells Int, 2011. **2011**: p. 704256.
16. Gronthos, S., et al., *Stem cell properties of human dental pulp stem cells.* Journal of Dental Research, 2002. **81**(8): p. 531-5.
17. Marchionni, C., et al., *Angiogenic potential of human dental pulp stromal (stem) cells.* Int J Immunopathol Pharmacol, 2009. **22**(3): p. 699-706.
18. Volponi, A.A., Y. Pang, and P.T. Sharpe, *Stem cell-based biological tooth repair and regeneration.* Trends Cell Biol, 2010. **20**(12): p. 715-22.
19. Morsczeck, C., et al., *Isolation of precursor cells (PCs) from human dental follicle of wisdom teeth.* Matrix Biol, 2005. **24**(2): p. 155-65.
20. Honda, M.J., et al., *Dental follicle stem cells and tissue engineering.* J Oral Sci, 2010. **52**(4): p. 541-52.
21. Kadar, K., et al., *Differentiation potential of stem cells from human dental origin - promise for tissue engineering.* J Physiol Pharmacol, 2009. **60 Suppl 7**: p. 167-75.
22. Kiraly, M., et al., *Simultaneous PKC and cAMP activation induces differentiation of human dental pulp stem cells into functionally active neurons.* Neurochem Int, 2009. **55**(5): p. 323-32.

23. Mantesso, A. and P. Sharpe, *Dental stem cells for tooth regeneration and repair.* Expert Opin Biol Ther, 2009. **9**(9): p. 1143-54.
24. Toma, C., et al., *Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart.* Circulation, 2002. **105**(1): p. 93-8.
25. Al-Khaldi, A., et al., *Therapeutic angiogenesis using autologous bone marrow stromal cells: improved blood flow in a chronic limb ischemia model.* Annals of Thoracic Surgery, 2003. **75**(1): p. 204-9.
26. Wu, Y.J., et al., *Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis.* Stem Cells, 2007. **25**(10): p. 2648-2659.
27. Oswald, J., et al., *Mesenchymal stem cells can be differentiated into endothelial cells in vitro.* Stem Cells, 2004. **22**(3): p. 377-84.
28. Chen, J., et al., *Kidney-derived mesenchymal stem cells contribute to vasculogenesis, angiogenesis and endothelial repair.* Kidney Int, 2008. **74**(7): p. 879-89.
29. Xu, Y., et al., *Umbilical cord-derived mesenchymal stem cells isolated by a novel explantation technique can differentiate into functional endothelial cells and promote revascularization.* Stem Cells Dev, 2010. **19**(10): p. 1511-22.
30. Kachgal, S. and A.J. Putnam, *Mesenchymal stem cells from adipose and bone marrow promote angiogenesis via distinct cytokine and protease expression mechanisms.* Angiogenesis, 2011. **14**(1): p. 47-59.
31. Kagiwada, H., et al., *Human mesenchymal stem cells as a stable source of VEGF-producing cells.* J Tissue Eng Regen Med, 2008. **2**(4): p. 184-9.
32. Chen, L., et al., *Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing.* Plos One, 2008. **3**(4): p. e1886.
33. Wu, Y., et al., *Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis.* Stem Cells, 2007. **25**(10): p. 2648-59.
34. Otsu, K., et al., *Concentration-dependent inhibition of angiogenesis by mesenchymal stem cells.* Blood, 2009. **113**(18): p. 4197-205.
35. Shi, S. and S. Gronthos, *Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp.* J Bone Miner Res, 2003. **18**(4): p. 696-704.
36. Nakashima, M., K. Iohara, and M. Sugiyama, *Human dental pulp stem cells with highly angiogenic and neurogenic potential for possible use in pulp regeneration.* Cytokine Growth Factor Rev, 2009. **20**(5-6): p. 435-40.
37. Gandia, C., et al., *Human dental pulp stem cells improve left ventricular function, induce angiogenesis, and reduce infarct size in rats with acute myocardial infarction.* Stem Cells, 2008. **26**(3): p. 638-45.
38. Bakopoulou, A., et al., *Comparative analysis of in vitro osteo/odontogenic differentiation potential of human dental pulp stem cells (DPSCs) and stem cells from the apical papilla (SCAP).* Archives of Oral Biology, 2011. **56**(7): p. 709-21.
39. Salehinejad, P., et al., *Comparison of different methods for the isolation of mesenchymal stem cells from human umbilical cord Wharton's jelly.* In Vitro Cell Dev Biol Anim, 2012. **48**(2): p. 75-83.
40. Struys, T., et al., *Ultrastructural and immunocytochemical analysis of multilineage differentiated human dental pulp- and umbilical cord-derived mesenchymal stem cells.* Cells Tissues Organs, 2011. **193**(6): p. 366-78.
41. Rebelatto, C.K., et al., *Dissimilar differentiation of mesenchymal stem cells from bone marrow, umbilical cord blood, and adipose tissue.* Exp Biol Med (Maywood), 2008. **233**(7): p. 901-13.
42. Jamal M., C.S., Goodis H., Karam S., *Dental stem cells and their potential role in regenerative medicine.* Journal of medical sciences, 2011. **4**(2): p. 53-61.
43. Liu, C.H. and S.M. Hwang, *Cytokine interactions in mesenchymal stem cells from cord blood.* Cytokine, 2005. **32**(6): p. 270-9.

44. Distler, J.H., et al., *Angiogenic and angiostatic factors in the molecular control of angiogenesis.* Q J Nucl Med, 2003. **47**(3): p. 149-61.
45. Moses, M.A., *The regulation of neovascularization of matrix metalloproteinases and their inhibitors.* Stem Cells, 1997. **15**(3): p. 180-9.
46. M., S.L.A.B., *ABT-210 oncolytic angiogenesis inhibitor.* Drugs of the future, 2005. **30**(11): p. 1081-1086.
47. Rusnati, M., et al., *Selective recognition of fibroblast growth factor-2 by the long pentraxin PTX3 inhibits angiogenesis.* Blood, 2004. **104**(1): p. 92-9.
48. Duh, E.J., et al., *Pigment epithelium-derived factor suppresses ischemia-induced retinal neovascularization and VEGF-induced migration and growth.* Invest Ophthalmol Vis Sci, 2002. **43**(3): p. 821-9.
49. Zhang, S.X., et al., *Pigment epithelium-derived factor downregulates vascular endothelial growth factor (VEGF) expression and inhibits VEGF-VEGF receptor 2 binding in diabetic retinopathy.* J Mol Endocrinol, 2006. **37**(1): p. 1-12.
50. Alexander, R.A., et al., *VEGF-induced endothelial cell migration requires urokinase receptor (uPAR)-dependent integrin redistribution.* Cardiovascular Research, 2012. **94**(1): p. 125-35.
51. Pro, B. and N.H. Dang, *CD26/dipeptidyl peptidase IV and its role in cancer.* Histol Histopathol, 2004. **19**(4): p. 1345-51.
52. Bagnato, A. and F. Spinella, *Emerging role of endothelin-1 in tumor angiogenesis.* Trends Endocrinol Metab, 2003. **14**(1): p. 44-50.
53. Zhuge, X., et al., *CXCL16 is a novel angiogenic factor for human umbilical vein endothelial cells.* Biochem Biophys Res Commun, 2005. **331**(4): p. 1295-300.
54. Li, A., et al., *IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis.* J Immunol, 2003. **170**(6): p. 3369-76.
55. Khabar, K.S., et al., *The alpha chemokine, interleukin 8, inhibits the antiviral action of interferon alpha.* J Exp Med, 1997. **186**(7): p. 1077-85.
56. Chang, S.W., et al., *Heat stress activates interleukin-8 and the antioxidant system via Nrf2 pathways in human dental pulp cells.* J Endod, 2009. **35**(9): p. 1222-8.
57. Silva, A.C., et al., *Interleukin-1 beta and interleukin-8 in healthy and inflamed dental pulps.* J Appl Oral Sci, 2009. **17**(5): p. 527-32.
58. Moon, M.H., et al., *Human adipose tissue-derived mesenchymal stem cells improve postnatal neovascularization in a mouse model of hindlimb ischemia.* Cell Physiol Biochem, 2006. **17**(5-6): p. 279-90.
59. Lee, M.Y., et al., *Angiogenesis in differentiated placental multipotent mesenchymal stromal cells is dependent on integrin alpha5beta1.* Plos One, 2009. **4**(10): p. e6913.
60. Oskowitz, A., et al., *Serum-deprived human multipotent mesenchymal stromal cells (MSCs) are highly angiogenic.* Stem Cell Res, 2011. **6**(3): p. 215-25.
61. Ribatti, D., et al., *In vivo time-course of the angiogenic response induced by multiple myeloma plasma cells in the chick embryo chorioallantoic membrane.* J Anat, 2003. **203**(3): p. 323-8.
62. Watanabe, K., et al., *Vasohibin as an endothelium-derived negative feedback regulator of angiogenesis.* J Clin Invest, 2004. **114**(7): p. 898-907.
63. Auerbach, R., et al., *Angiogenesis assays: a critical overview.* Clin Chem, 2003. **49**(1): p. 32-40.

Supplemental information

Table a: Major stimulators and inhibitors of angiogenesis and their role in the formation of new vessels [44]

Pro-angiogenic factors	Class	Function
Angiopoietin 1	Growth factor	Stimulation of endothelial cell sprouting Stimulation of vessel stabilization
Angiopoietin 2	Growth factor	Stimulation of endothelial cell migration and proliferation
CXC-chemokines with ELR motif	Chemokine	Stimulation of endothelial cell migration and proliferation
Epidermal growth factor (EGF)	Growth factor	Stimulation of endothelial cell proliferation
Fibroblast growth factor (FGF) family	Growth factor	Stimulation of endothelial cell migration and proliferation Upregulation of plasminogen activators Upregulation of adhesion molecules
Hepatocyte growth factor (HGF)	Growth factor	Stimulation of endothelial and smooth muscle cell migration and proliferation
Integrin	Receptor	Affect size of the lumen Stimulation of endothelial cell migration and attachment Reduction of endothelial cell apoptosis
Interleukin 8 (IL8)	Chemokine	Stimulation of endothelial cell migration, proliferation and survival
Matrix Metalloproteinases (MMPs)	Enzyme	Stimulation degradation extracellular matrix and thus release of growth factors out of the extracellular matrix
Monocyte chemotactic protein 1 (MCP-1)	Chemokine	Stimulation of endothelial cell migration Stimulation of monocyte recruitment
Vascular endothelial growth factor (VEGF)	Growth factor	Increases permeability of endothelial cells Stimulation of endothelial cell migration and proliferation Affect size of the lumen
Anti-angiogenic factors	**Class**	**Function**
Angiostatin	Enzyme	Decrease of endothelial cell migration, proliferation and tube formation Stimulation of endothelial cell apoptosis
CXC chemokines without ELR motif	Chemokine	Inhibition of FGF and VEGF binding to their receptors

Endostatin	Enzyme	Decrease of endothelial cell migration and proliferation Stimulation of endothelial cell apoptosis Inhibition of MMPs
Matrix Metalloproteinases (MMPs)	Enzyme	Generation of angiostatin

hDPSC isolation protocol

- Mechanically fractionate the pulp into pieces of 1-2mm³ (with scissors). ½ of the fractionated tissue will be plated in 6-well plates for the explants method, while the other ½ is processed for collagenase/dispase digestion. Put (or leave) the tissue pieces in an eppendorf tube.

<u>Collagenase dispase digestion</u>

- Digest the pulp in a solution of 3mg/ml collagenase type I and 4mg/ml dispase type II
    - Practically this means: adjust the volume to 500µl with the a-MEM basal medium
    - Add 35 µl out of the aliquots from the blue box in de freezer(in de freezer, in the blue box, there are aliquots with 3 mg collagenase and 4 mg dispase in 70µl)
    - Incubate for 1h at 37°C in the warm water bath
- Centrifugate for 5 minutes at 1200 rpm
- Resuspend the cells (is the pellet) in culture medium 1 ml
- Obtain single cell suspensions by passing the cells through a 100 µm strainer (put the 1 ml cell suspension and 2 ml medium through the strainer, this 3 ml has to be passed 5 times through the strainer)
- Seed cells in culture dishes and maintain under standard conditions (Incubate at 37°C with 5% CO_2)(just put 1ml in each well)
- Medium: Alfa MEM, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 10% fetal calf serum (FCS).
- Subconfluent cultures are harvested with 0.05% trypsine in PBS and seeded in a density of 100,000 cells in a small culture flask or 300,000 cells in a 75 cm² flask

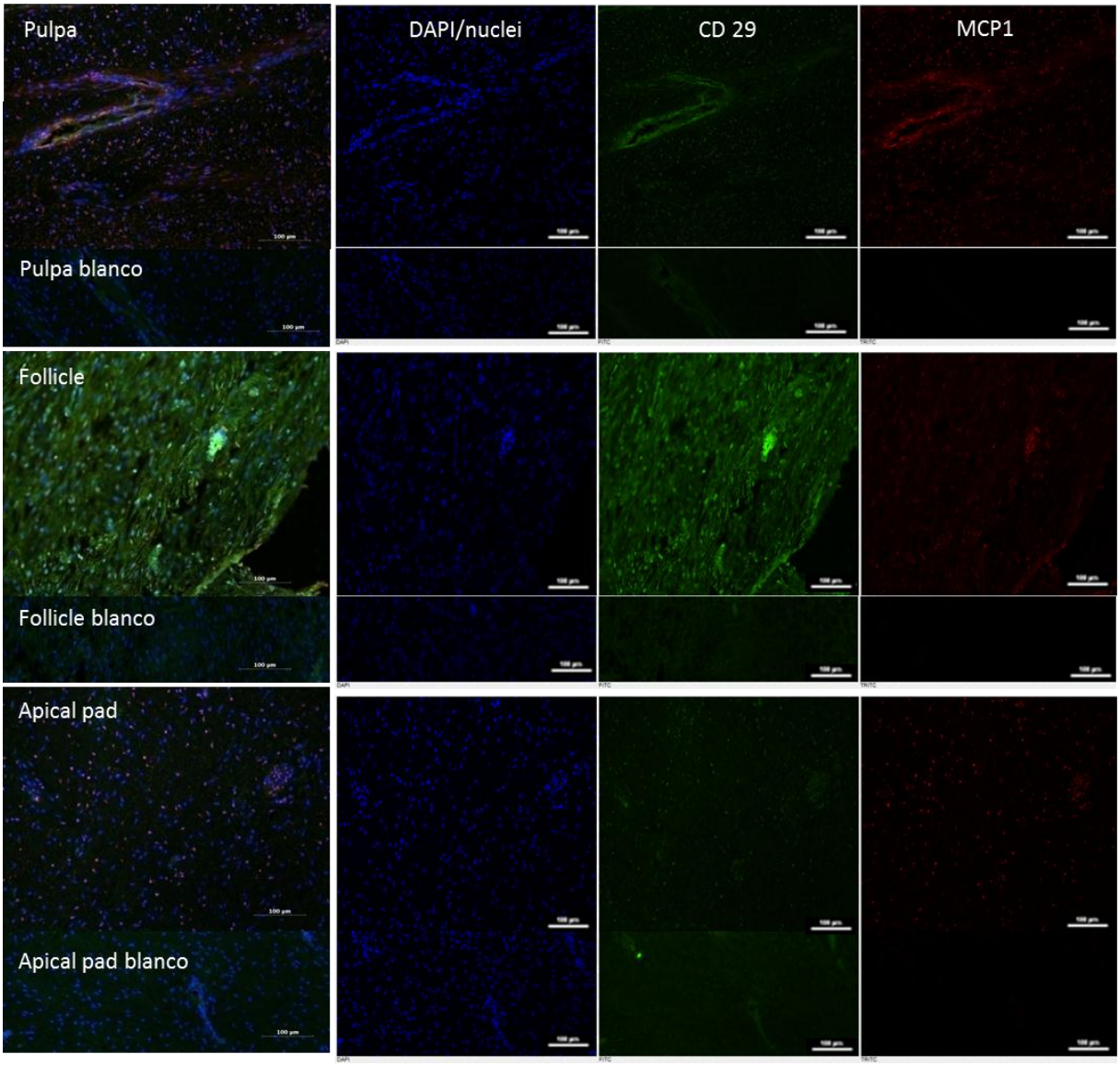


Figure a: Immunofluorescent staining on tissues against CD29 and MCP-1. This staining is a representative staining for 6 different patients (n=6). MCP-1 was evenly distributed throughout the 3 dental tissues. Scalebar= 100 µm.

Figure b: angiogenesis antibody array performed on 0.1% medium

Auteursrechtelijke overeenkomst

Ik/wij verlenen het wereldwijde auteursrecht voor de ingediende eindverhandeling:
Dental stem cells in angiogenesis and tissue engineering

Richting: **master in de biomedische wetenschappen-klinische moleculaire wetenschappen**
Jaar: **2012**

in alle mogelijke mediaformaten, - bestaande en in de toekomst te ontwikkelen - , aan de Universiteit Hasselt.

Niet tegenstaand deze toekenning van het auteursrecht aan de Universiteit Hasselt behoud ik als auteur het recht om de eindverhandeling, - in zijn geheel of gedeeltelijk -, vrij te reproduceren, (her)publiceren of distribueren zonder de toelating te moeten verkrijgen van de Universiteit Hasselt.

Ik bevestig dat de eindverhandeling mijn origineel werk is, en dat ik het recht heb om de rechten te verlenen die in deze overeenkomst worden beschreven. Ik verklaar tevens dat de eindverhandeling, naar mijn weten, het auteursrecht van anderen niet overtreedt.

Ik verklaar tevens dat ik voor het materiaal in de eindverhandeling dat beschermd wordt door het auteursrecht, de nodige toelatingen heb verkregen zodat ik deze ook aan de Universiteit Hasselt kan overdragen en dat dit duidelijk in de tekst en inhoud van de eindverhandeling werd genotificeerd.

Universiteit Hasselt zal mij als auteur(s) van de eindverhandeling identificeren en zal geen wijzigingen aanbrengen aan de eindverhandeling, uitgezonderd deze toegelaten door deze overeenkomst.

Voor akkoord,

Vanormelingen, Jessica

Datum: **11/06/2012**