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GENEESKUNDE

master in de biomedische wetenschappen: klinische moleculaire wetenschappen

Masterproef

The angiogenic properties and endothelial differentiation potential of human dental pulp stem cells

Promotor :
Prof. dr. Ivo LAMBRICHTS

Copromotor :
dr. Annelies BRONCKAERS

Petra Hilkens

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

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Preface

During the last 30 weeks, I had the opportunity to complete my internship at the Department of Morphology at Hasselt University. During these 8 months, I learned that science is not merely performing experiments, gathering facts and conducting critical analyses. Science is the result of minds thinking together, people working together and understanding each other. It is the result of a well-oiled machine that is called a team. And without the help of this team, this thesis wouldn't have come together. Therefore, I would like to thank all the people who made this past 30 weeks an unforgettable experience.

First of all, I would like to thank my promotor Prof. Dr. Ivo Lambrichts for giving me the opportunity to be part of his research group. Thank you for your kindness, your advice and valuable criticism, but most of all, thank you for your endless enthusiasm and making me always feel welcome.

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Last but certainly not least, I would like to thank my parents, my brother Gunther and of course the best boyfriend ever Brecht for their continuous encouragement and support, the reading of my thesis and for calming me down during stressful times.

Petra Hilkens; June 13th, 2011.

List of abbreviations

BDNF	Brain Derived Neurotrophic Factor
bFGF	Basic Fibroblast Growth Factor
BM-MSC	Bone Marrow-derived Mesenchymal Stem Cells
CSF	Colony Stimulating Factor
EC	Endothelial Cell
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
GDNF	Glial cell line Derived Neurotrophic Factor
hDPSC	Human Dental Pulp Stem Cells
HGF	Hepatocyte Growth Factor
IGF-1	Insulin-like Growth Fact
IGFBP-3	Insulin-like Growth Factor Binding Protein-3
IL-8	Interleukin-8
MBEC	Mouse Brain Endothelial Cells
MCP-1	Monocyte Chemotactic Protein-1
mMab	Mouse Monoclonal Antibody
MMP	Matrix Metalloproteinase
MSC	Mesenchymal Stem Cells
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NGF	Nerve Growth Factor
NO	Nitric Oxide
PAI-1	Plasminogen Activator Inhibitor-1
PECAM-1	Platelet Endothelial Cell Adhesion Molecule
PDGF	Platelet Derived Growth Factor
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
TGF-β	Transforming Growth Factor β
TIMP-1	Tissue Inhibitor of Matrix Metalloproteinase-1
TNF-α	Tumor Necrosis Factor α
TSP	Thrombospondin
uPA	Urinary Plasminogen Activator
VEGF	Vascular Endothelial Growth Factor

Abstract

Introduction: Angiogenesis, i.e. the formation of new capillaries from pre-existing blood vessels, plays an important role in wound healing and tissue engineering, but also in diseases such as stroke and myocardial infarction (MI). Unfortunately, traditional revascularization therapies are no longer an option for an increasing number of patients and the current stem cell therapies have lead to disappointing results in preclinical and clinical trials. Therefore, this study proposes human dental pulp stem cells (hDPSC) as a potential tool for vascular regeneration. Recent studies have indicated that hDPSC might have angiogenic properties. However, a more elaborate angiogenic profiling of these stem cells is required before any therapeutic application is possible. The goal of this study is to elucidate the angiogenic properties and the endothelial differentiation potential of hDPSC.

Materials & Methods: The angiogenic secretion profile of hDPSC was identified by means of RT-PCR and ELISA. In order to determine the biological effects of hDPSC on endothelial cells (EC), different *in vitro* models of angiogenesis were performed with hDPSC conditioned medium, such as an MTT assay (for proliferation), a transwell assay and a wound healing assay (for migration) and a hypoxia assay (for survival). After incubating hDPSC with five different induction media for several periods of time, their endothelial differentiation potential was determined by means of flow cytometry and transmission electron microscopy.

Results: hDPSC express pro-angiogenic as well as anti-angiogenic factors at mRNA and protein level, such as VEGF, IL-8, endostatin and PAI-1. Furthermore, the hDPSC conditioned medium significantly increased EC migration, as shown by the transwell and wound healing assay. However, the addition of neutralizing antibodies against VEGF and IL-8 did not significantly decrease EC migration, suggesting the participation of other angiogenic factors in the migration process. There was also no significant effect of hDPSC conditioned medium on EC proliferation and survival. In terms of endothelial differentiation, flow cytometric analysis indicated no endothelial marker expression in the tested conditions. Ultrastructural analysis showed no straightforward results. However, there were minor morphological changes suggestive of an endothelial differentiation potential.

Discussion and Conclusion: These results suggest that hDPSC have a predominant paracrine angiogenic effect *in vitro*, in particular on EC migration. In the future, the *in vitro* models of angiogenesis need to be performed with a human endothelial cell line in order to rule out potential species-related differences. Since hDPSC could not differentiate into endothelial cells under the given circumstances, an optimization of the applied differentiation protocols is also required in order to further determine the endothelial differentiation potential of hDPSC. If these stem cells are able to regenerate and/or to contribute to vascular networks, this will have great therapeutic potential, not only in terms of pulp regeneration, but also as a cell-based therapy for stroke and MI patients.

Samenvatting

Inleiding: Angiogenese, de vorming van capillairen uit bestaande bloedvaten, speelt niet alleen een belangrijke rol bij wondheling en weefsel engineering maar ook bij aandoeningen zoals beroertes en myocardiale infarcten (MI). Helaas is er een groeiend aantal patiënten dat geen baat meer heeft bij de traditionele methodes van revascularisatie en de huidige stamceltherapieën leveren niet de gewenste resultaten op in preklinische en klinische studies. Daarom stelt deze studie humane tandpulpastamcellen (hDPSC) voor als een nieuwe stamcelsoort voor vasculaire regeneratie. Recente studies hebben namelijk aangetoond dat hDPSC angiogene eigenschappen hebben. Toch is er nog een uitgebreide angiogene karakterisering van deze stamcellen nodig vooraleer enige therapeutische toepassing mogelijk is. Het doel van deze studie is dan ook de angiogene eigenschappen en het endotheliaal differentiatiepotentieel van hDPSC op te helderen.

Materiaal en Methoden: Het angiogeen secretieprofiel van hDPSC werd bepaald met behulp van RT-PCR en ELISA. Om de biologische effecten van hDPSC op het gedrag van endotheelcellen (EC) te onderzoeken werden er *in vitro* modellen van angiogenese uitgevoerd met hDPSC geconditioneerd medium, zoals een MTT assay (proliferatie), een transwell migratie assay en wondheling assay (migratie) en een hypoxia assay (overleving). Nadat de hDPSC met vijf verschillende inductiemedia werden ingeïncubeerd voor verschillende tijdsperiodes, werd het endotheliaal differentiatiepotentieel bepaald door middel van flow cytometrie en transmissie elektronenmicroscopie.

Resultaten: hDPSC brengen zowel pro-angiogene als anti-angiogene factoren tot expressie op mRNA en eiwitniveau, zoals VEGF, IL-8, endostatin en PAI-1. Het hDPSC geconditioneerd medium bleek de EC migratie significant te doen toenemen maar het uitblijven van een significant effect na het toevoegen van neutraliserende antilichamen tegen VEGF en IL-8 suggereert de betrokkenheid van andere angiogene factoren. Er was ook geen significant effect van het geconditioneerd medium op de EC proliferatie en overleving. Wat betreft de endotheliale differentiatie, toonde flow cytometrie geen duidelijke expressie van endotheliale merkers aan. De resultaten van de elektronenmicroscopie waren niet eenduidig maar zouden op een mogelijk endotheliaal proliferatiepotentieel kunnen wijzen.

Discussie en Conclusie: Deze resultaten suggereren dat hDPSC een hoofdzakelijk paracrien angiogeen effect uitoefenen *in vitro*, voornamelijk op EC migratie. Wat betreft de toekomst, dienen de *in vitro* modellen van angiogenese toegepast te worden op een menselijke endotheliale cellijn om potentiële soortgerelateerde verschillen uit te sluiten. Aangezien hDPSC niet kunnen differentiëren in endotheelcellen onder de gegeven omstandigheden, is er een optimalisatie van de differentiatieprotocols vereist om op die manier het endotheliaal differentiatiepotentieel van hDPSC verder te bepalen. Als deze stamcellen kunnen bijdragen aan vasculaire netwerken of zelfs kunnen genereren, dan is dit van groot therapeutisch belang, niet alleen in het veld van pulpregeneratie maar ook als mogelijke celgebaseerde therapie voor patiënten met een beroerte of MI.

1. Introduction

1.1 Stem cells

Stem cells can be found in different developmental stages and tissues and are defined by three main criteria, namely long-term self-renewal, multilineage potential and the ability of regenerating a certain tissue *in vivo*. Different populations of stem cells can be distinguished, such as totipotent, pluripotent, multipotent and unipotent stem cells. **Totipotent stem cells** are derived from a fertilized egg or zygote and are characterized by their capability of forming embryonic and extra-embryonic tissues, such as the placenta. **Pluripotent stem cells** on the other hand, such as embryonic stem cells, are derived from the inner cell mass of an early blastocyst. They are capable of differentiating into the three different germ layers and the germ cells. **Multipotent or adult stem cells** can be isolated from different adult organs and can give rise to various organ-specific cell types. They are responsible for the natural turn-over of tissues and/or organs. Examples are hematopoietic stem cells, mesenchymal stem cells and neural stem cells. **Unipotent stem cells** are stem cells with a limited self-renewal capacity. They are considered to be progenitor cells of one specific single cell type [1].

1.1.1 Mesenchymal stem cells

Mesenchymal stem cells (MSC) are adult stem cells that can be defined according to three criteria. Firstly, MSC are considered to be plastic-adherent when maintained under standard culture conditions. Secondly, these stem cells express certain markers such as CD105, CD90 and CD73, and they lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules. Thirdly, they are characterized by a so-called trilineage differentiation potential, i.e. differentiation into adipocytes, osteoblasts and chondrocytes (Figure 1) [2]. For a long time, adult stem cells were thought to differentiate into cells that are typical of the tissue of origin, but not into cells of non-related tissues. However, several studies have now indicated that certain types of adult stem cells are able to cross lineage boundaries and differentiate into atypical cell types. In other words, depending on the specific microenvironment, it seems that some adult stem cells are able to switch cell fate [1, 3]. For example, besides their characteristic trilineage differentiation potential, bone marrow-derived mesenchymal stem cells (BM-MSC) can also give rise to cardiac muscle cells, endothelial cells and neurons [1, 4].

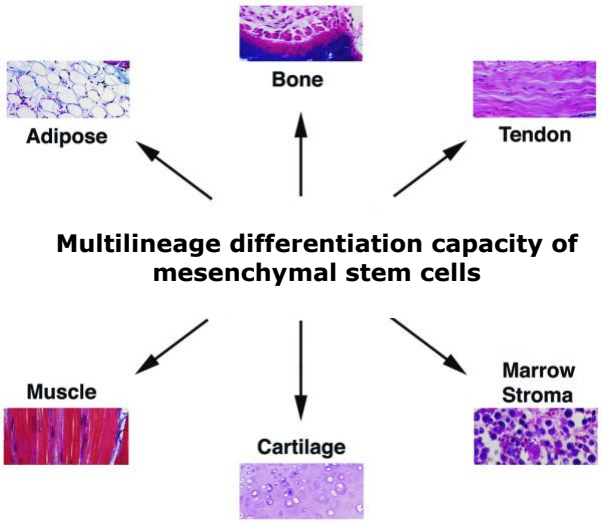


Figure 1: Multilineage differentiation capacity of mesenchymal stem cells. Mesenchymal stem cells are capable of differentiating into marrow stromal cells, chondrocytes, myocytes, adipocytes, osteoblasts and tenocytes. Adapted from: Tuan RS, Boland G, Tuli R. *Arthritis Research & Therapy* 2003, 5:32-45.

However, despite the numerous publications regarding this subject, the multilineage differentiation potential of BM-MSC is still under debate. Furthermore, the therapeutic benefits of these stem cells in the treatment of several disorders are offset by the relatively invasive isolation method, which stresses the need for an alternative source of adult stem cells [5, 6].

1.1.2 Human dental pulp stem cells

During tooth development, sequential and reciprocal interactions between oral epithelial cells and neural crest-derived mesenchymal cells regulate tooth morphogenesis and differentiation, which eventually results in the formation of an outer layer of enamel and an inner layer of primary dentin. Odontoblasts, the cells that are responsible for the formation of primary dentin, are also involved in the formation of reparative dentin in response to dental erosion and degradation. It has been postulated that these cells arise from precursor cells residing in the dental pulp, which is derived from the dental papilla and is infiltrated by blood vessels and nerve bundles [7, 8]. Gronthos *et al.* were the first to show the presence of a heterogeneous, clonogenic and highly proliferative cell population within the human dental pulp, namely the human dental pulp stem cells (hDPSC) [9]. Since then, a number of studies has been conducted to unravel the exact location, properties and differentiation capacities of these cells. With regard to the location of hDPSC within the mature dental pulp, several studies have mentioned the existence of three so-called stem cell niches. These include the undifferentiated mesenchymal cells or ‘sub-odontoblasts’, which are thought to reside in a cell-rich layer close

to the odontoblasts; a perivascular cell population which is associated with the pulpal vasculature; and a cell population in the central pulpal stroma which is positive for Notch-2, a signaling molecule with an important role in controlling stem cell fate [10].

In terms of the cellular properties and the differentiation potential of hDPSC, it seems that these cells can be considered as MSC. Not only are they plastic-adherent when maintained under standard culture conditions, but they also express several MSC markers such as CD29, CD44, CD90 and STRO-1 [7, 9, 11]. Furthermore, like BM-MSC, hDPSC are also characterized by a multilineage differentiation potential. Studies have indicated that hDPSC are not only capable of differentiating into odontoblasts *in vitro*, but they are also able to form an organized dentin-pulp-like complex lined with odontoblast-like cells, when seeded onto a scaffold and transplanted into immunocompromised mice [9, 12]. These observations suggest that hDPSC could play an important role in the repair of diseased and damaged dental tissues. Besides their potential role in tooth regeneration and repair, hDPSC could also be clinically applied in other domains since they are capable of differentiating into several other lineages. A number of studies has shown the capability of hDPSC of *in vitro* adipogenic, myogenic, osteogenic and chondrogenic differentiation [11, 13-15]. Several studies have also indicated that cultured dental pulp cells are able to protect and promote the survival of certain neurons *in vitro* and *in vivo* by producing neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF) [16, 17]. Moreover, besides this neuroprotective effect, these stem cells are also capable of differentiating into neuron-like cells *in vitro* and *in vivo* [18, 19]. According to recent studies, hDPSC also display certain angiogenic effects, which will be discussed in the following chapter.

1.2 Angiogenesis

Within the human body, three mechanisms of blood vessel formation can be distinguished, namely vasculogenesis, arteriogenesis and angiogenesis. During embryonic development, blood vessels form through **vasculogenesis**, i.e. the formation of a primitive network through *in situ* differentiation of migrated angioblasts into endothelial cells. **Angiogenesis**, i.e. the sprouting of new capillaries from pre-existing blood vessels, is the most studied and most predominant mechanism of blood vessel formation in the adult and is considered to be a complex but coordinated multi-step process. It involves vasodilatation, the degradation of extracellular matrix, the activation, proliferation and migration of endothelial cells, tube formation, and stabilization and remodeling of blood vessels (Figure 2). **Arteriogenesis** can

be defined as the rapid proliferation of pre-existing collateral arteries induced by shear stress in the case of an acute or chronic occlusion of a major artery [20, 21]. This study will focus on angiogenesis and its regulating factors.

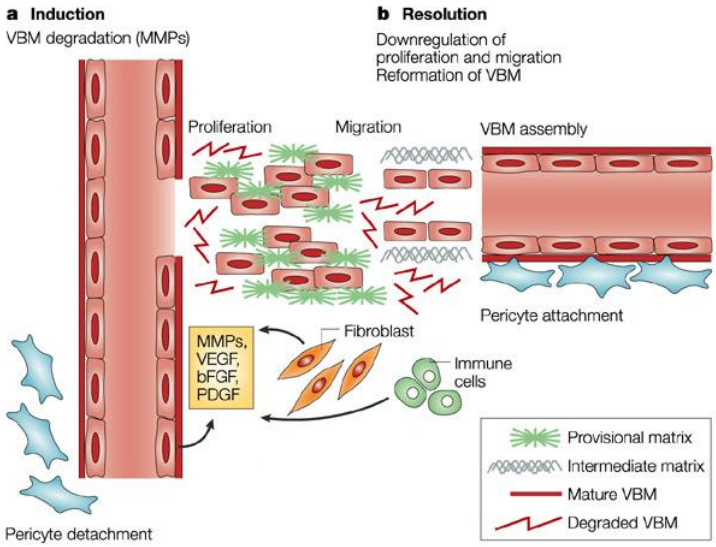


Figure 2: Cellular and humoral interactions during angiogenesis. Angiogenesis is a well-orchestrated process, which involves vasodilatation, the degradation of extracellular matrix, the activation, proliferation and migration of endothelial cells, tube formation, and stabilization and remodeling of blood vessels. Adapted from: Kalluri R. *Nature Reviews Cancer* 2003, 3: 422-433.

As mentioned earlier, angiogenesis is a well-orchestrated multi-step process and many regulating factors are involved, some of which play a dual role (Table 1) [22-26]. Within the healthy body, angiogenesis is controlled by a perfect balance of angiogenic activators and inhibitors. In general, the effect of the inhibitors is dominant over the stimulators, but when angiogenic growth factors are produced in excess of angiogenesis inhibitors, an 'angiogenic switch' takes place and the balance is tipped towards blood vessel growth [22].

Table 1: The major stimulators and inhibitors of angiogenesis and their associated functions.

Angiogenesis stimulating factors	
Angiogenin	Stimulation of EC proliferation.
Angiopoietin-1 (Ang-1)	Stimulation of EC sprouting. Vessel stabilization
Angiopoietin-2 (Ang-2)	Stimulation of EC proliferation, migration and sprouting in the presence of VEGF.
Basic Fibroblast Growth Factor (bFGF)	Stimulation of EC proliferation, migration and differentiation. Upregulation of plasminogen activators. Upregulation of integrins and other adhesion molecules.

CXC chemokines with ELR motif i.e. Interleukin-8 (IL-8)	Stimulation of EC proliferation and migration.
Colony Stimulating Factors (CSFs)	Stimulation of EC proliferation and migration.
Epidermal Growth Factor (EGF)	Stimulation of EC proliferation.
Erythropoietin	Stimulation of EC proliferation.
Hepatocyte Growth Factor (HGF)	Stimulation of EC and SMC proliferation and migration.
Insulin-like Growth Factor-1 (IGF-1)	Stimulation of EC proliferation. Induction of VEGF. Downregulation of EC apoptosis. Upregulation of plasminogen activators.
Insulin-like Growth Factor Binding Protein-3 (IGFBP-3)	Stimulation of EC motility and network formation.
Integrins	Stimulation of EC attachment and migration. Downregulation of EC apoptosis. Essential for FGF-induced angiogenesis.
Matrix Metalloproteinases (MMPs)	ECM degradation.
Monocyte Chemotactic Protein-1 (MCP-1)	Stimulation of monocyte recruitment. Stimulation of EC motility and differentiation. Upregulation of HIF-1α and VEGF.
Nitric Oxide (NO)	Stimulation of EC proliferation. Upregulation of vessel permeability. Induction of FGF-release
Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1)	Stimulation of EC aggregation, migration and tube formation. Vessel stabilization Essential for FGF-induced angiogenesis.
Platelet-Derived Growth Factor (PDGF)	Stimulation of SMC and PC proliferation. Vessel stabilization.
Transforming Growth Factor β (TGF-β)	Stimulation of tube formation *in vitro*. Vessel stabilization.
Tumor Necrosis Factor α (TNF-α)	Stimulation of EC migration. Stimulation of EC tube formation *in vitro*.
Urinary Plasminogen Activator (uPA)	Activation of plasmin to plasminogen: participation in ECM degradation.
Vascular Endothelial Growth Factor (VEGF)	Stimulation of EC proliferation and migration. Upregulation of vessel permeability. Downregulation of EC apoptosis. Upregulation of plasminogen activators. Upregulation of interstitial collagenase.

Angiogenesis inhibiting factors	
Angiopoietin-2 (Ang-2)	Vessel destabilization by antagonizing Angiopoietin-1 signaling. Upregulation of EC apoptosis.
Angiostatin	Downregulation of EC proliferation, migration and tube formation. Upregulation of EC apoptosis.
CXC chemokines without ELR motif	Inhibition of FGF and $VEGF_{165}$ receptor binding.
Endostatin	Downregulation of EC proliferation and migration. Upregulation of EC apoptosis. Inhibition of MMPs.
Insulin-like Growth Factor Binding Protein-3 (IGFBP-3)	Anti-angiogenic effects in cancer development: Induction of EC apoptosis (?) Downregulation and inhibition of MMP-9 and VEGF.
Matrix Metalloproteinases (MMPs)	Generation of angiostatin.
Plasminogen Activator Inhibitor-1 (PAI-1)	Inhibitor of uPA.
Thrombospondin-1 and 2 (TSP-1/2)	Downregulation of EC migration. Upregulation of EC apoptosis.
Tissue Inhibitor of Matrix Metalloproteinase-1 (TIMP-1)	Inhibitor of MMPs.
Transforming Growth Factor β (TGF-β)	Downregulation of EC proliferation and migration. Downregulation of plasminogen activators. Upregulation of TIMPs. Upregulation of EC apoptosis.
Tumor Necrosis Factor α (TNF-α)	Inhibition of FGF-induced EC proliferation *in vitro*. Upregulation of EC apoptosis.

The angiogenic process plays an important role in wound healing and tissue engineering, because without the well-orchestrated humoral and cellular interactions that lead to blood vessel development, cutaneous wounds would progress to non-healing chronic wounds and newly transplanted tissue would experience a lack of oxygen and nutrients and eventually become necrotic. Furthermore, blood supply is also of vital importance in ischemic diseases such as stroke and myocardial infarction (MI) [27]. However, since traditional methods of revascularization are no longer an option for an increasing number of patients, stem cells have been postulated as a possible alternative method for the regeneration of vascular networks [6].

1.2.1 Angiogenesis and stem cells

Several kinds of stem cells have already been proposed as potential candidate cells for vascular regeneration, namely endothelial progenitor cells, MSC, embryonic or induced

pluripotent stem cells, adipose-derived stem cells and cardiac stem cells. Studies have indicated that these cells exert a positive effect on angiogenesis, either by secreting pro-angiogenic factors, by differentiating into endothelial cells or by exerting both effects. However, most of these stem cells have lead to disappointing results in pre-clinical and clinical trials [6].

1.2.1.1 Angiogenesis and mesenchymal stem cells

With regard to the angiogenic properties of MSC, studies have indicated that these stem cells have a positive impact on angiogenesis, either (i) by secreting paracrine pro-angiogenic factors or (ii) by differentiating into endothelial cells themselves. By performing an antibody array analysis, Kagiwada *et al.* showed that BM-MSC express pro-angiogenic as well as anti-angiogenic factors such as IL-6, IL-8, TIMP-1, angiogenin, MCP-1 and VEGF [28]. Other angiogenic factors that are secreted by MSC are: bFGF, MMPs, uPA, HGF and TNFα, although the angiogenic secretion profile seems to be dependent on the tissue of origin [29, 30]. Besides this paracrine crosstalk, MSC also seem to modulate the angiogenic response by establishing direct cell contact with endothelial cells [31]. In terms of endothelial differentiation, BM-MSC as well as umbilical cord-derived MSC and kidney-derived MSC are capable of differentiating into endothelial cells *in vitro* and *in vivo* and of forming capillary beds [4, 32-34]. In contrast, Au *et al.* showed that BM-MSC are not capable of differentiating into endothelial cells but instead behave like and differentiate into functional perivascular cells (pericytes) *in vivo* in order to regulate tissue perfusion [35]. Besides the numerous publications stating a pro-angiogenic impact of MSC, a study of Otsu *et al*. showed that BM-MSC are also capable of inhibiting angiogenesis in a concentration-dependent manner by producing reactive oxygen species [36]. These observations, taken together with the limited success of MSC in clinical studies and the painful and invasive isolation procedures stress the need for the identification of novel stem cells with a potential therapeutic application in vascular medicine [5, 6].

1.2.1.2 Angiogenesis and human dental pulp stem cells

Recent studies have indicated that hDPSC are able to secrete pro-angiogenic factors such as VEGF, bFGF, PDGF and CSF under basal conditions, after hypoxic treatment or after pulp injury [37-41]. Furthermore, these stem cells also express certain endothelial markers at a basal level, such as vascular cell adhesion molecule 1 (VCAM-1), CD105, CD146, VEGFR1 and VEGFR2 [9, 38, 42]. Moreover, Marchionni *et al.* showed that hDPSC treated with

VEGF are capable of endothelial differentiation *in vitro*, as indicated by their tube forming capability and an increased expression of VEGF receptors and von Willebrand factor (vWF) [42]. However, despite the functional improvements observed after injection of these stem cells into rats with a coronary artery ligation, there was no proof of *in vivo* differentiation into endothelial, smooth muscle or cardiac cells. This could indicate a predominant paracrine effect of the hDPSC [43]. In contrast, a study of d'Aquino *et al.* indicated that a subset of hDPSC was able to form vascularized bone tissue *in vivo*, after co-differentiation into osteoblasts and endotheliocytes [15]. These observations suggest that more elaborate profiling of hDPSC is required to determine their exact role in angiogenesis. For this reason, the goal of this study is to elucidate the angiogenic properties and the endothelial differentiation potential of hDPSC. The study comprises the following research questions: are hDPSC capable of inducing angiogenesis, and secondly, are they capable of forming blood vessels themselves? In order to determine whether hDPSC are able to induce angiogenesis, elaborate angiogenic profiling will be conducted to identify which pro- and anti- angiogenic factors they secrete. After performing an antibody array for angiogenesis-related proteins, preliminary data from our laboratory indicate that hDPSC express pro-angiogenic (MMP-9, uPA, IL-8, HGF, VEGF and MCP-1) as well as anti-angiogenic factors (PAI-1, TIMP-1, TSP and endostatin) at protein level. However, these results need to be validated by means of RT-PCR and ELISA. Furthermore, several *in vitro* models of angiogenesis will be applied to test the impact of hDPSC on the behavior of endothelial cells during the different steps of the angiogenic process. With regard to the endothelial differentiation potential, the *in vitro* endothelial differentiation capacity of hDPSC will be evaluated by means of transmission electron microscopy (morphological changes) and flow cytometry (expression of endothelial markers), after applying different protocols as described in literature. Given the literature and the preliminary data from our laboratory, it is expected that hDPSC will exert a positive effect on angiogenesis, either by secreting pro-angiogenic factors, by differentiating into endothelial cells, or by exerting both effects. If hDPSC are able to regenerate and/or to contribute to vascular networks, this will have great therapeutic potential, not only in terms of dental caries treatment or pulp regeneration but also as a cell-based therapy for patients who suffer from stroke, MI or other ischemic diseases. Furthermore, hDPSC could be used as a tool to promote wound healing in, for example, diabetes patients or to promote the formation of new blood vessels in patients who received a tissue transplant.

2. Materials and Methods

2.1 Isolation and culturing of human dental pulp stem cells

Pulp tissue was obtained with informed consent from human third molars that were extracted for therapeutic or orthodontic reasons at Ziekenhuis Maas en Kempen, Bree. After extraction, the teeth were mechanically fractioned and the dental pulp was isolated with a forceps. hDPSC were isolated according to two different methods, namely enzymatic digestion and the explant method [44]. When applying enzymatic digestion, dental pulp was minced into little fragments, which were incubated for 30 min. with a 4 mg/ml collagenase/dispase solution (Roche Diagnostics, Vilvoorde, Belgium). Cell suspensions were obtained by passing the digested tissue fragments through a 70 µm cell strainer (BD, Erembodegem, Belgium) and were cultured afterwards in a 6-well plate in Minimal Essential Medium Alpha (αMEM) (Invitrogen, Merelbeke, Belgium) supplemented with 100 U/ml Penicillin and 100 µg/ml Streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen) and 10% Fetal Bovine Serum (FBS, Biochrom AG, Berlin, Germany) (further referred to as standard hDPSC culture medium), and maintained at 37°C, 5% CO_2. When applying the explant method, dental pulp was minced into little fragments which were cultured in a 6-well plate in standard hDPSC culture medium, and maintained at 37°C, 5% CO_2. With both isolation methods, the culture medium was changed every 2-3 days and the cultures were monitored regularly with an inverted microscope (Nikon Eclipse TS100). When confluent, the cells were recovered from the culture plate by treatment with 0.05% trypsin/EDTA (Invitrogen) and sub-cultured for further experiments. Unless mentioned otherwise, explant hDPSC were used in all experiments.

2.2 Human dental pulp stem cell conditioned medium

hDPSC were seeded at 20.000 cells/cm² in standard hDPSC culture medium. After 24h of culturing, hDPSC were rinsed with PBS and incubated with standard hDPSC culture medium supplemented with 0,1% FBS instead of 10% FBS. After 48h or 72h of incubation, the medium was collected and stored at -80°C.

2.3 Culturing of mouse brain endothelial cells

Mouse brain endothelial cells (MBEC) were kindly provided by Prof. Dr. S. Liekens of the Rega Institute for Medical Research (Leuven, Belgium). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) Glutamax^{TM} I (Invitrogen, Merelbeke, Belgium) supplemented with 100 U/ml Penicillin and 100 µg/ml Streptomycin (Invitrogen),

and 10% FBS (Biochrom AG, Berlin, Germany) (further referred to as standard MBEC culture medium) and maintained at 37°C, 5% CO_2. The culture medium was changed every 2-3 days and when confluent, the cells were recovered from the culture flasks with 0.05% trypsin/EDTA (Invitrogen) and sub-cultured for further experiments.

2.4 Reverse Transcriptase Polymerase Chain Reaction

hDPSC were seeded at 4000 cells/cm². When confluent, cells were trypsinized, washed with PBS and a cell pellet was made. Total RNA was extracted from the hDPSC pellets as described by the RNeasy Plus Mini Kit (Qiagen, Venlo, the Netherlands). 700 ng of total RNA was reverse transcribed into cDNA according to the manufacturer's instructions of the Reverse Transcription System (Promega, Leiden, the Netherlands). Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was performed by means of Taq DNA Polymerase (1U/µl) (Roche Diagnostics, Vilvoorde, Belgium) according to the protocol in table 2. All primers were supplied by Eurogentec S.A. (Seraing, Belgium) and are listed in table 3. Afterwards, samples were loaded onto a 1,2% agarose gel (Invitrogen, Merelbeke, Belgium), together with a 100 bp ladder (Invitrogen)

Table 2: Composition of PCR reaction mix and applied PCR program

PCR mix per sample (µl)		**PCR program**		
10x PCR buffer	2,5	1x	5 minutes	94°C
Forward primer (25 µM)	1	35x	1 minute	95°C
Reverse primer (25 µM)	1		1 minute	60°C
dNTPs (2mM)	0,25		45 seconds	72°C
Taq polymerase	0,75	1x	10 minutes	72°C
MilliQ	18,5		∞	4°C

Table 3: Primer sequences for Reverse Transcriptase Polymerase Chain Reaction

Gene	**Primer**	**Sequence**	**Product size (bp)**	**Tm (°C)**
Angiogenic factors				
Endostatin	Forward	ATG-CTG-ACA-TTC-ACC-TGC-C	174	58
	Reverse	ATG-AAG-TCA-GCA-CCT-GCT-GG		62
HGF	Forward	TTC-ATG-ATG-TCC-ACG-GAA-CA	575	58
	Reverse	TTG-TAT-TGG-TGG-GTG-CTT-CA		58
IGFBP-3	Forward	TTG-CAC-AAA-AGA-CTG-CCA-AG	275	58
	Reverse	CAA-CAT-GTG-GTG-AGC-ATT-CC		60
IL-8	Forward	AGG-GTT-GCC-AGA-TGC-AAT-AC	420	60
	Reverse	AAA-CCA-AGG-CAC-AGT-GGA-AC		60
MCP-1	Forward	AAG-CAG-AAG-TGG-GTT-CAG-GA	300	60
	Reverse	GCA-ATT-TCC-CCA-AGT-CTC-TG		60

PAI-1	Forward	ATA-CTG-AGT-TCA-CCA-CGC-CC	320	62
	Reverse	GTG-GAG-AGG-CTC-TTG-GTC-TG		64
TIMP-1	Forward	GCT-TCT-GGC-ATC-CTG-TTG-TT	462	60
	Reverse	TTT-GCA-GGG-GAT-GGA-TAA-AC		58
uPA	Forward	GCC-ATC-CCG-GAC-TAT-ACA-GA	417	62
	Reverse	AGG-CCA-TTC-TCT-TCC-TGG-GT		60
VEGF	Forward	CCT-TGC-TGC-TCT-ACC-TCC-AC	280	64
	Reverse	ATC-TGC-ATG-GTG-ATG-TTG-GA		58
Housekeeping genes				
β-actin	Forward	AAA-TCT-GGC-ACC-ACA-CCT-TC	185	56
	Reverse	AGA-GGC-GTA-CAG-GGA-TAG-CA		56
β2-Microglobulin	Forward	CTC-ACG-TCA-TCC-AGC-AGA-GA	213	56
	Reverse	CGG-CAG-GCA-TAC-TCA-TCT-TT		56
Gus B	Forward	AGC-CAG-TTC-CTC-ATC-AAT-GG	160	56
	Reverse	GGT-AGT-GGC-TGG-TAC-GGA-AA		56

2.5 Enzyme-linked Immunosorbent Assay (ELISA)

ELISAs were performed on hDPSC conditioned medium (48h/72h) in order to determine the concentration of the following angiogenic factors: bFGF (BioLegend, Antwerp, Belgium), endostatin (RayBiotech, Inc., Boechout, Belgium), IGFBP3 (RayBiotech, Inc.), IL-8 (BioLegend) , MCP-1 (PeproTech, London, UK), PAI-1(Invitrogen), TIMP-1 (PeproTech), uPA (Boster Biological Technology, Ltd., Antwerp, Belgium), VEGF (PeproTech). Unless mentioned otherwise, all ELISAs were performed on hDPSC conditioned medium (passage 1-3) of at least 6 different patients according to manufacturer's instructions.

2.6 Immunocytochemistry

Immunocytochemistry was performed using the DAB Envision™ Kit (Dako, Heverlee, Belgium) (See supplemental materials and methods S1.1). The primary antibodies that were used are listed in table 4. The staining was examined using a Nikon Eclipse 80i microscope with complementary camera. Images were digitally processed with Nikon EclipseNet Imaging Software (Excel® Technologies, Inc., Enfield, Connecticut, USA).

Table 4: Primary antibodies for immunohistochemistry

Antibody	Type *	Dilution	Supplier
bFGF	mMab	1:100	Sigma-Aldrich, Bornem, Belgium.
HGF	mMab	1:50	Santa Cruz Biotechnology Inc., Heidelberg, Germany

(*) mMab: mouse monoclonal antibody.

2.7 *In vitro* angiogenesis assays

2.7.1 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

MBEC were seeded at 10.000 or 5000 cells/cm² in a 96-well plate in standard MBEC culture medium. After 24h of culturing, the cells were rinsed with PBS and incubated with the appropriate conditions, i.e. hDPSC conditioned medium (passage 1-3, 48h), standard hDPSC culture medium (as a positive control) or αMEM supplemented with 0,1% FBS (as a negative control). After 72h of incubation, the different media were removed and 500 µg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in standard hDPSC culture medium was added to the wells. After 4h of incubation, the MTT solution was removed and a DMSO - glycine solution was added in order to allow reduction to formazan. The absorbance was measured at a wavelength of 540-550 nm with a Benchmark microplate reader (Bio-Rad Laboratories, Nazareth Eke, Belgium).

2.7.2 Transwell migration assay

MBEC were seeded in translucent ThinCert™ tissue culture inserts (pore size 8 µm; Greiner Bio-One, Wemmel, Belgium) at 12.500 cells/cm² in αMEM supplemented with 0,1% FBS and placed in 24-well insert companion plates of which a number of wells were previously seeded with hDPSC at 50 000 cells/cm² and incubated with the same medium after 24h of culturing. Other conditions in the wells beneath were: hDPSC conditioned medium (passage 1-5, 48h) with or without 1 µg/ml of neutralizing VEGF or IL-8 antibodies (R&D Systems, Abingdon, United Kingdom), 100 ng/ml of recombinant human IL-8 or VEGF (ImmunoTools, Friesoythe, Germany), with or without 1 µg/ml of neutralizing VEGF or IL-8 antibodies, standard hDPSC culture medium (as a positive control) or αMEM supplemented with 0,1% FBS (as a negative control). At 7h post-seeding, the inserts were washed in PBS, fixed with 4% paraformaldehyde and stained with hematoxylin in order to allow analysis with AxioVision software, edition 4.6.3 (Carl Zeiss NV-SA, Zaventem, Belgium). Pictures were taken with an inverted microscope (Nikon Eclipse TS100), equipped with a ProgRes® C3 digital microscope camera (Jenoptik, Germany).

2.7.3 Wound healing assay

MBEC were seeded at 52.000 cells/cm² in a 6-well plate in standard MBEC culture medium. After 24h of culturing, two wounds were created by manually scraping the cellular monolayer with a 200 µl pipette tip. Cells were rinsed with PBS and incubated with the appropriate conditions, i.e. hDPSC conditioned medium (passage 1-5, 48h) with or without 1 µg/ml of

neutralizing VEGF or IL-8 antibodies (R&D Systems, Abingdon, United Kingdom), 100 ng/ml recombinant human IL-8 or VEGF (ImmunoTools, Friesoythe, Germany) with or without 1 µg/ml of neutralizing VEGF or IL-8 antibodies, standard hDPSC culture medium (as a positive control) or αMEM supplemented with 0,1% FBS (as a negative control). 4µg/ml Mitomycin C (Sigma-Aldrich, Bornem, Belgium) was added to each condition in order to inhibit cell proliferation. After 24h or 48h of incubation, cells were fixed in 4% paraformaldehyde and stained with hematoxylin. Pictures were taken with an inverted microscope (Nikon Eclipse TS100), equipped with a ProgRes® C3 digital microscope camera (Jenoptik, Germany) and analyzed with AxioVision software, edition 4.6.3 (Carl Zeiss NV-SA, Zaventem, Belgium).

2.7.4 Hypoxia assay

MBEC were seeded at 50.000 cells/cm² in two 6-well plates in standard MBEC culture medium. After 24h of culturing, the cells were rinsed in PBS and incubated with the appropriate media, i.e. hDPSC conditioned medium (passage 1-5, 72h) or αMEM supplemented with 0,1% FBS (as a control). One 6-well plate was cultured under standard culture conditions (normoxia), while the other one was placed inside a BD Gaspak™ EZ Anaerobe Gas Generating Pouch System with indicator (BD, Erembodegem, Belgium). After 24h or 48h of culturing, the cells were recovered from the culture plates with 0.05% trypsin/EDTA and prepared for an Annexin V-FITC/Propidium Iodide staining as described by the Annexin V-FITC Apoptosis Detection kit (eBioscience, Vienna, Austria). The staining was analyzed by means of the BD FACSCalibur™ System (BD), using BD CellQuest Pro™ Software.

2.8 Endothelial differentiation

hDPSC (passage 1-2) of two different patients were seeded at 5000 cells/cm² in a 6-well plate or on 13 mm Thermanox™ plastic cover slips (Nunc, Belgium) in a 24-well plate in standard hDPSC culture medium. After 24h of culturing, the cells were rinsed in PBS and incubated with the appropriate media as described in literature (Table 5). After 10 or 21 days of incubation, the cells in the 6-well plates were recovered from the culture plates with 0.05% trypsin/EDTA and characterized by flow cytometry (BD FACSCalibur™ System) using anti-human monoclonal antibodies directly conjugated to fluorescein isothiocyanate (CD44, CD31, CD34 and CD54 (ImmunoTools, Friesoythe, Germany)) or phycoerythrin (VEGFR2 (BioLegend, Antwerp, Belgium)). The cells were labeled according to standard protocols.

Matched labeled isotypes were used as controls. Data were acquired and analyzed with BD CellQuest Pro™ Software. The cells on Thermanox™ plastic cover slips were fixed in 2% glutaraldehyde (Laborimpex, Brussels, Belgium) in 0,05 M sodium cacodylate buffer (Aurion, Wageningen, The Netherlands) (pH=7,3) at 4°C, after which they were analyzed with a transmission electron microscope (Philips EM208) equipped with a Morade Soft Imaging System camera. Images were digitally processed by ITEM-FEI software (Olympus SIS, Münster, Germany). The same protocol was repeated for an incubation period of 7, 10 and 21 days with enzymatically digested hDPSC (passage 1-2) of two different patients. Cells were only incubated with control medium and method 1 (Table 5). Marker expression was assessed after 7 days of incubation, while potential morphological changes were determined after 10 and 21 days of incubation.

Table 5: Methods for endothelial differentiation

Method	Medium
Control	DMEM (4,5 g/ml glucose, Invitrogen, Merelbeke, Belgium) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin, 2 mM L-glutamine and 2% FBS (Biochrom AG, Berlin, Germany).
Method 1 [42]	DMEM (4,5 g/ml glucose) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin, 2 mM L-glutamine, 2% FBS (Biochrom AG) and 50 ng/ml VEGF (ImmunoTools, Friesoythe, Germany).
Method 2 [4]	Clonetics® EGM®-2 Endothelial Cell Growth Medium-2 (Lonza, Verviers, Belgium) supplemented with 2% FBS.
Method 3 [33]	DMEM Glutamax™ I (Invitrogen) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin, 5% FBS, 100 ng/ml VEGF, 50 ng/ml EGF (ImmunoTools) and 1 µg/ml hydrocortisone (Invitrogen).
Method 4 [45]	Indirect co-culture with MBEC (1:1 ratio) on transparent ThinCert™ tissue culture inserts (pore size 0,4 µm; Greiner Bio-One) in DMEM (4,5 g/ml glucose) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin, 2 mM L-glutamine and 2% FBS.
Method 5 [46]	Clonetics® EGM®-2 Endothelial Cell Growth Medium-2 supplemented with 2% FBS and 50 ng/ml VEGF.

2.9 Statistical analysis

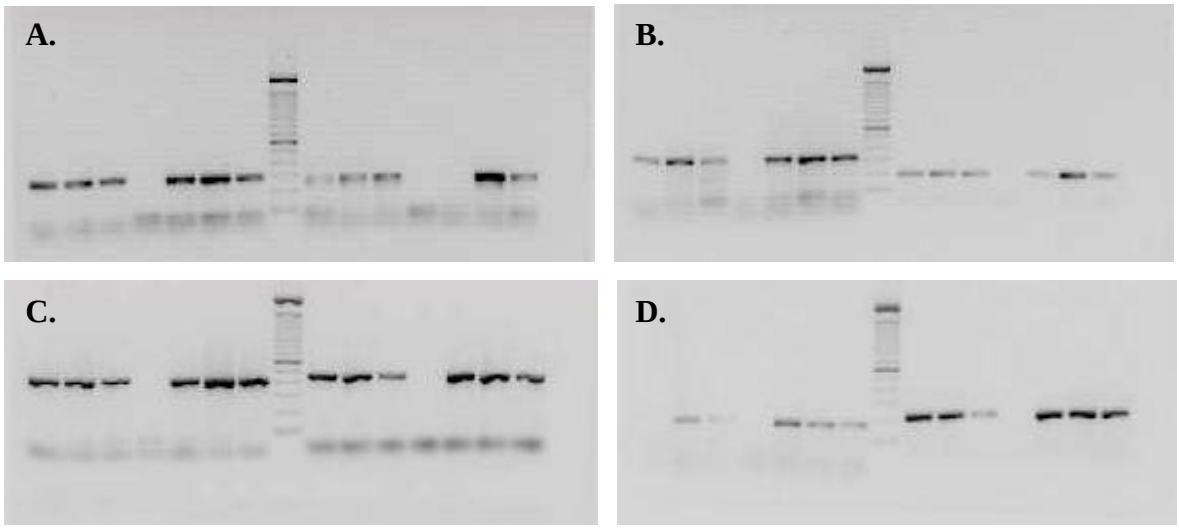
Statistical analysis was performed using Graphpad Prism 4 Software (Graphpad, California, USA). After controlling for normality by means of a D'Agostino & Pearson omnibus normality test, comparisons between control and experimental groups were made by means of a Kruskall-Wallis test or a one way ANOVA, while applying a Dunn's multiple comparison or Bonferonni's multiple comparison post-hoc test respectively. Differences were considered statistically significant at P-values $\leq 0,05$. All data were expressed as mean ± SD.

3. Results

3.1 Determination of the angiogenic secretion profile of hDPSC

3.1.1 Expression of angiogenic factors at mRNA level

In order to assess the expression of angiogenic factors at mRNA level, RT-PCR was performed on cDNA samples of 6 different patients (Fig. 3). With regard to VEGF, there was a clear expression of this pro-angiogenic factor in all 6 patients (280 bp, fig. 3A). MCP-1 on the other hand, was not expressed in all patient samples and there was a clear difference in expression level between patients (300 bp, fig. 3A). A difference in expression level of anti-angiogenic factor PAI-1 was also present in all 6 patient samples, although less distinct (320 bp, fig. 3B). Housekeeping gene β-actin was consistently expressed in all 6 patient samples, with a minor increased expression level in one patient (185 bp, fig.3B). Pro-angiogenic factor uPA (417 bp, fig. 3C) and anti-angiogenic factor TIMP-1 (462 bp, fig. 3C) were clearly expressed in all 6 patient samples, although there were some minor differences in expression level between the different patients. Not all patient samples showed a clear expression of anti-angiogenic factor endostatin (174 bp, fig. 3D), while housekeeping gene β2-Microglobulin was consistently expressed by all patients, except for a minor decrease in expression level in one sample (213 bp, fig. 3D). As was the case with endostatin, patient samples also displayed a differential expression of pro-angiogenic factor Il-8 (420 bp, fig. 3E). IGFBP-3 on the other hand, was expressed by all 6 patients and only showed minor differences in expression level (275 bp, fig. 3E). Only 4 patients showed an expression of pro-angiogenic factor HGF (575 bp, fig. 3F), while housekeeping gene Gus B was consistently expressed in all samples, except for a decreased expression level in one patient (160 bp, fig. 3F).



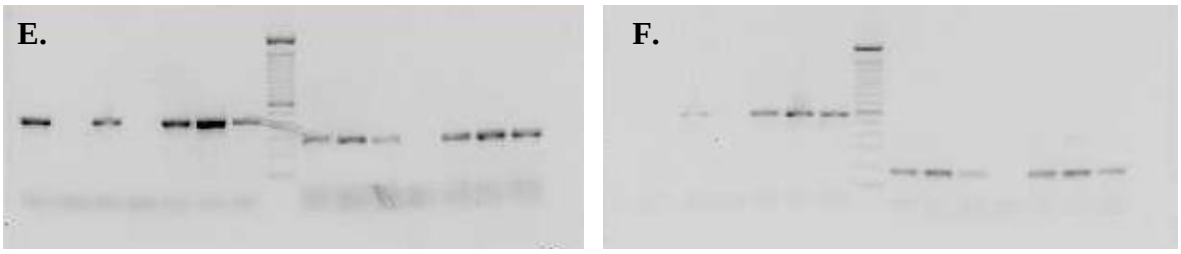


Figure 3: RT-PCR - expression of angiogenic factors at mRNA level. RT-PCR was performed on cDNA samples of 6 different patients. All samples were loaded on a 1,2% agarose gel, together with a 100 bp ladder. Empty lanes in the middle represent no template controls. A. Left: VEGF (280 bp). Right: MCP-1 (300 bp). B. Left: PAI-1 (320 bp). Right: β-actin (185 bp). C. Left: uPA (417 bp). Right: TIMP-1 (462). D. Left: endostatin (174 bp). Right: β2-Microglobulin (213 bp). E. Left: IL-8 (420 bp). Right: IGFBP-3 (275 bp). F. Left: HGF (575). Right: Gus B (160 bp).

3.1.2 Expression of angiogenic factors at protein level

Besides the expression of angiogenic factors at mRNA, it is also important to determine whether hDPSC are capable of expressing and/or secreting angiogenic factors at protein level. Therefore, multiple ELISAs for different angiogenic factors were performed on the hDPSC conditioned medium of at least 6 different patients (except for TIMP-1, of which the secretion was only measured in the conditioned medium of 3 different patients). The abovementioned angiogenic factors were all secreted in hDPSC conditioned medium of 48h and 72h. The concentration of angiogenic factors showed an increasing trend in all samples of hDPSC conditioned medium of 72h, except for IL-8 and uPA, of which the concentration decreased after 72h. The measured concentrations are compared with the serum concentrations in healthy persons as described in literature, together with the ED50 values as described in literature or by different biotechnology companies (Table 6).

Table 6: ELISA - secretion of angiogenic factors in hDPSC conditioned medium

Angiogenic factor	hDPSC conditioned medium (pg/ml)	Serum of healthy persons	ED50
bFGF	Too low to be measured	1,466 ± 1,89 pg/ml [47]	0,05 - 2 ng/ml
Endostatin	48h: 271 ± 93 72h: 419 ± 107	22,2 ± 2,1 ng/ml [48]	550 ng/ml [49]
IGFBP3	48h: 2449 ± 1583 72h: 3856 ± 2562	39 ± 7 ng/ml [50]	0,2 - 1,5 µg/ml
IL-8	48h: 745 ± 614 72h: 503 ± 512	5,2 ± 0,8 pg/ml [51]	10 - 100 ng/ml
MCP-1	48h: 233 ± 147 72h: 281 ± 113	155,2 ± 43,5 pg/ml [52]	5 - 15 ng/ml
PAI-1	48h: 2408 ± 1518 72h: 2743 ± 1437	N/A	N/A

TIMP-1	48h: 7708 ± 1584 72h: 9547 ± 1897	73,5 ± 14,2 pg/ml [53]	N/A
uPA	48h: 380 ± 467 72h: 279 ± 243	362 ± 90 ng/ml [54]	N/A
VEGF	48h: 327 ± 154 72h: 477 ± 202	227 ± 247 pg/ml [47]	1-2 ng/ml

Since there was no ELISA kit commercially available for HGF and the secretion of bFGF in hDPSC conditioned medium was too low to be measured, immunocytochemistry against these factors was carried out (Fig. 4). HGF (Fig. 4 A, B) and bFGF (Fig. 4 C, D) were both clearly expressed at protein level by hDPSC.

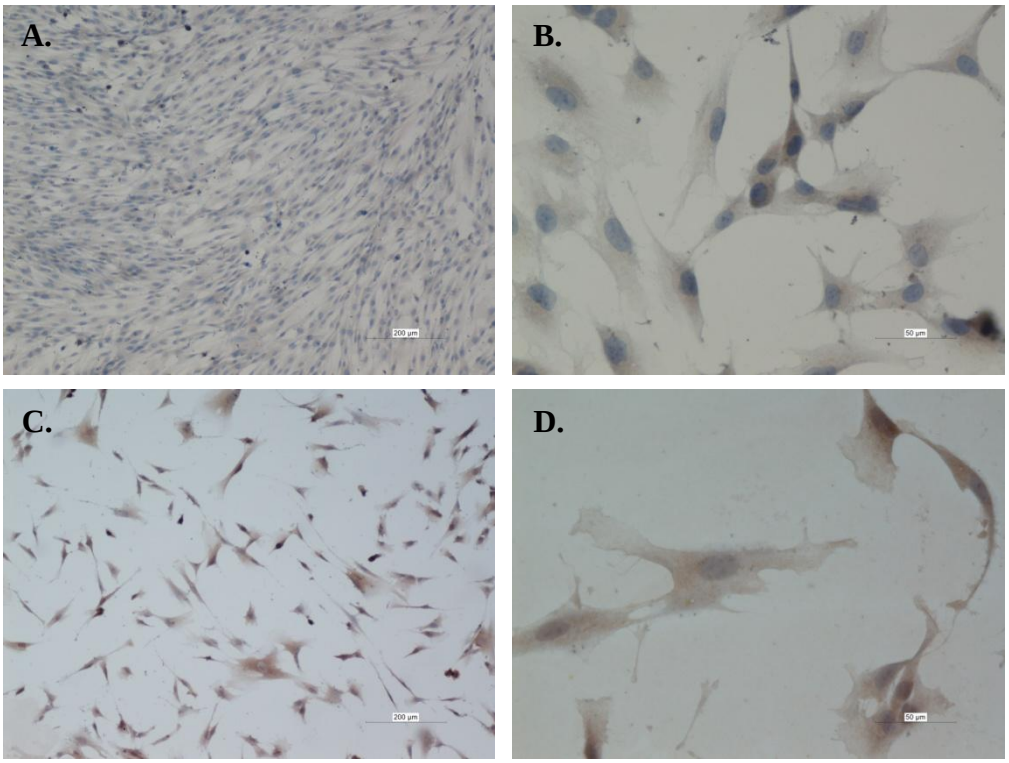


Figure 4: Immunocytochemistry - expression of HGF and bFGF at protein level. Immunocytochemistry was performed on hDPSC with mouse monoclonal antibodies against HGF (1:50) and bFGF (1:100). hDPSC were positive for both factors. A. HGF. Scale bar = 200 µm. B. HGF. Scale bar = 50 µm. C. bFGF. Scale bar = 200 µm. D. bFGF. Scale bar = 50 µm.

3.2 *In vitro* models of angiogenesis

Since previous results indicated that hDPSC expressed and/or secreted angiogenic factors, the potential paracrine impact of hDPSC on the behavior of endothelial cells, and thus angiogenesis, was determined by applying several *in vitro* models of angiogenesis.

3.2.1 MTT assay

In order to assess whether hDPSC had an impact on the proliferation of MBEC, an MTT assay was performed with different samples of hDPSC conditioned medium. Compared to the negative control situation, hDPSC conditioned medium had no significant effect on the proliferation of MBEC, when seeded at 10.000 cells/cm². The measured absorbance was comparable with the absorbance values of the negative control (Fig. 5). The same results were obtained when MBEC were seeded at 5.000 cells/cm² (See supplemental data S2.1).

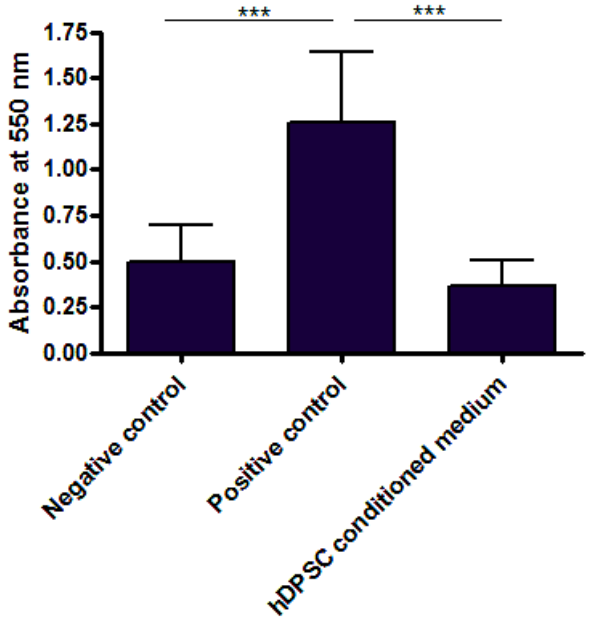


Figure 5: MTT assay - the potential impact of hDPSC on the proliferation of MBEC. MBEC were seeded at 10 000 cells/cm² and were incubated for 72h with hDPSC conditioned medium, standard hDPSC culture medium (positive control) or standard hDPSC culture medium supplemented with 0,1% FBS instead of 10% FBS (negative control). Afterwards an MTT assay was performed. There was no significant effect of hDPSC conditioned medium on the proliferation of MBEC compared to the negative control situation. Data were analyzed with a one way ANOVA followed by a Bonferonni's multiple comparison post-hoc test. Data are represented as mean ± SD and correspond to n = 4 assays. *** = P-value < 0,001.

3.2.2 Transwell migration assay

Besides proliferation, the migration of endothelial cells is another important step in the process of blood vessel formation. The potential impact of hDPSC on the migration of MBEC was tested by means of a transwell migration assay. After 7h of incubation with hDPSC conditioned medium, there was a significant increase of MBEC migration compared to the negative control situation (Fig. 6A, E). An even more pronounced increase in migration was observed when MBEC were incubated with hDPSC (Fig. 6B, E).

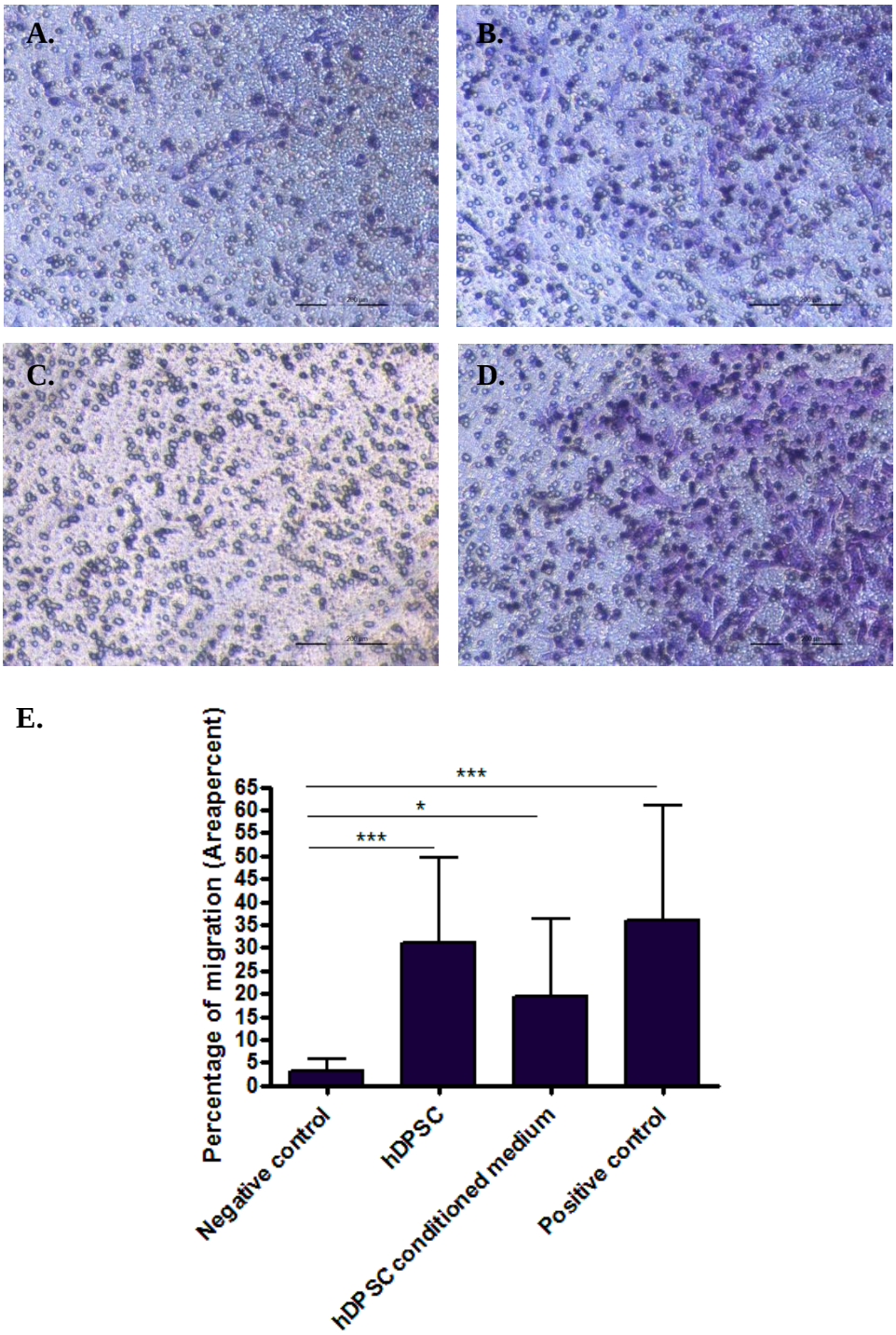


Figure 6: Transwell migration assay - the potential impact of hDPSC on the migration of MBEC. Inserts with MBEC were incubated for 7h with hDPSC conditioned medium, hDPSC, standard hDPSC culture medium (positive control) or standard hDPSC culture medium supplemented with 0,1% FBS instead of 10% FBS (negative control). Pictures were taken with an inverted microscope and analyzed with AxioVision Software. hDPSC as well as hDPSC conditioned medium had a significant effect on the migration of MBEC compared to the control situation. A. hDPSC conditioned medium. B. hDPSC. C. Control. D. Positive control. E. Percentage of migration (Areapercent) in different conditions. Data were analyzed with a one way ANOVA followed by a Bonferonni's multiple comparison post-hoc test. Data are represented as mean ± SD and correspond to n = 6 assays. * = P-value < 0,05. *** = P-value < 0,001. Scale bars = 200 µm.

According to literature, both VEGF and IL-8 play an important role in the promotion of endothelial cell migration [22]. Therefore, recombinant human VEGF and IL-8 were added to the negative control medium in order to determine their impact on the migration of MBEC. After 7h of incubation with recombinant VEGF, there was no significant increase in the migration of MBEC compared to the negative control situation. However, a positive trend was observed (Fig. 7A). This trend was also observed when MBEC were incubated with recombinant IL-8 (Fig. 7B). (For pictures, see supplemental data S2.2).

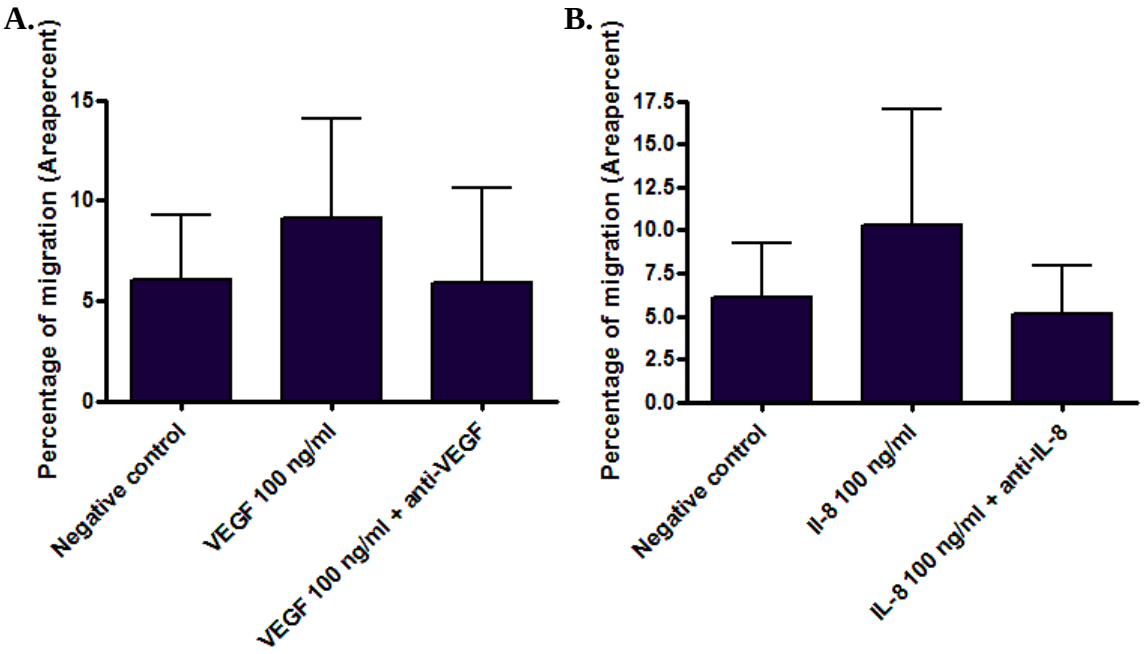


Figure 7: Transwell migration assay - the potential impact of recombinant human VEGF and IL-8 on the migration of MBEC. Inserts with MBEC were incubated for 7h with standard hDPSC culture medium supplemented with 0,1% FBS instead of 10% FBS (negative control), negative control medium supplemented with recombinant VEGF or IL-8 or negative control medium supplemented with recombinant VEGF or IL-8 and neutralizing antibodies against VEGF or IL-8. Recombinant VEGF as well as IL-8 showed a positive trend towards increased MBEC migration compared to the negative control situation. A. Percentage of migration (Areapercent) after addition of recombinant VEGF. B. Percentage of migration (Areapercent) after addition of recombinant IL-8. Data were analyzed with a Kruskall-Wallis test followed by a Dunn's multiple comparison post-hoc test. Data are represented as mean ± SD and correspond to n = 4 assays.

In order to determine a potential synergistic effect of recombinant human VEGF and IL-8 on the migration of MBEC, the endothelial cells were incubated with both factors together. After 7h of incubation, there was no significant increase of migration of MBEC compared to the negative control situation or recombinant VEGF or IL-8 alone (Fig. 8).

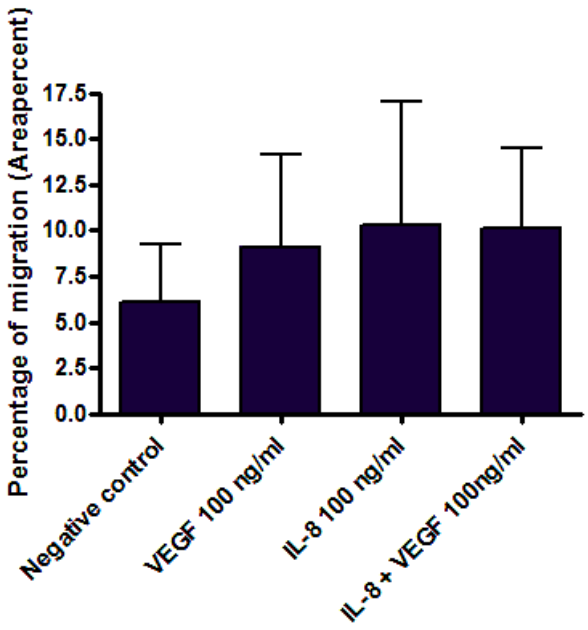


Figure 8: Transwell migration assay - the potential synergistic impact of recombinant VEGF and IL-8 on the migration of MBEC. Inserts with MBEC were incubated for 7h with standard hDPSC culture medium supplemented with 0,1% FBS instead of 10% FBS (negative control), negative control medium supplemented with recombinant VEGF or IL-8 or negative control medium supplemented with both recombinant factors. The combination of recombinant VEGF and IL-8 caused no significant increase of MBEC migration compared to the negative control situation or recombinant VEGF or IL-8 alone. Data were analyzed with a Kruskall-Wallis test followed by a Dunn's multiple comparison post-hoc test. Data are represented as mean ± SD and correspond to n = 4 assays.

Since the previous results have indicated that VEGF and IL-8 could potentially cause an increase of endothelial cell migration, antibodies against these pro-angiogenic factors were added to hDPSC and hDPSC conditioned medium in order to determine whether these factors had a predominant role in the migration process. After 7h of incubation, the addition of neutralizing antibodies against VEGF to hDPSC caused a significant decrease of the migration of MBEC (Fig. 9A). This was not the case when neutralizing antibodies against IL-8 were added to hDPSC. However, a trend towards decreased migration was observed (Fig. 9B). The addition of neutralizing antibodies against VEGF or IL-8 to hDPSC conditioned medium caused no significant decrease in the migration of MBEC. The observed migration was comparable with the migration caused by the hDPSC conditioned medium (Fig. 9C, D). (For pictures, see supplemental data S2.3).

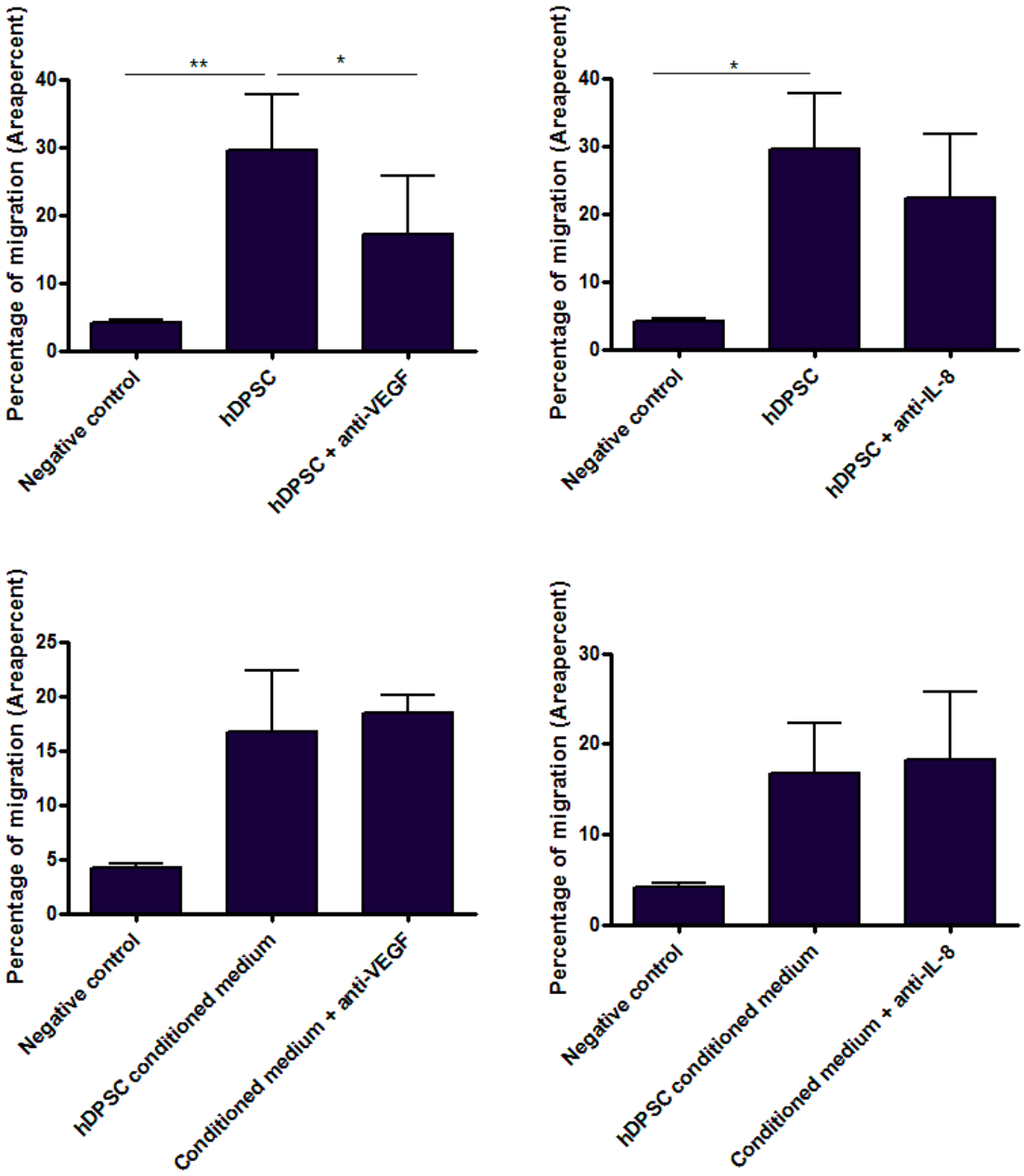


Figure 9: Transwell migration assay - the potential impact of neutralizing antibodies against VEGF or IL-8 on the migration of MBEC. Inserts with MBEC were incubated for 7h with standard hDPSC culture medium supplemented with 0,1% FBS instead of 10% FBS (negative control), hDPSC with or without neutralizing antibodies against VEGF or IL-8 or hDPSC conditioned medium with or without neutralizing antibodies against VEGF or IL-8. The neutralizing antibodies against VEGF only caused a significant decrease in MBEC migration when added to hDPSC. The neutralizing antibodies against IL-8 caused no significant decrease in MBEC migration, although a trend towards decreased migration was observed when added to hDPSC. A. hDPSC + anti-VEGF. B. hDPSC + anti-IL-8. C. hDPSC conditioned medium + anti-VEGF. D. hDPSC conditioned medium + anti-IL-8. Data (A) were analyzed with a one way ANOVA followed by a Bonferonni's multiple comparison post-hoc test or (B-D) with a Kruskall-Wallis test followed by a Dunn's multiple comparison post-hoc test. Data are represented as mean ± SD and correspond to n = 2 assays. * = P-value < 0,05. ** = P-value < 0,01.

3.2.3 Wound healing assay

Another way of studying migration is by looking at directional migration. In order to test the potential impact of hDPSC on the directional migration of MBEC, a wound healing assay was performed. After 24h of incubation, hDPSC conditioned medium significantly increased the migration of MBEC compared to the negative control situation (Fig. 10D, E). This effect was maintained after 48h of incubation (See supplemental data S2.4).

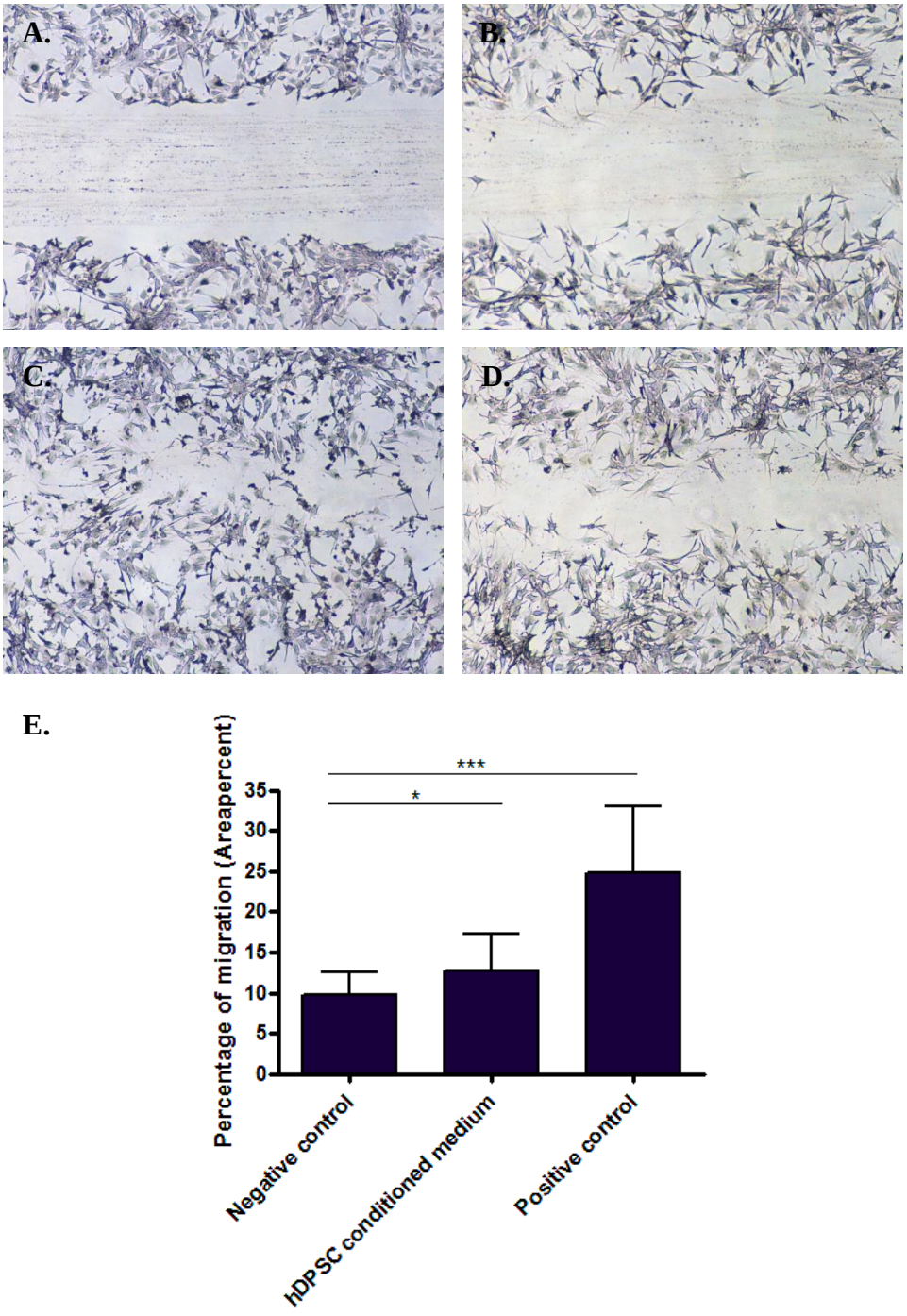


Figure 10: Wound healing assay - the potential impact of hDPSC on the directional migration of MBEC after 24h of incubation. A scratch within a monolayer of MBEC was incubated with hDPSC conditioned medium, standard

hDPSC culture medium (positive control) or standard hDPSC culture medium supplemented with 0,1% FBS instead of 10% FBS (negative control). Pictures were taken with an inverted microscope and analyzed with AxioVision Software. hDPSC conditioned medium has a significant effect on the migration of MBEC as compared to the negative control situation. A. Before. B. Negative control. C. Positive control. D. hDPSC conditioned medium. E. Percentage of directional migration (Areapercent) in different conditions. Data were analyzed with a Kruskall-Wallis test followed by a Dunn's multiple comparison post-hoc test. Data are represented as mean ± SD and correspond to n = 8 assays. * = P-value < 0,05. *** = P-value < 0,001. All pictures were taken at 40x magnification.

Comparable with the transwell migration assay, recombinant human VEGF and IL-8 were added to the negative control medium in order to determine their impact on the directional migration of MBEC. After 24h of incubation, recombinant VEGF caused a significant increase in the directional migration of MBEC, compared to the negative control situation (Fig. 11A). The addition of recombinant IL-8, however, did not have a significant impact on the directional migration of MBEC. The amount of migration was comparable with the migration observed in the negative control condition. Moreover, after the addition of neutralizing antibodies against IL-8 a significant increase in MBEC migration was observed compared to the condition with recombinant IL-8 alone (Fig. 11B). (For pictures, see supplemental data S2.5).

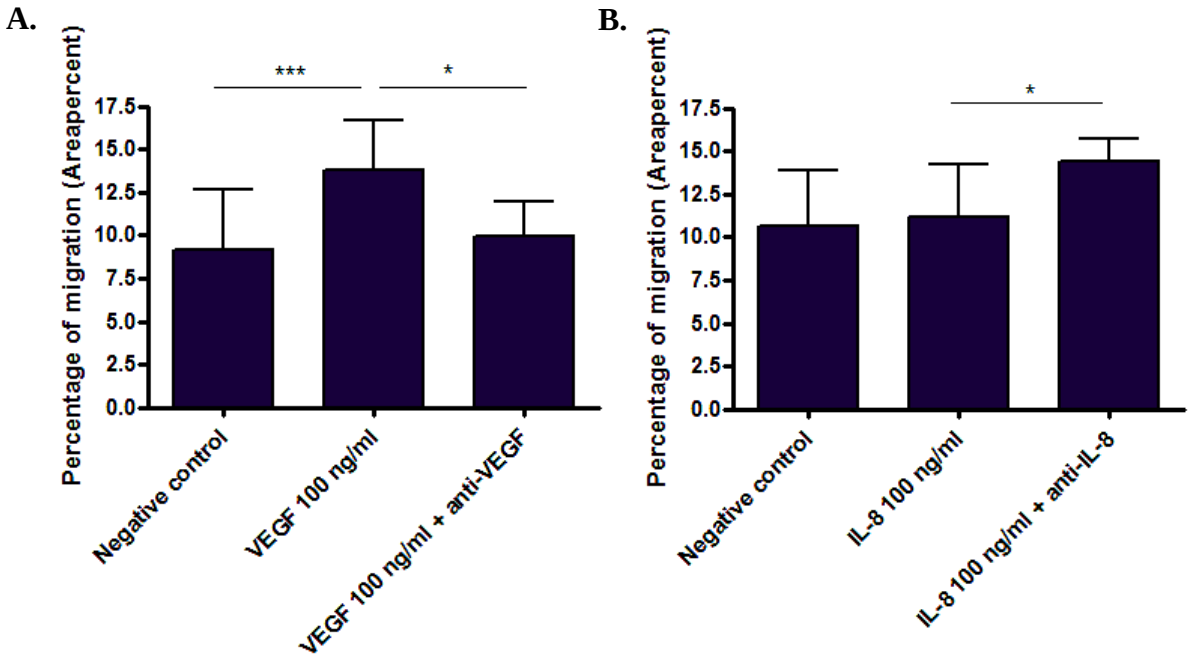


Figure 11: Wound healing assay - the potential impact of recombinant human VEGF and IL-8 on the directional migration of MBEC after 24h of incubation. A scratch within a monolayer of MBEC was incubated with standard hDPSC culture medium supplemented with 0,1% FBS instead of 10% FBS (negative control), negative control medium supplemented with recombinant VEGF or IL-8 or negative control medium supplemented with recombinant VEGF or IL-8 and neutralizing antibodies against VEGF or IL-8. A. Percentage of directional migration (Areapercent) after addition of recombinant VEGF. Recombinant VEGF had a significant impact on the directional

migration of MBEC as compared to the negative control situation. B. Percentage of directional migration (Areapercent) after addition of recombinant IL-8. Recombinant IL-8 had no significant effect on MBEC migration. However, the addition of anti-IL-8 caused a significant increase in MBEC migration. Data (A) were analyzed with a one way ANOVA followed by a Bonferonni's multiple comparison post-hoc test or (B) with a Kruskall-Wallis test followed by a Dunn's multiple comparison post-hoc test. Data are represented as mean ± SD and correspond to n = 4 assays (A) or n = 5 assays (B). * = P-value < 0,05. *** = P-value < 0,001.

Since the previous results indicated that recombinant VEGF had significantly increased the directional migration of MBEC, neutralizing antibodies against this pro-angiogenic factor were added to the hDPSC conditioned medium in order to determine a potential predominant role of VEGF in the migration process. Since the transwell migration assay showed a potential impact of recombinant IL-8 on MBEC migration, neutralizing antibodies against this factor were also added to hDPSC conditioned medium. After 24h of incubation, the neutralizing antibodies against VEGF and IL-8 caused no significant decrease of MBEC migration. The amount of migration was comparable in all three conditions (Fig. 12A, B). (For pictures, see supplemental data S2.6).

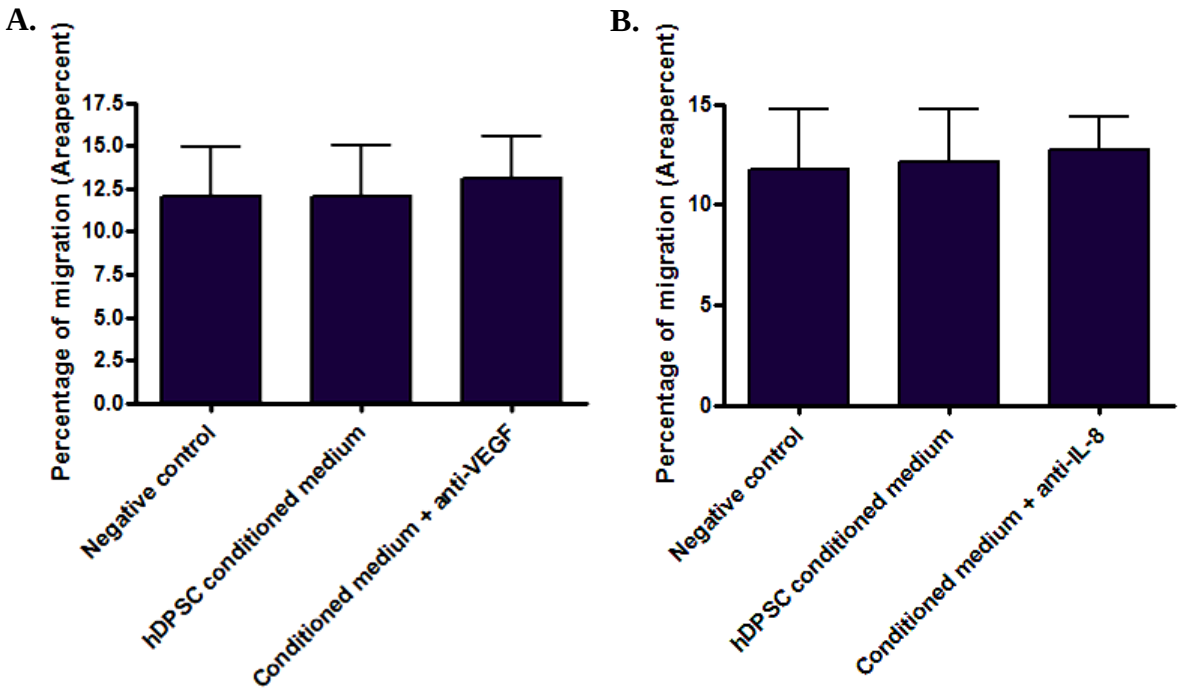


Figure 12: Wound healing assay - the potential impact of neutralizing antibodies against VEGF or IL-8 on the directional migration of MBEC. A scratch within a monolayer of MBEC was incubated for 24h with standard hDPSC culture medium supplemented with 0,1% FBS instead of 10% FBS (negative control) or hDPSC conditioned medium with or without neutralizing antibodies against VEGF or IL-8. The neutralizing antibodies against VEGF and IL-8 had no significant effect on the directional migration of MBEC. A. hDPSC conditioned medium + anti-VEGF. B. hDPSC conditioned medium + anti-IL-8. Data (A) were analyzed with a Kruskall-Wallis test followed by a Dunn's multiple comparison post-hoc test or (B) with a one way ANOVA followed by a Bonferonni's multiple comparison post-hoc test. Data are represented as mean ± SD and correspond to n = 4 assays. (A) or n = 3 assays (B).

3.2.4 Hypoxia assay

According to literature, angiogenic factors could also have an impact on endothelial survival and apoptosis [22]. In order to determine the potential impact of hDPSC on endothelial cell survival a hypoxia assay was performed. After 24h of incubation with hDPSC conditioned medium under normoxic and hypoxic conditions, there was no significant difference in the amount of living, necrotic, early apoptotic and late apoptotic cells, compared to the control situation (Fig. 13A, B). After 24h of incubation with hDPSC under normoxia on the other hand, a trend towards an increased amount of living cells and a decreased amount of necrotic cells was observed (Fig. 13C). However, this effect was not maintained when MBEC were incubated under hypoxic conditions. The amounts of cells were comparable between control and hDPSC (Fig. 13D).

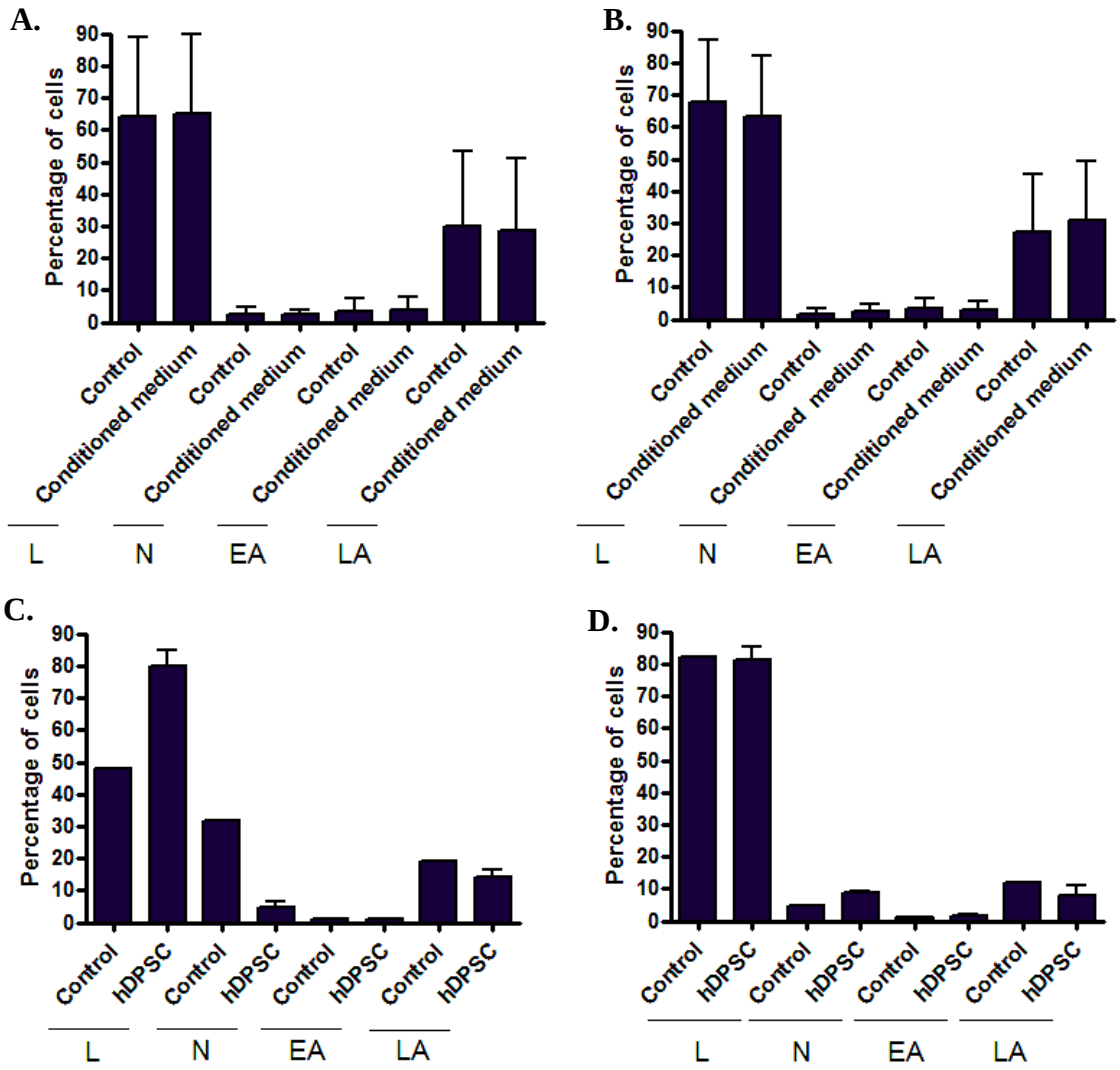
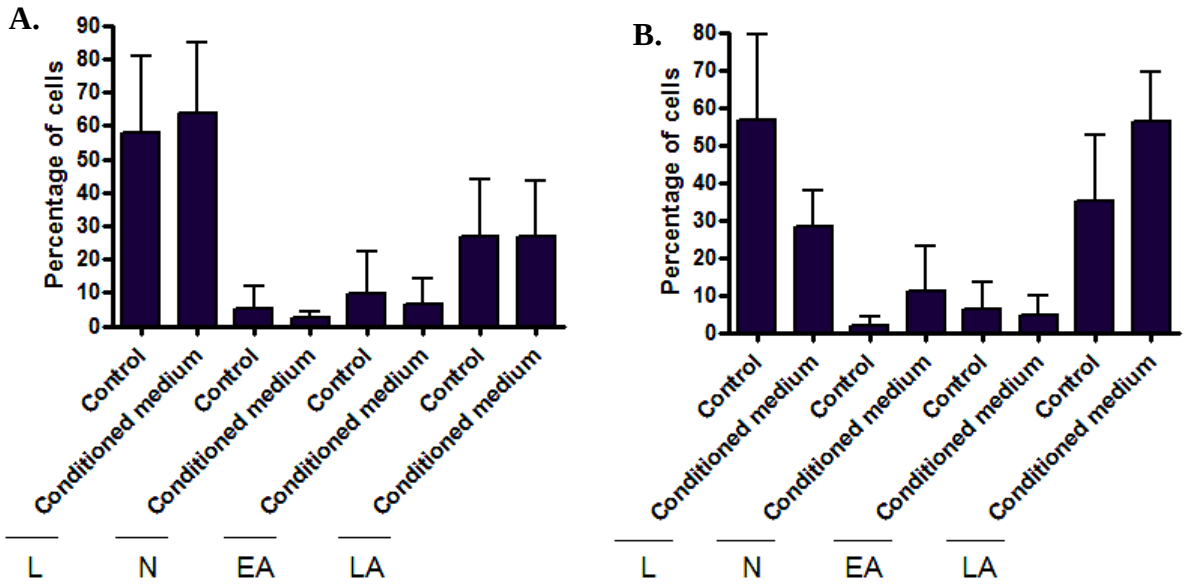


Figure 13: Hypoxia assay - the potential impact of hDPSC on the survival of MBEC after 24h of incubation. MBEC were incubated under normoxia and hypoxia with standard hDPSC culture medium supplemented with 0,1% FBS instead of 10% FBS (negative control), hDPSC conditioned medium or hDPSC. Afterwards, an Annexin V-Propidium

Iodide staining was performed and cell percentages were determined by means of flow cytometry. L= living cells. N = necrotic cells. EA = early apoptotic cells. LA = late apoptotic cells. hDPSC conditioned medium as well as hDPSC had no significant effect on the survival of MBEC, except for a protective trend seen in the normoxia-hDPSC condition. A. Normoxia: hDPSC conditioned medium. B. Hypoxia: hDPSC conditioned medium. C. Normoxia: hDPSC. D. Hypoxia: hDPSC. Data were analyzed with a Kruskall-Wallis test followed by a Dunn's multiple comparison post-hoc test. Data are represented as mean ± SD and correspond to n = 2 assays. (A, B) or n = 1 assay (C, D)

In order to maximize the potential impact of hDPSC on the survival of MBEC, the hypoxia assay was also performed with a 48h incubation period. After 48h of incubation with hDPSC conditioned medium under normoxia, there was no significant difference in the amount of living, necrotic, early apoptotic and late apoptotic cells, compared to the control situation (Fig. 14A). However, when incubated under hypoxic conditions, the hDPSC conditioned medium showed a trend towards a decreased amount of living cells and an increased amount of necrotic and late apoptotic cells (Fig. 14B). After 48h of incubation with hDPSC under normoxia, the amounts of cells were comparable between control and hDPSC, except for minor trend towards decreased early apoptotic cells in the hDPSC condition (Fig. 14C). When MBEC were incubated with hDPSC under hypoxia, the amount of living cells was comparable between control and hDPSC, although there was a decrease compared to the normoxia condition. Furthermore, hDPSC showed a trend towards an increased amount of necrotic cells and a decreased amount of early and late apoptotic cells (Fig. 14D).



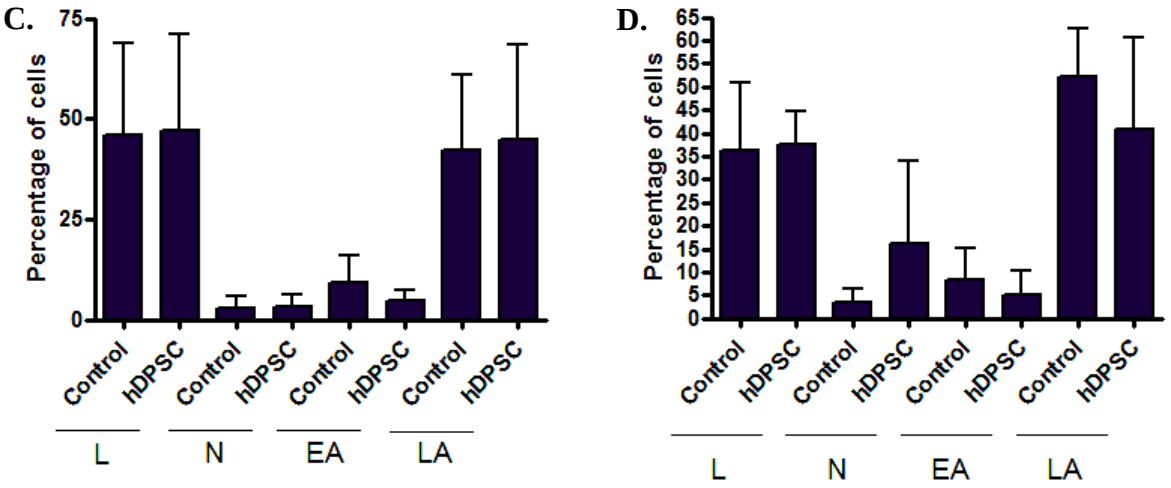


Figure 14: Hypoxia assay - the potential impact of hDPSC on the survival of MBEC after 48h of incubation. MBEC were incubated under normoxia and hypoxia with standard hDPSC culture medium supplemented with 0,1% FBS instead of 10% FBS (negative control), hDPSC conditioned medium or hDPSC. Afterwards, an Annexin V-Propidium Iodide staining was performed and cell percentages were determined by means of flow cytometry. L= living cells. N = necrotic cells. EA = early apoptotic cells. LA = late apoptotic cells. hDPSC conditioned medium as well as hDPSC had no significant effect on the survival of MBEC, except for a trend towards dying cells seen in the hypoxia-hDPSC conditioned medium condition and a minor protective trend in the hypoxia-hDPSC condition. A. Normoxia: hDPSC conditioned medium. B. Hypoxia: hDPSC conditioned medium. C. Normoxia: hDPSC. D. Hypoxia: hDPSC. Data were analyzed with a Kruskall-Wallis test followed by a Dunn's multiple comparison post-hoc test. Data are represented as mean ± SD and correspond to n = 2 assays.

3.3 Determination of the endothelial differentiation potential of hDPSC

Besides the potential paracrine effects of hDPSC on the behavior of MBEC, it is also important to assess whether these stem cells are capable of forming blood vessels themselves by, for example, differentiating into endothelial cells.

3.3.1 Endothelial marker expression

In order to assess the endothelial differentiation potential of hDPSC, the endothelial marker expression was determined by means of flow cytometry after incubating the stem cells with several induction media for different periods of time (Table 5). After 10 days of incubation, there was no significantly increased expression of endothelial markers CD31, CD34 and VEGFR2 when comparing the control situation with the different induction media, although a trend towards increased endothelial marker expression was observed with method 4 (Fig. 15A-C). With regard to stem cell marker CD44, there was no significant difference in expression level between control and induction media. However, method 2 showed a minor negative trend in CD44 expression (Fig. 15D).

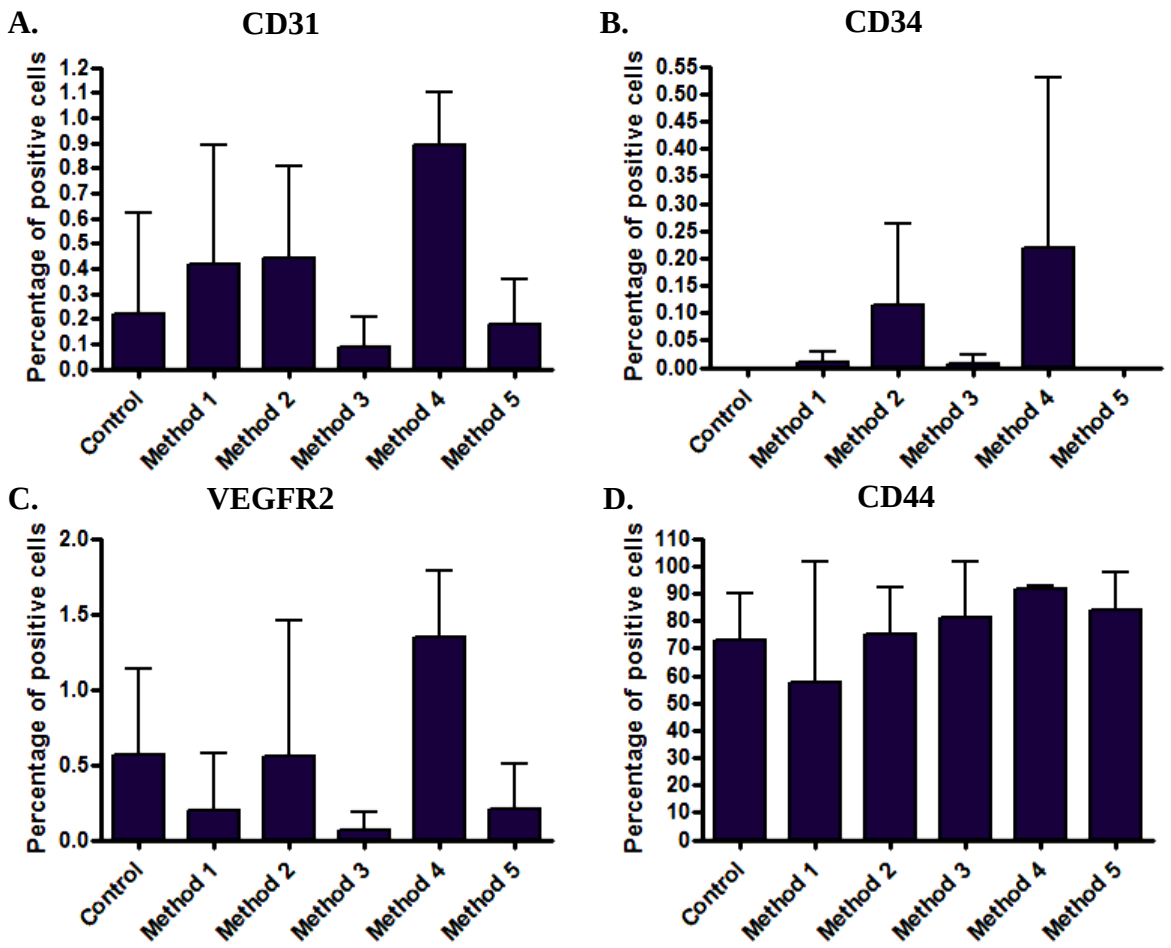


Figure 15: The endothelial differentiation potential of hDPSC at protein level after 10 days of incubation. hDPSC were incubated with several induction media and after 10 days, the expression levels of CD31, CD34, VEGFR2 and CD44 were determined by means of flow cytometry. There were no significant differences in marker expression between control and induction media. A. CD31. B. CD34. C. VEGFR2. D. CD44. Data were analyzed with a Kruskall-Wallis test followed by a Dunn's multiple comparison post-hoc test. Data are represented as mean ± SD and correspond to n = 2 assays.

After 21 days of incubation, the cultures incubated with method 4 loosened so they had to be discarded. With regard to the remaining conditions, there was no significant increase in the expression level of the aforementioned endothelial markers compared to the control condition. However, method 5 showed a minor positive trend in terms of CD31 and CD34 expression (Fig. 16A-C). Although the general CD44 expression level decreased in comparison to the 10 days incubation period, there was no significant difference between the control situation and the induction media. Method 3 did show a trend towards increased CD44 expression (Fig. 16D).

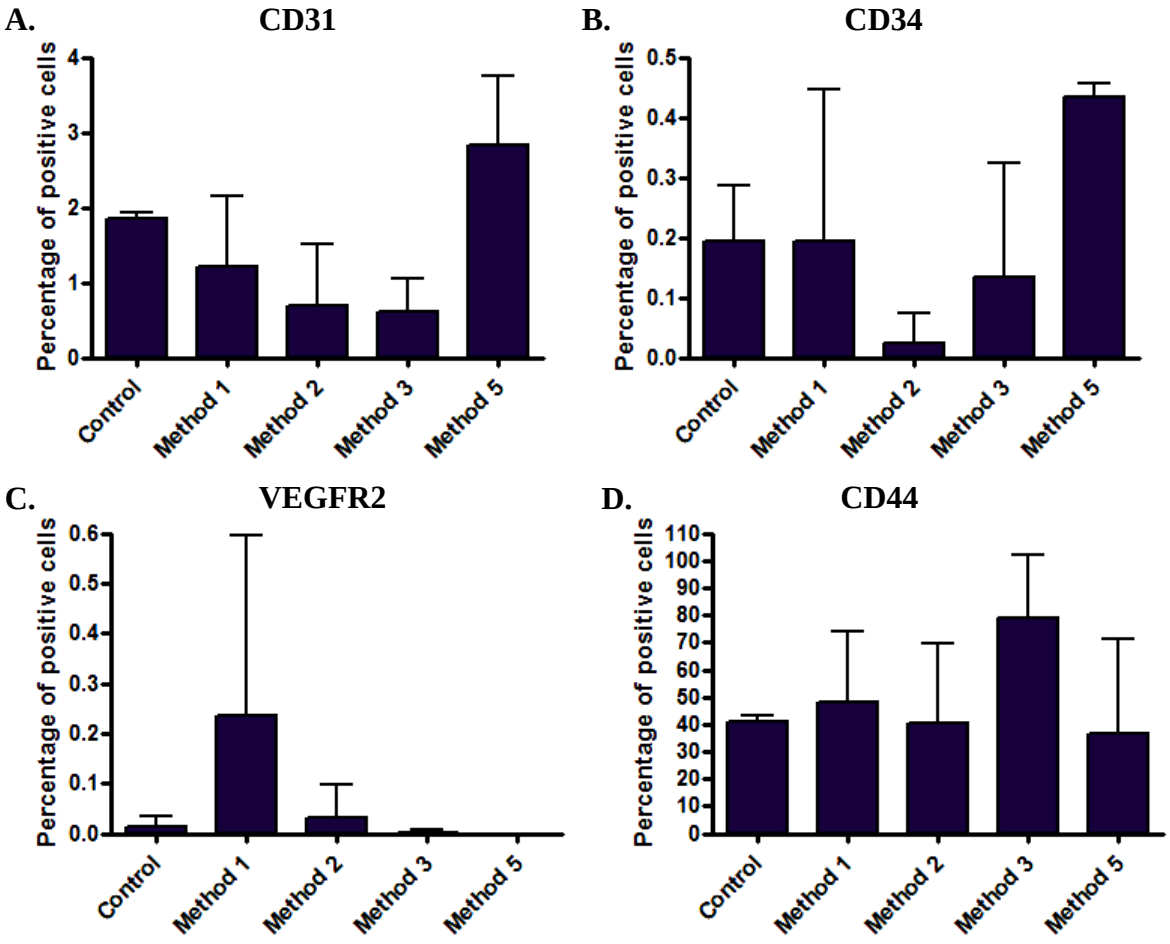
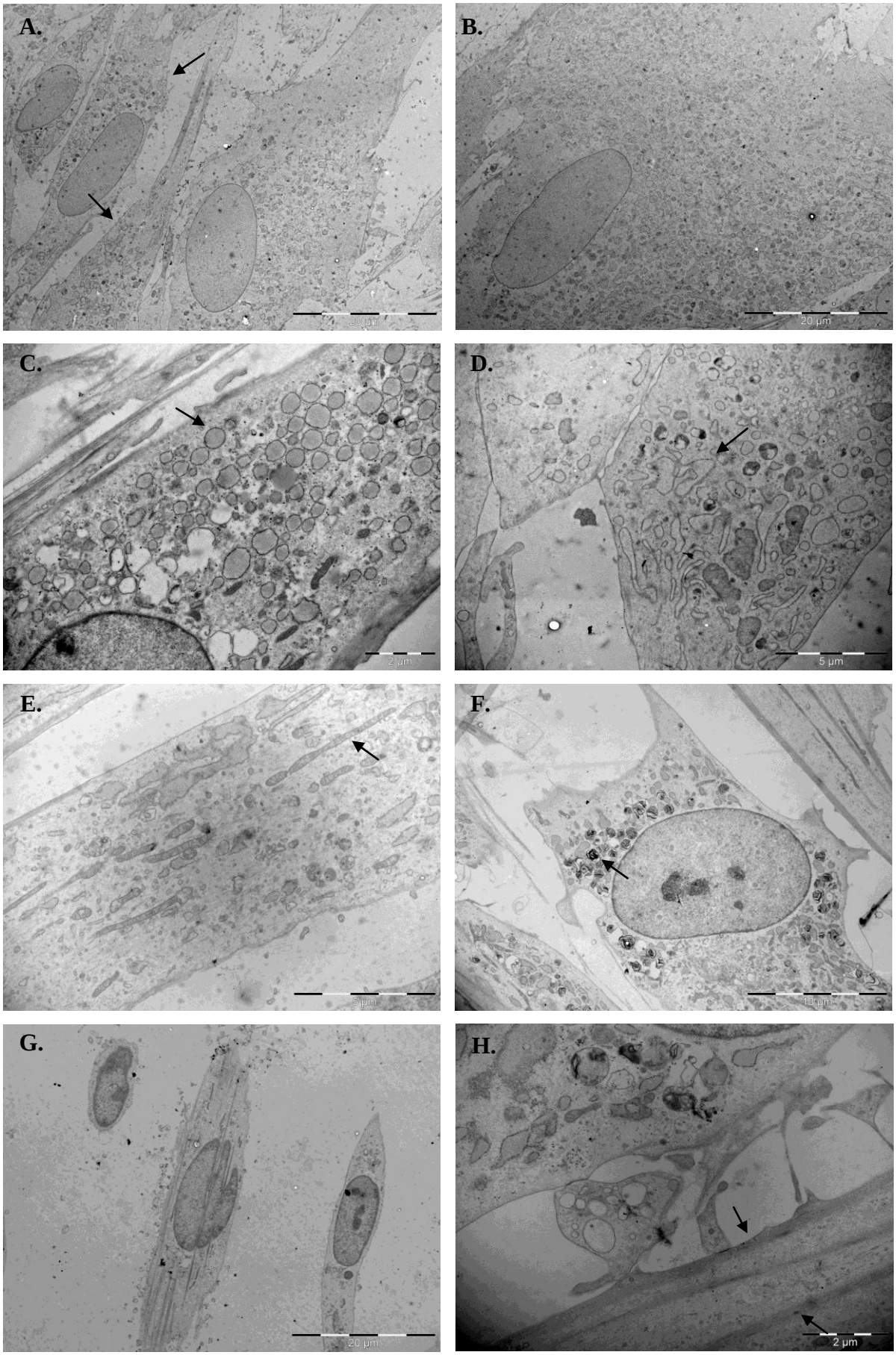


Figure 16: The endothelial differentiation potential of hDPSC at protein level after 21 days of incubation. hDPSC were incubated with several induction media and after 21 days, the expression levels of CD31, CD34, VEGFR2 and CD44 were determined by means of flow cytometry. There were no significant differences in marker expression between control and induction media. A. CD31. B. CD34. C. VEGFR2. D. CD44. Data were analyzed with a Kruskall-Wallis test followed by a Dunn's multiple comparison post-hoc test. Data are represented as mean ± SD and correspond to n = 2 assays.

Since hDPSC are a heterogeneous stem cell population, the use of different isolation methods could yield different subsets of hDPSC [44]. Therefore, explant hDPSC and enzymatically digested hDPSC could display different angiogenic properties. In order to reproduce the applied protocol of Marchionni *et al.*, the first research group who mentioned the *in vitro* endothelial differentiation potential of hDPSC, enzymatically digested hDPSC were incubated with control medium and method 1[42]. After 7 days of incubation, expression of endothelial markers CD31, CD34, CD54 and stem cell marker CD44 was assessed (See supplemental data S2.7).

3.3.2 Morphological changes

Despite the absence of significant endothelial marker expression, potential morphological changes of the incubated cells were assessed at the ultrastructural level by means of transmission electron microscopy. With regard to the explant hDPSC, there were no clear differences between the used methods or the two incubation periods of 10 and 21 days. The cells seemed to have a variable morphology; while a subset of cells displayed an elongated, fibroblast-like appearance (Fig. 17A, arrow), other cells had a more irregular shape (Fig. 17A). In terms of cell organelle content, there was no difference between the used induction media and the control situation; all cells appeared to be very active and rich in organelles, which were spread throughout the whole cell body (Fig. 17A, B). Concerning organelle morphology, all cells seemed to have a dilated rough endoplasmic reticulum (RER), although the shape differed from patient to patient from a more circular appearance (Fig. 17C, arrow) to a more irregular shape (Fig. 17D, arrow). The cells also contained elongated mitochondria (Fig. 17E, arrow). Furthermore, a great subset of cells contained numerous multilamellar vesicles (Fig. 17F, arrow). Regardless of the used medium, certain cells also displayed invaginated nuclei (Fig. 17G). Furthermore, in a great subset of cells actin fibers were visibly present, though mostly condensed in the periphery of the cells (Fig. 17H, arrows). Secretion of collagen fibers by certain cells was also observed, there was however no difference between the control situation and the different methods used (Fig. 17I). There also appeared to be clear contact between the cells in certain cases (Fig. 17J). The only apparent difference between the control situation and the different induction media was the presence of electron-dense granules in a few cells of the inducing conditions, this was however a rare event (Fig. 17K). With regard to the enzymatically digested hDPSC, the cells appeared similar to the explant hDPSC, although there was a clear difference between the different incubation periods (10 days and 21 days) on the one hand and the control situation and the induction media on the other hand, in terms of the aforementioned electron-dense granules (See supplemental data S2.7).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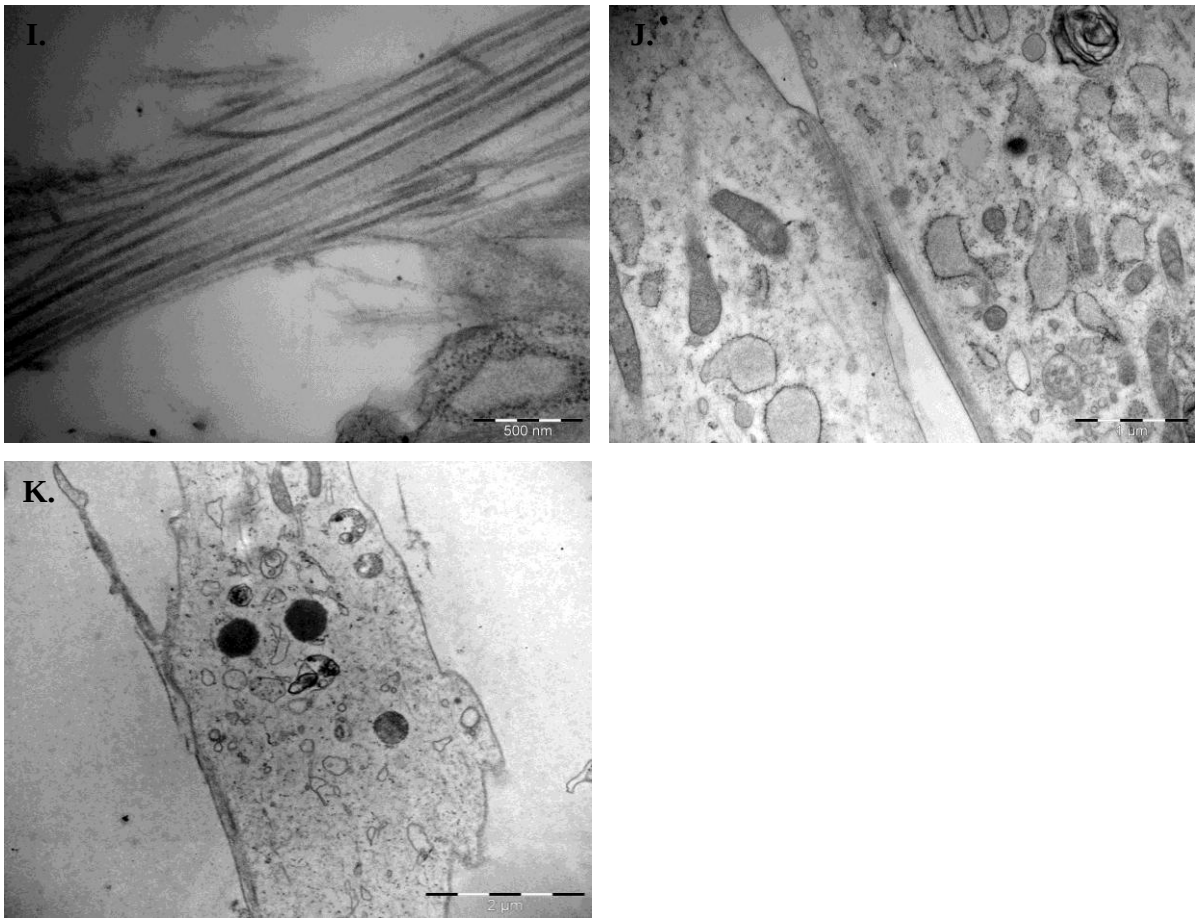

Figure 17: The endothelial differentiation potential of hDPSC at ultrastructural level after 10 days or 21 days of incubation. hDPSC were incubated with several induction media and after 10 or 21 days, the potential morphological changes at the ultrastructural level were assessed by means of transmission electron microscopy. A. Cellular morphology. Scale bar = 20 µm. B. Cell organelles. Scale bar = 20 µm. C. Dilated rough endoplasmic reticulum: circular appearance (arrow). Scale bar = 2 µm. D. Dilated rough endoplasmic reticulum: irregular shape (arrow). Scale bar = 5 µm. E. Mitochondria: elongated appearance (arrow). Scale bar = 5 µm. F. Multilamellar vesicles (arrow). Scale bar: 10 µm. G. Invaginated nuclei. Scale bar = 20 µm. H. Condensed actin fibers (arrows). Scale bar: 2 µm. I. Collagen fibers. Scale bar = 500 nm. J. Cell-cell contact. Scale bar = 1 µm. K. Electron-dense granules. Scale bar = 2 µm.

4. Discussion

Within the adult human body, stem cell niches have been identified in a number of tissues, including skin, hair follicles, bone marrow, intestine, brain, pancreas and also dental pulp. Since the maintenance and regulation of stem cell populations is tightly controlled by their local microenvironment, stem cell niches often appear to be highly vascularized sites. Within the dental pulp several stem cell niches have been identified, one of them being a perivascular niche. This perivascular localization of hDPSC could not only be important for population maintenance and regulation, but could also indicate a possible predisposition of these stem cells towards an endothelial or pericyte lineage. According to recent studies, hDPSC not only express classical MSC markers such as CD29, CD90 and STRO-1, the majority of the stem cells also displays a basal expression of endothelial markers CD146 and CD105, and pericyte marker 3G5 [38, 55, 56]. Furthermore, hDPSC also express VEGFR1 and VEGFR2, which could indicate responsiveness towards VEGF [42]. With regard to the *in vitro* endothelial differentiation potential of these stem cells, several studies have indeed indicated an increased expression of endothelial markers such as CD31, CD34 and von Willebrand factor after exposure to media containing VEGF [42, 56]. However, publications regarding the *in vivo* endothelial differentiation potential of hDPSC have yielded contradictory results, suggesting a more paracrine pro-angiogenic effect of hDPSC [15, 43]. In order to determine the contribution of hDPSC to vascular (re)generation, an elaborate angiogenic profiling of these stem cells is required. Therefore, the goal of this study was to elucidate the angiogenic properties and endothelial differentiation potential of hDPSC.

With regard to the angiogenic secretion profile of hDPSC, preliminary results from our laboratory indicated that hDPSC expressed and/or secreted pro-angiogenic (MMP-9, uPA, IL-8, VEGF, bFGF and MCP-1) as well as anti-angiogenic factors (PAI-1, TIMP-1, TSP and endostatin) (data not shown). However, the results of the performed antibody array needed to be validated by means of RT-PCR and ELISA. In order to determine the angiogenic secretion profile at mRNA level, RT-PCR was performed on samples of 6 different patients. Results indicated that hDPSC express pro-angiogenic (VEGF, MCP-1, uPA, IL-8 and HGF) as well as anti-angiogenic factors (PAI-1, TIMP-1 and endostatin) and one angiogenic factor with a dual role (IGFBP-3). A subset of these factors, namely VEGF, HGF and MCP-1, had already been demonstrated at mRNA level in a $CD31^-/CD146^-$ subpopulation of DPSC. Furthermore, this study also showed a minor expression at mRNA level of MMP-9 and stromal-derived factor-1 (SDF-1), the latter being a pro-angiogenic factor which collaborates with VEGF in the process of neovascularization [57, 58]. However, the DPSC that were studied were of

porcine origin, so potential species-related differences have to be taken into account. Other studies indicated that hDPSC also express other pro-angiogenic factors at mRNA level, namely macrophage colony stimulating factor (M-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF) and stem cell factor (SCF), the latter only being expressed in a subset of cells and which has been shown to promote the survival, migration and capillary tube formation of human umbilical vein endothelial cells (HUVECs) *in vitro* [39, 59, 60]. In comparison, human BM-MSC express pro-angiogenic (VEGF, MCP-1, bFGF, EGF, PDGF, HGF, IL-8, Ang1,TNFα, MMPs and uPA) as well as anti-angiogenic factors (TIMP-1/2) and factors with a dual role (Ang2, TGFβ) at mRNA level [61-64]. hDPSC are a heterogeneous population and together with factors such as age, dental health and the amount of orthodontic force, which al have their impact on growth factor homeostasis, this could account for the observed variability in gene expression [65-68]. In order to determine the angiogenic secretion profile of hDPSC at protein level, ELISAs were performed on the hDPSC conditioned medium of at least 6 different patients. Results indicated that all factors which were expressed at mRNA level were also secreted in hDPSC conditioned medium of 48h and 72h. The concentration of angiogenic factors showed an increasing trend in all samples of hDPSC conditioned medium of 72h, except for IL-8 and uPA, of which the concentration decreased after 72h. Since the cells are incubated for relatively long time period with low serum-containing medium (0,1% FBS), this could have its effect on cellular homeostasis [56]. Therefore, protein breakdown or a defect in the cellular translation machinery could be a potential explanation for the observed decrease of certain proteins. However, this does not explain why the hDPSC conditioned medium only shows a decreased content of certain factors and not its entire protein content. With regard to the secretion of bFGF, analysis indicated that the concentration within the hDPSC conditioned medium was too low to be measured. However, an immunostaining indicated that bFGF is expressed at protein level. These observations suggest that bFGF is merely expressed by hDPSC, but not secreted in the cellular supernatant. In contrast, a study of Tran-Hung *et al.* showed a high level of bFGF secretion by hDPSC under basal conditions. However, these results could possibly be biased since the analyzed medium contained 10% FBS [40]. Since there was no ELISA kit for HGF available, an immunostaining was performed which confirmed the expression of HGF at protein level. When comparing the measured protein concentrations with the available ED50 values from literature, the measured concentrations seemed to be significantly lower. However, the ED50 only gives an indication of the protein concentration required for a half-maximum response. The angiogenic factors can still exert an effect at lower concentrations.

Furthermore, hDPSC conditioned medium is not merely a collection of proteins with different ED50 values, it has to be considered as a whole of angiogenic factors that work together and form a discrete balance between stimulation and inhibition of angiogenesis [22]. Regarding the angiogenic secretion profile of BM-MSC, a study of Chen *et al.* indicated that these stem cells express and/or secrete pro-angiogenic (IL-8, MCP-1, SCF, SDF-1, EGF, IGF-1, Ang1, VEGF, PDGF, CSFs, HGF), anti-angiogenic (TIMP-1) as well as factors with a dual role (TGFβ, TNFα, IGFBP-3) at protein level [69].

Besides the angiogenic secretion profile, the potential impact of hDPSC on the behavior of endothelial cells was also determined. Since angiogenesis is a multistep process, several *in vitro* models of angiogenesis were performed in order to mimic the most important steps within the angiogenic process. The potential impact of hDPSC on the proliferation of MBEC was determined by means of a MTT assay. Despite the presence of proliferation-promoting factors in the hDPSC conditioned medium, such as VEGF and IL-8, there was no significant increase of MBEC proliferation. The absorbance levels were comparable between the control condition and the hDPSC conditioned medium. There is a possibility that the concentration of the aforementioned factors in the conditioned medium was not high enough to induce a clear increase in proliferation. Another explanation may lie in the fact that proliferation-inhibiting factors are also present in de medium; these factors could have a predominant effect compared to the pro-angiogenic factors and tip the angiogenic balance towards an inhibitory state. In contrast, Iohara *et al.* demonstrated a significant increase in HUVEC proliferation after incubating the cells with conditioned medium of porcine pulp-derived $CD31^-$ $CD146^-$ stem cells [57]. However, besides considering the potential species-related differences swine versus human, one also has to keep in mind that this study only focused on a subset of DPSC which could potentially have a higher angiogenic potential compared to DPSC in general [38]. Furthermore, species-related differences also have to be taken into account when studying the behavior of endothelial cells. Differential expression of antigens, receptors and chemokines between, for example, human and murine endothelial cells could lead to differences in cellular reactivity [70, 71]. Moreover, since antigen expression and the responsiveness to growth factors and inhibitors may vary with the tissue of origin, brain endothelial cells will not necessarily respond the same as umbilical vein endothelial cells [71]. In comparison, the studies regarding the effect of BM-MSC conditioned medium on the proliferation of endothelial cells also yielded contrasting results; one publication indicated a significant effect of BM-MSC conditioned medium on the proliferation of HUVECs, while another one mentioned a clear lack of a proliferation-promoting effect. However, it should be

noted that the applied experimental protocols differed significantly between studies [69, 72].

During angiogenesis, endothelial cells migrate along a chemotactic gradient of angiogenesis-inducing factors [20]. The migration of endothelial cells in response to angiogenic factors or stem cells *in vitro* can be assessed by means of different assays, one of them being the modified Boyden chamber assay or transwell migration assay [71]. In order to test the potential impact of hDPSC on the migration of endothelial cells, inserts with MBEC were incubated for 7h with hDPSC or hDPSC conditioned medium. The results of this assay indicated that hDPSC conditioned medium as well as hDPSC themselves, significantly increased MBEC migration, although hDPSC had a more pronounced stimulating effect. These results are consistent with the aforementioned ELISA analysis, which showed the presence of potent chemoattractants of endothelial cells in the hDPSC conditioned medium, such as VEGF, IL-8 and MCP-1. The more pronounced effect of hDPSC could probably be explained by the continuous release of angiogenic factors by hDPSC, leading to a more concentrated 'conditioned' medium which probably allowed for a maximal effect of the migration-promoting factors present. Another explanation for this effect could be sought in the preservation of the hDPSC conditioned medium; since the medium is stored at -80°C for an extended period of time, this could lead to breakdown of the containing proteins and a thus a reduced concentration of migration-promoting angiogenic factors. The observations made in this study regarding the effect of hDPSC on the migration of endothelial cells, have not been mentioned elsewhere. In comparison, studies with regard to the angiogenic effects of BM-MSC indicated the chemotactic effects of BM-MSC conditioned medium on HUVECs and human uterus microvascular endothelial cells (HUMECs) [69, 72].

According to literature, several angiogenic factors have an impact on the migration of endothelial cells, two of them being VEGF and IL-8 [22]. In order to test the impact of these factors on the migration of MBEC, a transwell migration assay was conducted with recombinant human VEGF and IL-8, factors which had already been shown to significantly increase the migration of human brain endothelial cells (HBEC) [73, 74]. The results of this assay showed that both recombinant VEGF and IL-8 did not significantly increase the migration of MBEC, although a clear trend towards increased migration was observed. However, the addition of recombinant VEGF and IL-8 together did not significantly increase MBEC migration compared to VEGF or IL-8 alone, indicating the absence of synergism between both factors.

Since both VEGF and IL-8 are secreted by hDPSC, the transwell migration assay was performed with neutralizing antibodies against these pro-angiogenic factors, in order to

determine whether they had a predominant role in MBEC migration. When MBEC were incubated with hDPSC in combination with neutralizing antibodies against VEGF, a significant decrease in migration was observed. In contrast, the neutralizing antibodies against IL-8 only caused a trend towards decreased migration, indicating a more predominant role of VEGF in the promotion of MBEC migration. However, the neutralizing antibodies against VEGF did not abolish migration completely, suggesting the participation of other migration-promoting factors such as bFGF, MCP-1, HGF and CSF, some of which are known to be secreted by hDPSC [39, 41]. In contrast to the clear effect of neutralizing antibodies when added to hDPSC, there was no effect on MBEC migration when neutralizing antibodies were added to the hDPSC conditioned medium. This discrepancy could potentially be explained by the fact that hDPSC themselves generate a more concentrated version of 'conditioned' medium or that VEGF and IL-8 secreted in the hDPSC conditioned medium are potentially below the concentration required to observe effects and thus be inhibited. Furthermore, the lack of an inhibitory effect of the neutralizing antibodies and the comparable levels of migration between the hDPSC conditioned medium and the 'inhibitory' condition suggest the participation and potential predominant role of other migration-promoting factors in the hDPSC conditioned medium. In comparison, the same effects were observed when adding neutralizing antibodies against VEGF to BM-MSC conditioned medium, also suggesting a too low concentration of VEGF. However, no comparison was made between BM-MSC and BM-MSC conditioned medium [72].

Another important aspect of endothelial cell migration is directional migration, which plays an important role during wound healing *in vivo*. In a wound healing assay, a confluent monolayer of endothelial cells is "wounded" and the endothelial cells are migrating back in order to reform the monolayer. Therefore, this assay is considered to represent one aspect of wound healing *in vivo* [71]. In order to test the potential impact of hDPSC on the directional migration of MBEC, a wound healing assay was performed with hDPSC conditioned medium. The results of this assay indicated that, after 24h or 48h of incubation, hDPSC conditioned medium significantly increased the directional migration of MBEC compared to the control situation. As observed in the transwell migration assay, these results are consistent with the performed ELISA analysis, which indicated the presence of endothelial migration-promoting factors in the hDPSC conditioned medium, such as VEGF, IL-8 and MCP-1. The effects with regard to the effect of hDPSC on the directional migration of endothelial cells have not been mentioned elsewhere. However, a recent study has indicated that DPSC from human deciduous teeth could enhance wound healing of a skin defect *in vivo*, although it was

not mentioned whether this was due to promotion of endothelial cell migration [75]. In comparison, recent studies have indicated that BM-MSC enhanced wound healing *in vivo*, through promotion of re-epithelialization, cell infiltration and angiogenesis [64, 69]. Comparable with the transwell migration assay, MBEC were incubated with recombinant human VEGF and IL-8 in order to test the impact of these factors on directional migration. In contrast to the transwell migration assay, migration analysis indicated that recombinant VEGF significantly increased the directional migration of MBEC, while recombinant IL-8 did not affect directional migration. The amount of migration was comparable between the control condition and the IL-8 condition. A potential explanation for the discrepancy between the results of the transwell migration assay and the wound healing assay with regard to recombinant human IL-8 could be that IL-8 does not play a role in directional migration, while other factors such as VEGF do. Moreover, it should be noted that, although the MBEC in both migration assays are incubated with the same conditions, the addition of Mitomycin C in the wound healing assay is a differing factor between both assays. Mitomycin C is a fungal toxin which inhibits DNA synthesis and is normally used to rule out the effect of cell proliferation in migration assays. However, since Mitomycin C affects DNA synthesis, genes that play a role in the molecular regulation of migration could also be corrupted. Furthermore, literature indicated that Mitomycin C can inhibit endothelial monolayer regeneration by mechanisms independent of cell proliferation and DNA synthesis [76]. These data suggest that Mitomycin C indeed accounts for the discrepancy between both migration assays, although the toxin does not seem to affect the migration-promoting effect of VEGF. Another observation which was unexpected and could not be explained was the increase in MBEC migration after addition of a neutralizing antibody against recombinant IL-8.

Despite the absence of a clear effect of recombinant IL-8 on directional migration, neutralizing antibodies against this factor were still added to the hDPSC conditioned medium since there was an effect of IL-8 in the transwell migration assay. Besides the addition of anti-IL-8 antibodies, the wound healing assay was also performed with neutralizing antibodies against VEGF in order to determine a potential predominant role of one of these factors. Migration analysis indicated that the neutralizing antibodies against VEGF and IL-8 caused no significant decrease of MBEC migration. Moreover, the amount of migration was comparable in all three conditions. This lack of an inhibitory effect suggests that VEGF as well as IL-8 do not play a predominant role in the regulation of directional MBEC migration, which could explain the comparable levels of migration between hDPSC conditioned medium with or without neutralizing antibodies against VEGF and IL-8. However, the levels of

migration were comparable between all three conditions, which could only be explained by the discrete angiogenic balance of pro-angiogenic and anti-angiogenic factors in the hDPSC conditioned medium that is potentially tipped towards an inhibitory state, not allowing more migration than the amount observed at baseline level in the control condition. Since previous analysis of 8 assays indicated that hDPSC conditioned medium significantly increased directional MBEC migration compared to the control situation, while the levels of migration are comparable after conducting 4 assays, future repetition of the experimental protocol could also increase the overall effect of hDPSC conditioned medium.

Besides proliferation and migration, the overall survival rate of endothelial cells also plays an important role during the regulation of angiogenesis. According to literature, several angiogenic factors have an impact on endothelial cell survival [22]. Some of these factors are also present in the hDPSC conditioned medium, namely VEGF, IGFBP-3, TSP and endostatin. In order to determine the potential impact of hDPSC on survival of MBEC, a hypoxia assay was performed. After 24h of incubation with hDPSC conditioned medium under normoxic and hypoxic conditions, there were no significant differences in the amount of living, necrotic, early apoptotic and late apoptotic cells, compared to the control situation. Furthermore, there were no differences between normoxia and hypoxia with regard to the amount of living, necrotic and apoptotic cells. These data suggest that a 24h incubation period under hypoxic conditions was insufficient to induce apoptosis. However, after 48h of incubation under hypoxia, the hDPSC conditioned medium showed a trend towards a decreased amount of living cells and an increased amount of necrotic and late apoptotic cells. A potential explanation could be that after 48h hypoxia MBEC start to secrete certain proteins that, in combination with the proteins present in the hDPSC conditioned medium, lead to apoptosis. In contrast, at study of Iohara *et al.* demonstrated a significant decrease in HUVEC apoptosis after incubating the cells with conditioned medium of porcine pulp-derived $CD31^-$ $CD146^-$ stem cells [57]. However, besides considering the abovementioned species-related and tissue-related differences, one also has to keep in mind that the authors applied a significantly different experimental protocol to induce apoptosis, which could lead to different results. According to literature, hypoxia enhances the angiogenic potential of hDPSC by increasing the expression of hypoxia-inducible transcription factor - 1α (HIF-1α) and consequently VEGF [37]. Since VEGF plays an important role in promoting the survival of endothelial cells, this suggests that incubating MBEC with hDPSC under hypoxic conditions would lead to a significantly increased VEGF secretion by hDPSC and a subsequent significant increase in MBEC survival compared to control situation and the normoxia

condition. After 24h of incubation with hDPSC under normoxic conditions, a trend towards an increased amount of living cells compared to the control situation was observed. Since the control condition consists of serum-free (0,1% FBS) medium and contains significantly less growth factors than the amount secreted by hDPSC, this observation could be expected. However, this effect was not maintained when MBEC were incubated under hypoxia. The percentage of living, necrotic and apoptotic cells were comparable between the control condition and hDPSC. Furthermore, the amount of living cells of the hDPSC condition under normoxia was comparable with the amount observed in the control situation and the hDPSC under hypoxia. Again, this could indicate that a 24h incubation period under hypoxic conditions is insufficient to induce apoptosis. After 48h of incubation under hypoxia, the amount of living cells decreased in comparison to the normoxia condition but there was no difference between the hDPSC and the control situation. Compared to the control situation and the normoxia condition, hDPSC showed a trend towards an increased amount of necrotic cells which could partly account for the observed decrease in the amount of living cells. Furthermore, the percentage of late apoptotic cells also increased compared to the normoxia condition, although the hDPSC showed a trend towards a protective effect compared to the control situation. The discrepancy between the latter and hDPSC conditioned medium could be explained by the increase in angiogenic potential due to hypoxia. The fact that hDPSC probably produce a more concentrate 'conditioned' medium could also play a role but then there would also have been a clear difference between the control situation and hDPSC under normoxic conditions. In comparison, BM-MSC conditioned medium of hypoxic BM-MSC significantly decreased hypoxia-induced apoptosis of human aortic endothelial cells (HAEC) [77]. In contrast, a study of Otsu *et al.* showed a concentration-dependent inhibition of angiogenesis by rat BM-MSC, partly due to induction of endothelial apoptosis [36]. With regard to the angiogenic secretion profile and paracrine effects of hDPSC, it can be concluded that these stem cells express and secrete a whole array of pro-angiogenic and anti-angiogenic factors. hDPSC have a significant effect on MBEC migration, but not on proliferation or cell survival. In the future, it would be useful to conduct the aforementioned assays with a human endothelial cell line, in order to rule out potential species-related differences. Furthermore, the predominant regulating factors need to be determined with neutralizing antibodies in order to potentially manipulate the angiogenic effect of hDPSC in the future.

As already mentioned, hDPSC do not only display paracrine angiogenic effects; recent studies have indicated that these stem cells are also capable of *in vitro* endothelial differentiation [42, 56]. In order to assess the endothelial differentiation potential of explant hDPSC, the stem

cells were incubated with different induction media adopted from literature [4, 33, 42, 45, 46]. After 10 and 21 days of incubation, endothelial marker expression was assessed by means of flow cytometry. If the endothelial differentiation succeeded, a clear increase in endothelial marker expression together with a clear decrease in stem cell marker expression was expected. However, flow cytometric analysis indicated that there was no significant increase in the expression levels of CD31, CD34 and VEGFR2 after 10 or even 21 days of incubation. Furthermore, despite the observed decrease in the general expression level of stem cell marker CD44 after 21 days of incubation, there were no significant differences between the control situation and the different induction media. The fact that the level of CD44 expression also decreased in the control situation indicates that the culture conditions, i.e. cellular confluency and medium, have an influence on the stem cell properties of the cells. Therefore, culture conditions have to be carefully monitored and possibly be adjusted during future experiments. In contrast, Marchionni *et al*. indicated an increase in the expression of endothelial markers CD54, VEGFR1, VEGFR2 and von Willebrand factor after incubating hDPSC for 7 days with induction medium [42]. In an attempt to reproduce these results, flow cytometry was performed after incubating enzymatically digested hDPSC for 7 days with the described induction medium. Preliminary analysis indicated that there were no significant differences between the control situation and the induction medium with regard to expression of endothelial markers such as CD31, CD34 and CD54. Furthermore, while the expression levels of CD31 and CD34 were below 1%, 65-70 % of control as well as 'differentiated' cells were positive for CD54 expression. This discrepancy with the published results could potentially be explained by the low serum-containing (2% FBS) control medium that was used compared to the 10% FBS- containing medium that was used in the article. These data suggest that hDPSC could not differentiate into endothelial cells under the given circumstances. In comparison, 3 out of 5 protocols applied in this study were already applied on MSC of different origin and all lead to endothelial differentiation [4, 33, 45]. It should be noted that a very recent study of Karbanova *et al.* mentioned an upregulation of CD31, CD34, CD105 and von Willebrand factor after incubating hDPSC for 7 days with endothelial induction medium. Furthermore, the authors mentioned a clear effect of cell density and serum content on the endothelial marker expression [56].

Despite the lack of endothelial marker expression, ultrastructural analysis was performed in order to assess potential morphological changes. Normally, hDPSC are characterized by large euchromatic nuclei, with one or more prominent nucleoli. Within the cytoplasm, an organelle-rich perinuclear zone and an electron-lucent peripheral region without any organelles can be

distinguished. With regard to organelle content, the perinuclear region contains elongated mitochondria, dilated RER cisternae and some Golgi apparatus. Furthermore, the electron-lucent peripheral region contains vesicles, vacuoles and bundles of microfilaments, the latter being most commonly observed along the cell's edges. No extracellular matrix deposition or specialized cell junctions are present [78]. In contrast, ultrastructural analysis of the explant hDPSC in this study showed that, after 10 or 21 days of incubation with control or endothelial induction media, the cells were characterized by large, sometimes invaginated, euchromatic nuclei. Bundles of microfilaments were present in most of the cells, although condensed along the cell's edges. Within the cytoplasm, there were no clear subcellular compartments; the organelles were spread through the whole cytoplasm. With regard to organelle morphology, the shape of the clearly dilated RER varied among cells; cisternae morphology varied from a circular appearance to a more irregular shape. This circular dilated RER, together with the numerous multilamellar vesicles observed in a great subset of control as well as 'differentiated' hDPSC, were also observed by Struys *et al.* although in adipogenic differentiated hDPSC [78]. Furthermore, a small subset of cells displayed specialized cell junctions and secreted collagen fibers. In most studies of hDPSC, secretion of collagen fibers is considered to be a hallmark of odontogenic and osteogenic differentiation [14, 56, 78]. With regard to the enzymatically digested hDPSC, cellular and organelle morphology was similar to the explant hDPSC. However, there was a clear difference between both incubation periods on the one hand, and the control situation and induction medium on the other hand. After 21 days of incubation, most of the 'differentiated' cells and only a minor subset of control hDPSC showed the presence of electron-dense granules, which resemble the lipid droplets observed by Struys *et al.* in adipogenic differentiated umbilical cord-derived mesenchymal stem cells (UC-MSC) [78]. However, these electron-dense granules could also contain glycosaminoglycans, which can only be ascertained after conducting an Alcian Blue staining. Furthermore, one cell showed a structure which could potentially be a basal lamina, although an immunostaining against the different components of the basal lamina, such as laminin, fibronectin and type IV collagen, has to be performed to confirm this observation. Taken together, the ultrastructural analysis of 'endothelial differentiated' hDPSC showed no straightforward results. The cells lacked Weibel-Palade bodies, which are typical for endothelial cells, but displayed specialized cell junctions and a potential basal lamina, which are also present in endothelial cells [79]. Furthermore, the cells showed typical hallmarks of adipogenic, osteogenic, odontogenic and chondrogenic differentiated hDPSC and UC-MSC [14, 56, 78]. In comparison, ultrastructural analysis of endothelial differentiated BM-MSC

showed the presence of Weibel-Palade bodies, pinocytotic vesicles and specialized cell junctions between the cells [80]. With regard to the endothelial differentiation potential of hDPSC, it can be concluded that hDPSC are not able to differentiate into endothelial cells under the given circumstances, since they lacked endothelial marker expression and showed no straightforward morphological changes. In the future it would be useful to optimize the currently used protocols, since most of them are applied on BM-MSC, or adopt other protocols from literature. Furthermore, expression of endothelial markers such as, VEGFR1/2, CD31, CD34 and von Willebrand factor, needs to assessed at protein as well as mRNA level.

At the beginning of the study it was hypothesized that hDPSC would have a positive impact on angiogenesis, either by secreting pro-angiogenic factors, by differentiating into endothelial cells, or by exerting both effects. The results partly cover the hypothesis since hDPSC have a predominant paracrine effect on angiogenesis, in particular on the migration but not the proliferation or survival of endothelial cells.

5. Conclusion and Synthesis

Angiogenesis, i.e. the formation of new capillaries from pre-existing blood vessels, plays an important role in wound healing and tissue engineering, but also in diseases such as stroke and myocardial infarction. Unfortunately, traditional revascularization therapies are no longer an option for an increasing number of patients and the current stem cell therapies have lead to disappointing results in preclinical and clinical trials. Therefore, this study proposes human dental pulp stem cells (hDPSC) as a potential tool for vascular regeneration. Recent studies have indicated that hDPSC might have angiogenic properties. However, a more elaborate angiogenic profiling of these stem cells is required before any therapeutic application is possible.

The present study comprised two different parts, the first one being the assessment of the paracrine angiogenic impact of hDPSC. The angiogenic secretion profile of these stem cells was determined at mRNA and protein level by means of RT-PCR and ELISA. Although there was clear variability between patient samples, hDPSC expressed and/or secreted pro-angiogenic (bFGF, IGFBP-3, IL-8, HGF, MCP-1, uPA and VEGF) as well as anti-angiogenic factors (endostatin, PAI-1, TIMP-1) at mRNA and protein level. In order to assess the biological effects of hDPSC on the behavior of endothelial cells, *in vitro* models of angiogenesis were performed in order to mimic the different steps of the angiogenic process. In contrast to literature, hDPSC had no impact on the proliferation of endothelial cells, as was shown by means of an MTT assay. This lack in effect in comparison to literature can probably be attributed to species-related differences, since MBEC were used in this assay. Another explanation could be that the balance of angiogenic factors within the hDPSC conditioned medium was not favorable for proliferation. This study was the first to show a significant impact of hDPSC on the (directional) migration of endothelial cells. As there was significant effect on MBEC migration after the addition of neutralizing antibodies against VEGF and IL-8, these factors probably do not play a predominant role in the migration process. Although a minor protective trend was observed after 48h, hDPSC had no significant impact on the survival of MBEC, as indicated by means of a hypoxia assay. This could probably be attributed to the concentration of secreted angiogenic factors, which was probably too low to observe a clear effect.

The second part of this study focused on the endothelial differentiation potential of hDPSC. After incubating the cells for 10 or 21 days with different induction media, flow cytometric analysis showed no significant increase in the expression levels of endothelial markers CD31, CD34 and VEGFR2. Despite the general decrease in CD44 expression after 21 days of

incubation, there was no significant difference between the control situation and the applied induction media. Potential morphological changes were assessed at ultrastructural level but showed no straightforward results. Control hDPSC as well as 'differentiated' hDPSC displayed characteristics of odontogenic, adipogenic, osteogenic and chondrogenic differentiation. The cells only displayed minor endothelial-like characteristics, such as pinocytotic vesicles and a potential basal lamina. In contrast to explant hDPSC, most 'differentiated' enzymatically digested hDPSC showed electron-dense granules within their cytoplasm. An explanation for this discrepancy may lie in the different isolation method, which can yield different populations of hDPSC. In contrast to literature, these results suggest that hDPSC are not able to differentiate into endothelial cells under the given circumstances. However, a very recent study showed successful endothelial differentiation of hDPSC after applying a new protocol. Furthermore, since most of the applied protocols were used in studies of MSC, optimization of these protocols is required before a firm conclusion can be drawn. In comparison, BM-MSC also express angiogenic factors at mRNA and protein level and have a clear paracrine impact on endothelial cell migration, while the studies regarding the impact on endothelial proliferation and survival have yielded contrasting results. Furthermore, these stem cells are capable of *in vitro* endothelial differentiation. However, since hDPSC are isolated very easily from extracted third molars without any risk to the patient, have a higher proliferative and immunomodulatory capacity than BM-MSC and retain their multilineage differentiation capacity after cryopreservation, these stem cells display several advantages over BM-MSC with regard to future *in vivo* use and clinical applications.

In conclusion, this study provides new evidence regarding the angiogenic properties of hDPSC. hDPSC secrete a whole array of pro-angiogenic and anti-angiogenic factors and seem to have a predominant paracrine effect on angiogenesis, in particular on the migration of endothelial cells but not on endothelial cell proliferation and survival. Furthermore, protein analysis and electron microscopy results suggest that hDPSC are not able to differentiate into endothelial cells under the given circumstances, although no firm conclusion can be drawn at this moment. With regard to future experiments, it would be useful to conduct the described *in vitro* models with a human endothelial cell line, in order to rule out potential species-related differences. The predominant regulating factors within these assays also need to be determined in order to potentially manipulate the angiogenic effects of hDPSC in the future. Furthermore, optimization of existing differentiation protocols and application of newly described differentiation methods is required in order fully characterize the endothelial differentiation potential of hDPSC.

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Supplemental Information

S1 Supplemental Materials and Methods

S1.1 Immunocytochemistry

Fixation of cells

- Remove the medium and rinse the cells with PBS (0,01 M, pH 7,2).
- Add 4% paraformaldehyde and incubate for 20 minutes at room temperature.
- Remove the fixative and rinse the cells 3 to 4 times with PBS (0,01 M, pH 7,2).

Immunostaining

- Rinse the fixed cells with PBS (0,01 M, pH 7,2).
- When needed, permeabilize the cells with 0,05% Triton in PBS for 30 minutes at 4° C.
- Rinse the cells 3 times with PBS (0,01 M, pH 7,2) for 1 to 2 minutes.
- Block aspecific binding sites with 3% normal goat serum (Dako, Heverlee, Belgium) in PBS for 20 minutes at room temperature.
- Rinse the cells 3 times with PBS (0,01 M, pH 7,2) for 1 to 2 minutes.
- Add 300 µl of the appropriate primary antibody to each well. Dilution and incubation temperature depend on the used antibody. Incubate for 1 hour.
- Rinse the cells 4 times with PBS (0,01 M, pH 7,2) for 2 to 4 minutes.
- Add peroxidase-labeled polymer-conjugated secondary antibody (Dako, Heverlee, Belgium): goat anti-mouse or goat anti-rabbit immunoglobulin. Incubate for 30 minutes at room temperature.
- Rinse the cells 4 times with PBS (0,01 M, pH 7,2) for 2 to 4 minutes.
- Add 200-250 µl substrate-chromogen to each well: 1 ml of substrate + 1 drop of DAB chromogen (Dako, Heverlee, Belgium). Incubate for 1 to 10 minutes (remove when brown staining becomes visible).
- Rinse the cells 3 to 4 times with distilled water.
- Counterstain the cells with hematoxylin for 8 to 10 minutes at room temperature.
- Rinse the cells a couple of times with tap water.
- Rinse the cells with distilled water.
- Mount the glass cover slips with Aquatex (Merck, Brussels, Belgium) on microscope slides (Thermo Scientific, Erembodegem, Belgium)

S2 Supplemental data and results

S2.1 MTT assay

Compared to the negative control situation, hDPSC conditioned medium had no significant effect on the proliferation of MBEC, when seeded at 5 000 cells/cm². The measured absorbance was comparable with the absorbance values of the negative control (Fig. S1).

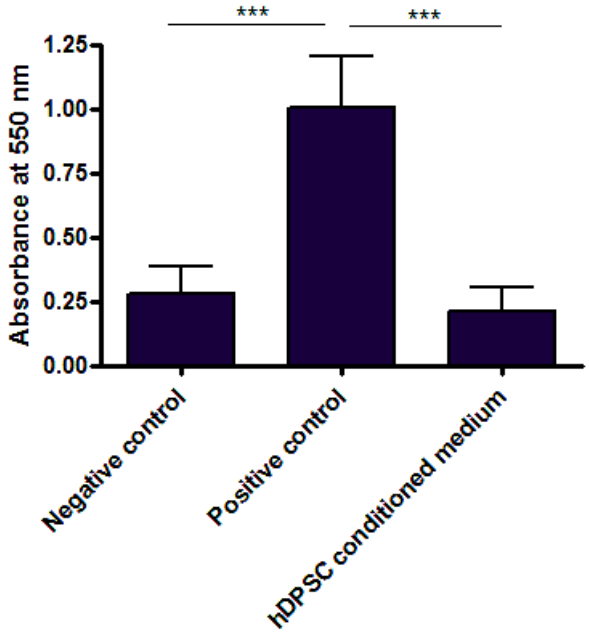


Figure S1: MTT-assay - the potential impact of hDPSC on the proliferation of MBEC. MBEC were seeded at 5 000 cells/cm² and were incubated for 72h with hDPSC conditioned medium, standard hDPSC culture medium (positive control) and standard hDPSC culture medium supplemented with 0,1% FBS instead of 10% FBS (negative control), afterwards an MTT assay was performed. There was no significant effect of hDPSC conditioned medium on the proliferation of MBEC compared to the negative control situation. Data were analyzed with a one way ANOVA followed by a Bonferonni's multiple comparison post-hoc test. Data are represented as mean ± SD and correspond to n = 4 assays. *** = P-value < 0,001.

S2.2 Transwell migration assay: the potential impact of recombinant human VEGF or IL-8

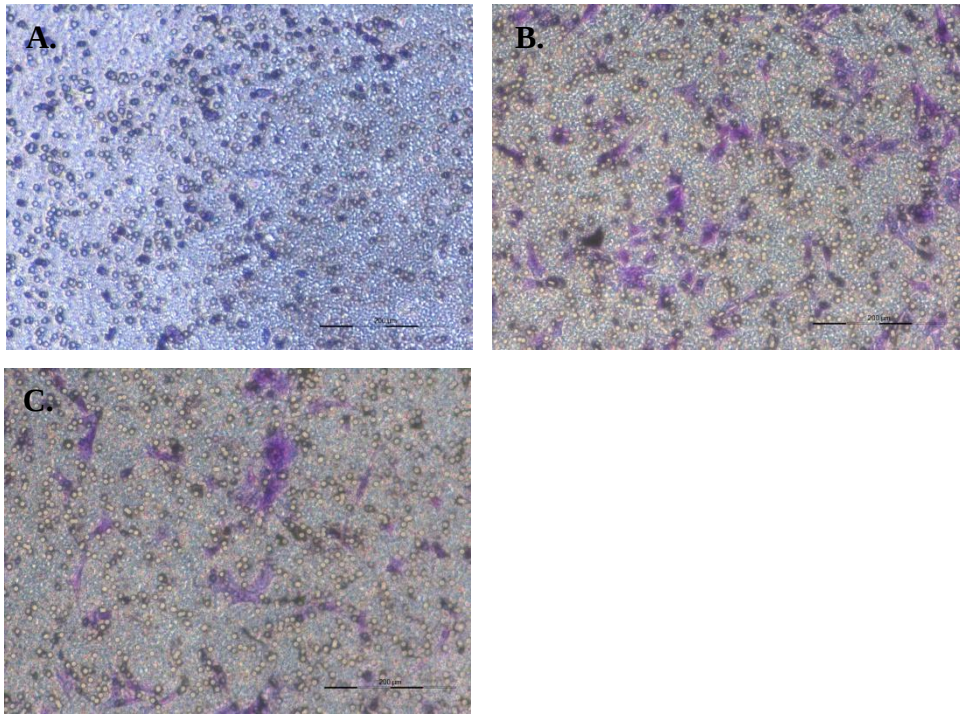
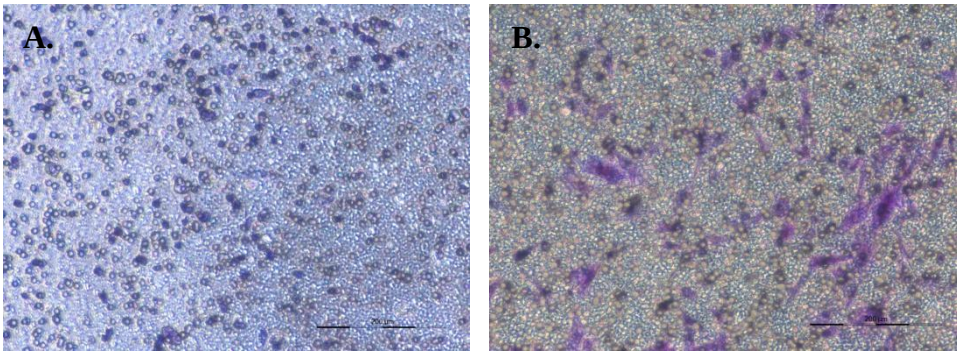


Figure S2: Transwell migration assay - the potential impact of recombinant VEGF on the migration of MBEC. Inserts with MBEC were incubated for 7h with standard hDPSC culture medium supplemented with 0,1% FBS instead of 10% FBS (negative control), negative control medium supplemented with recombinant VEGF or negative control medium supplemented with recombinant VEGF and neutralizing antibodies against VEGF. Pictures were taken with an inverted microscope and analyzed with AxioVision Software. A. Negative control. B. Recombinant VEGF. C. Recombinant VEGF + anti-VEGF. Scale bars = 200 µm.



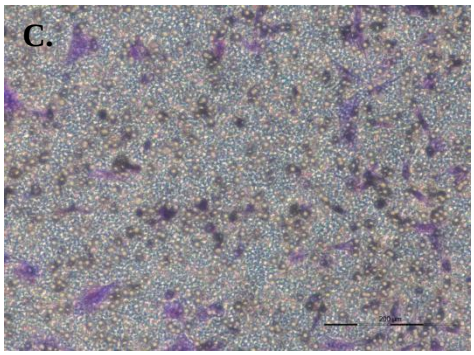
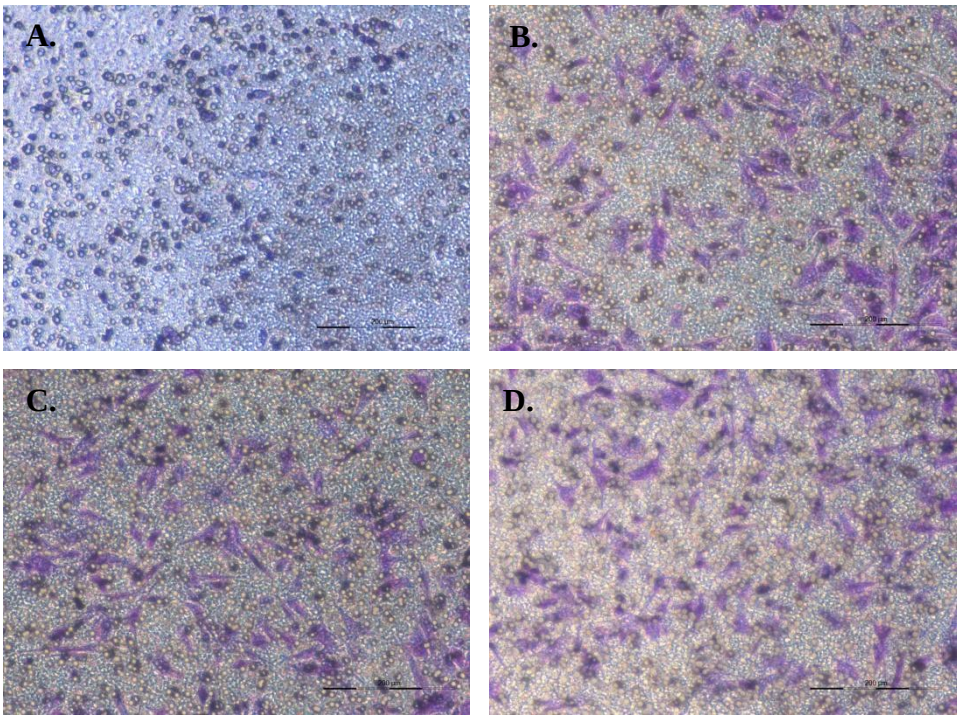


Figure S3: Transwell migration assay - the potential impact of recombinant IL-8 on the migration of MBEC. Inserts with MBEC were incubated for 7h with standard hDPSC culture medium supplemented with 0,1% FBS instead of 10% FBS (negative control), negative control medium supplemented with recombinant IL-8 or negative control medium supplemented with recombinant IL-8 and neutralizing antibodies against IL-8. Pictures were taken with an inverted microscope and analyzed with AxioVision Software. A. Negative control. B. Recombinant IL-8. C. Recombinant IL-8 + anti-IL-8. Scale bars = 200 µm.

S2.3 Transwell migration assay: the potential impact of neutralizing antibodies against VEGF or IL-8



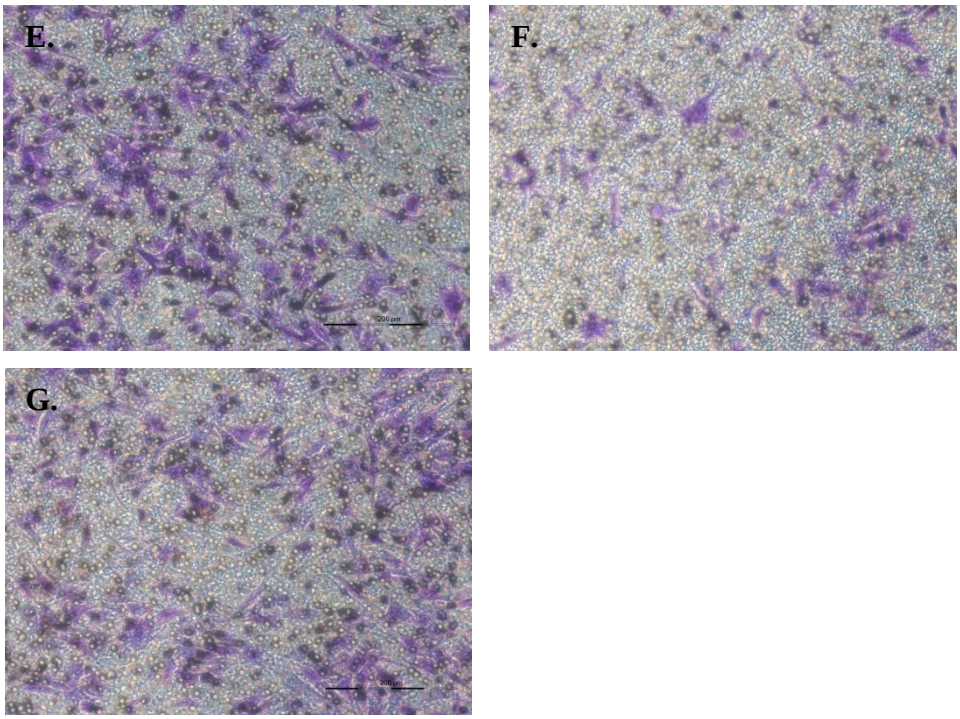
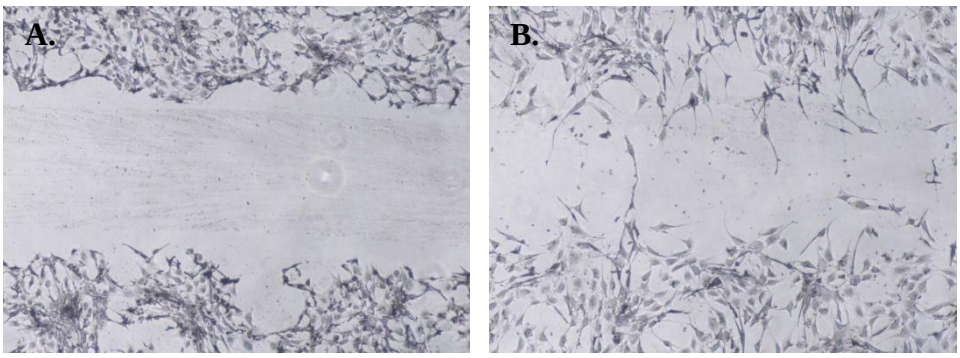


Figure S4: Transwell migration assay - the potential impact of neutralizing antibodies against VEGF or IL-8 on the migration of MBEC. Inserts with MBEC were incubated for 7h with standard hDPSC culture medium supplemented with 0,1% FBS instead of 10% FBS (negative control), hDPSC with or without neutralizing antibodies against VEGF or IL-8 or hDPSC conditioned medium with or without neutralizing antibodies against VEGF or IL-8. A. Negative control B. hDPSC conditioned medium. C. hDPSC conditioned medium + anti-VEGF. D. hDPSC conditioned medium + anti-IL-8. E. hDPSC. F. hDPSC + anti-VEGF. G. hDPSC + anti-IL-8. Scale bars = 200 µm.

S2.4 Wound healing assay: the potential impact of hDPSC after 48h of incubation

After 48h of incubation, hDPSC conditioned medium significantly increased the migration of MBEC compared to the negative control situation (Fig. S5).



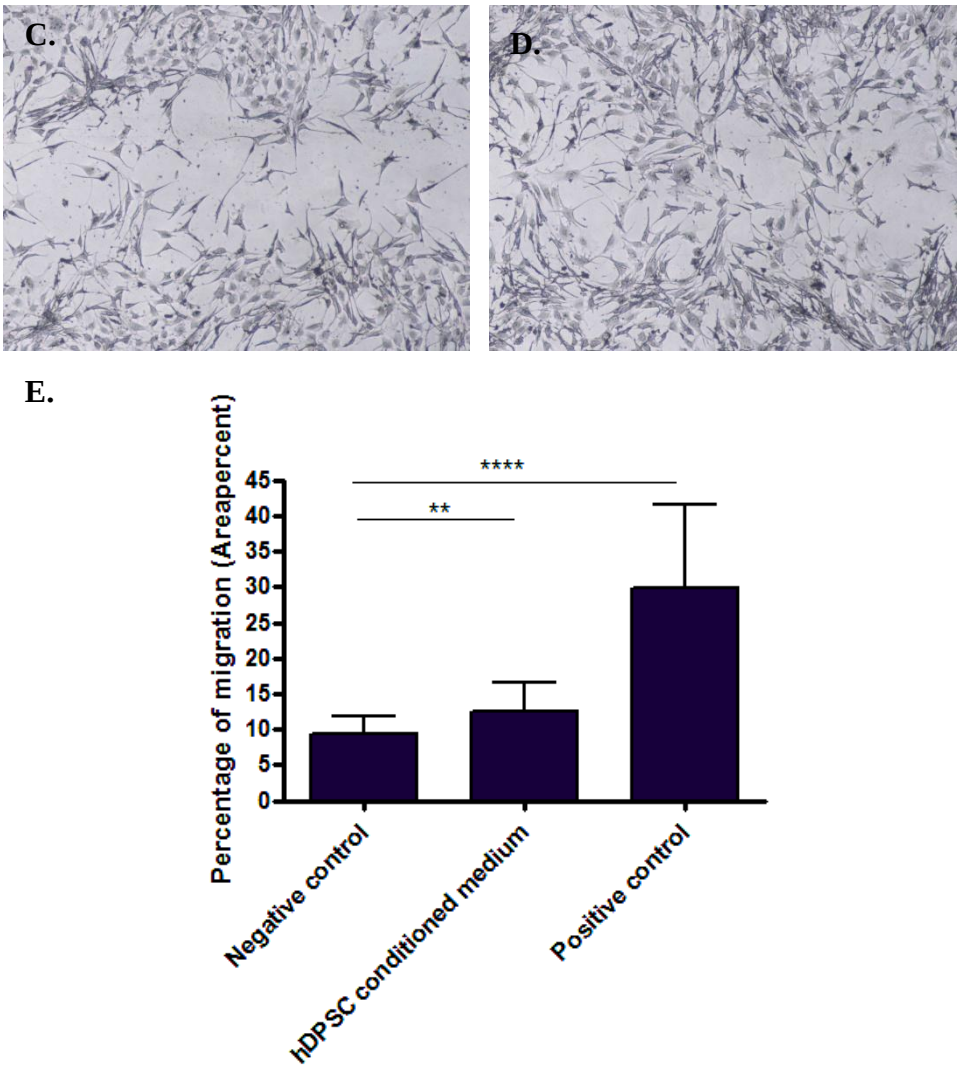


Figure S5: Figure 10: Wound healing assay - the potential impact of hDPSC on the directional migration of MBEC after 48h of incubation. A scratch within a monolayer of MBEC was incubated with hDPSC conditioned medium, standard hDPSC culture medium (positive control) or standard hDPSC culture medium supplemented with 0,1% FBS instead of 10% FBS (negative control). Pictures were taken with an inverted microscope and analyzed with AxioVision Software. hDPSC conditioned medium had a significant effect on the migration of MBEC as compared to the negative control situation. A. Before. B. Negative control. C. hDPSC conditioned medium. D. Positive control. E. Percentage of directional migration (Areapercent) in different conditions. Data were analyzed with a unpaired t-test. Data are represented as mean ± SD and correspond to n = 5 assays. * = P-value < 0,05. **** = P-value < 0,0001. All pictures were taken at 40x magnification.

S2.5 Wound healing assay: the potential impact of recombinant human VEGF or IL-8

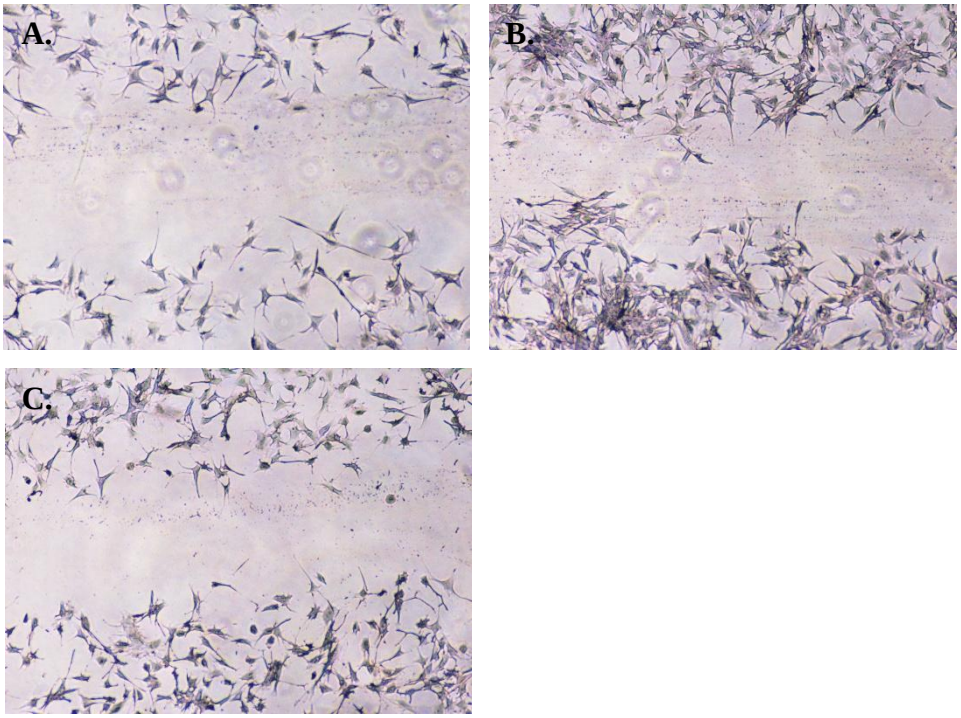
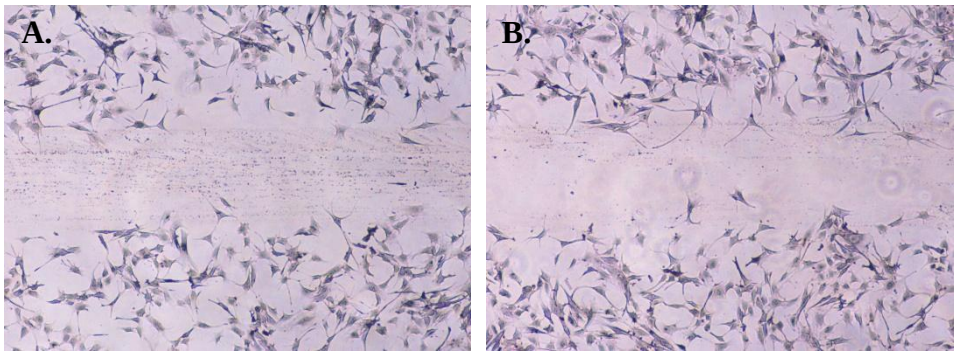


Figure S6: Wound healing assay - the potential impact of recombinant VEGF on the directional migration of MBEC. A scratch within a monolayer of MBEC was incubated for 24h with standard hDPSC culture medium supplemented with 0,1% FBS instead of 10% FBS (negative control), negative control medium supplemented with recombinant VEGF or negative control medium supplemented with recombinant VEGF and neutralizing antibodies against VEGF. Pictures were taken with an inverted microscope and analyzed with AxioVision Software. A. Negative control. B. Recombinant VEGF. C. Recombinant VEGF + anti-VEGF. All pictures were taken at 40x magnification.



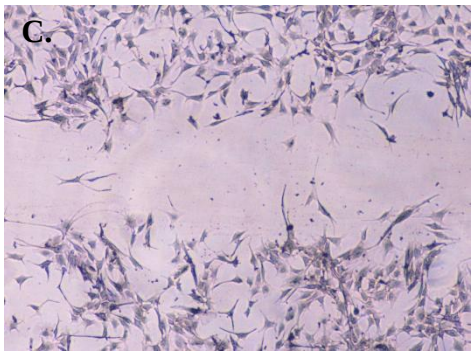


Figure S7: Wound healing assay - the potential impact of recombinant IL-8 on the directional migration of MBEC. A scratch within a monolayer of MBEC was incubated for 24h with standard hDPSC culture medium supplemented with 0,1% FBS instead of 10% FBS (negative control), negative control medium supplemented with recombinant IL-8 or negative control medium supplemented with recombinant IL-8 and neutralizing antibodies against IL-8. Pictures were taken with an inverted microscope and analyzed with AxioVision Software. A. Negative control. B. Recombinant IL-8. C. Recombinant IL-8 + anti-IL-8. All pictures were taken at 40x magnification.

S2.6 Wound healing assay: the potential impact of neutralizing antibodies against VEGF or IL-8

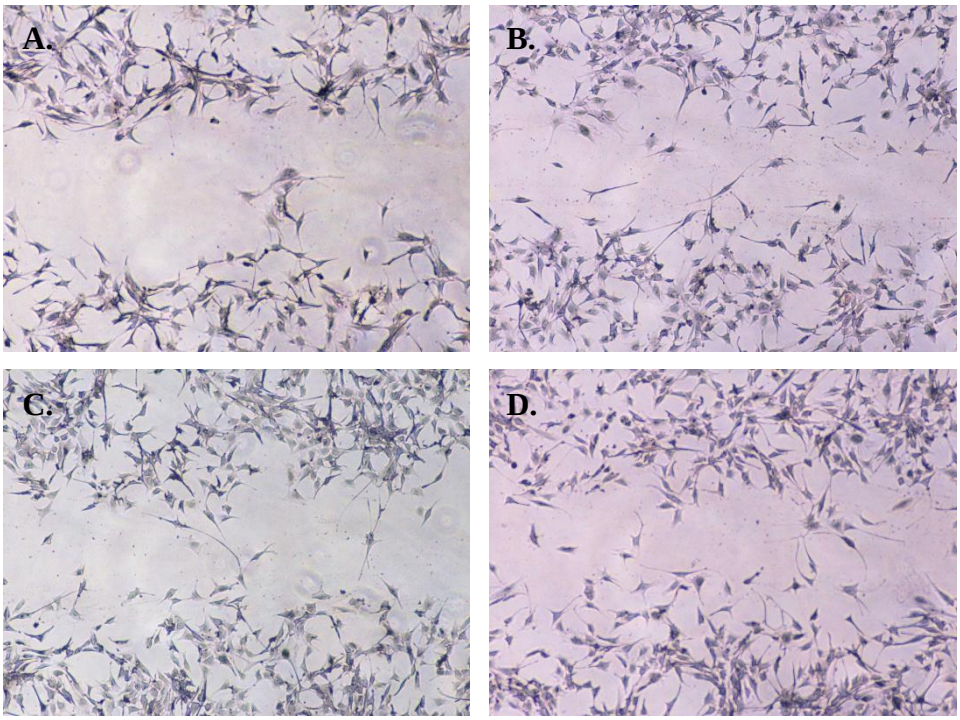


Figure S8: Wound healing assay - the potential impact of neutralizing antibodies against VEGF or IL-8 on the directional migration of MBEC. A scratch within a monolayer of MBEC was incubated for 24h with standard hDPSC culture medium supplemented with 0,1% FBS instead of 10% FBS (negative control) or hDPSC conditioned medium with or without neutralizing antibodies against VEGF or IL-8. A. Negative control B. hDPSC conditioned medium. C. hDPSC conditioned medium + anti-VEGF. D. hDPSC conditioned medium + anti-IL-8. All pictures were taken at 40x magnification.

S2.7 The endothelial differentiation potential of enzymatically digested hDPSC

In order to reproduce the results of Marchionni *et al.*, enzymatically digested hDPSC were incubated for 7 days with control medium or endothelial induction medium [42]. Preliminary data suggest that, after 7 days of incubation, there is no difference between the control situation and induction medium regarding the expression of endothelial markers CD31, CD34, CD54 and stem cell marker CD44 (Fig. S9).

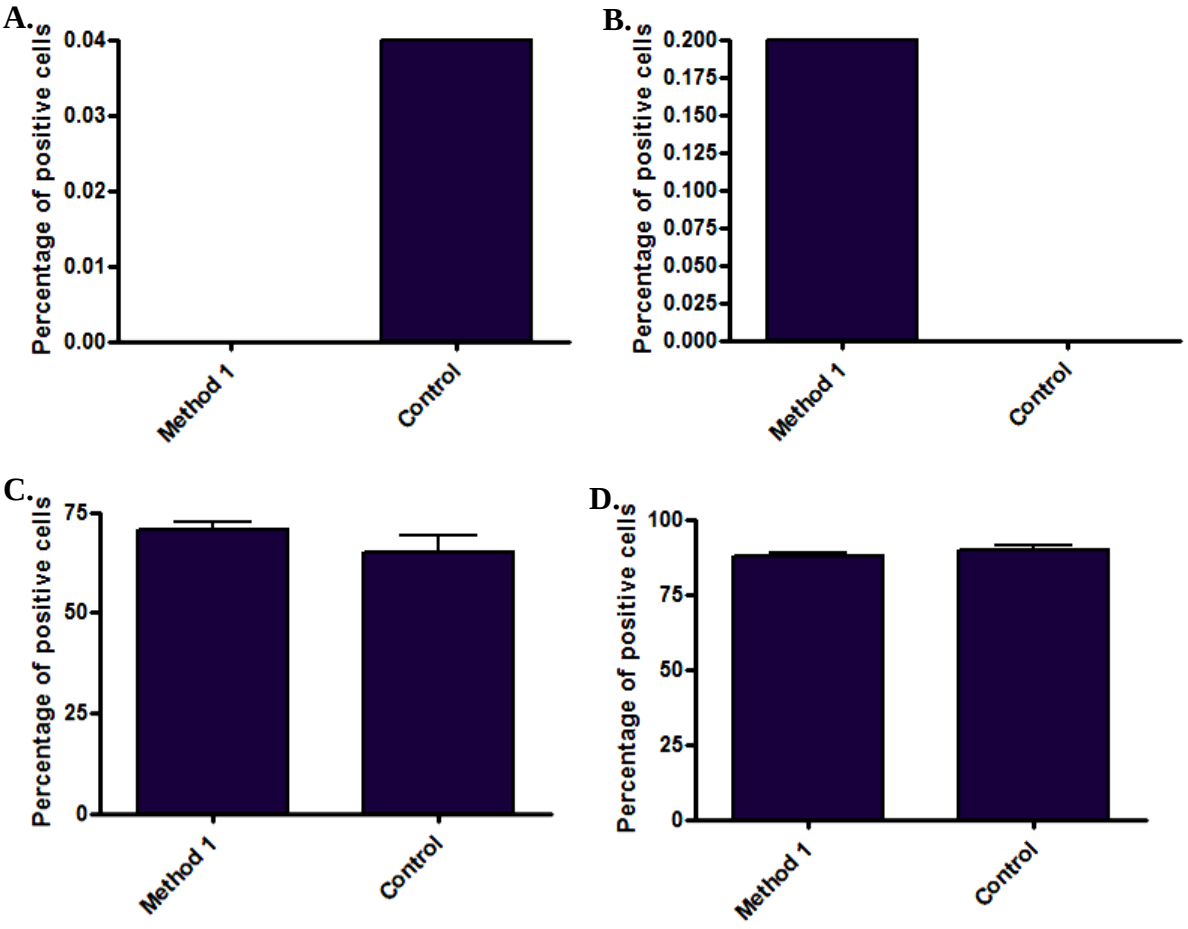
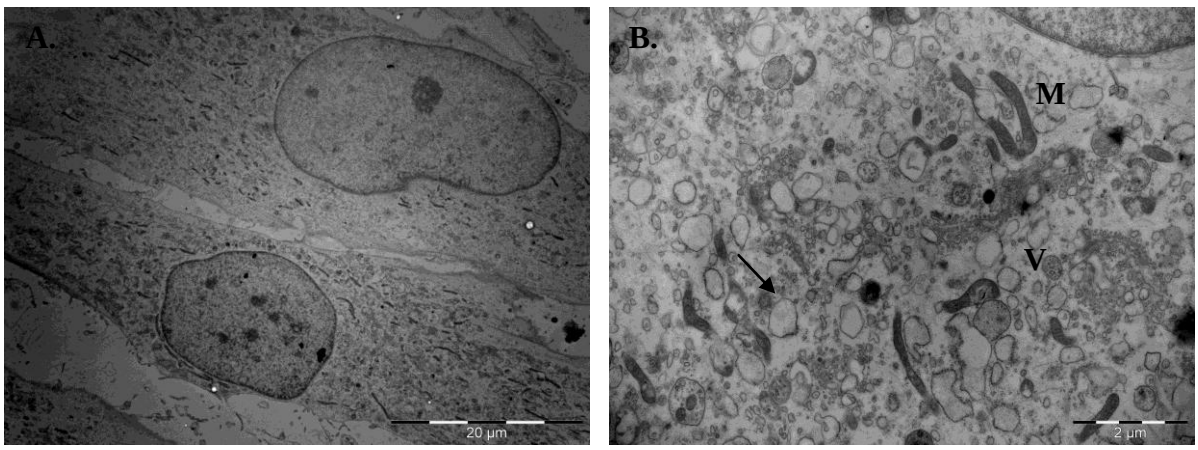
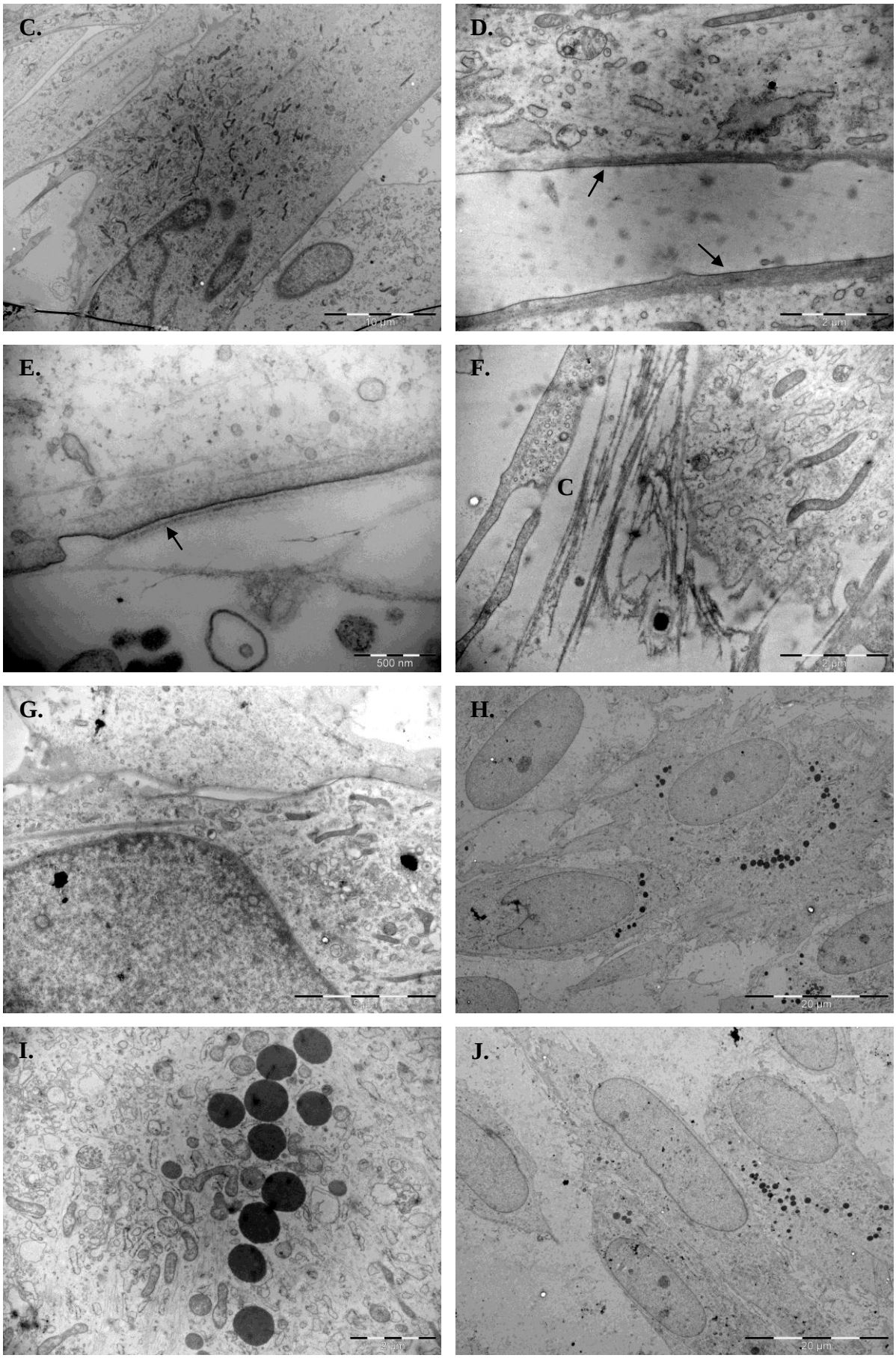


Figure S9: The endothelial differentiation potential of hDPSC at protein level after 7 days of incubation. hDPSC were incubated with control medium and induction medium and after 7 days, the expression levels of CD31, CD34, CD54 and CD44 were determined by means of flow cytometry. There were no significant differences in marker expression between control and induction media. A. CD31. B. CD34. C. CD54. D. CD44. Data could not be statistically analyzed since the protocol was only conducted once with hDPSC of two different patients. Data are represented as mean ± SD and correspond to n = 1 assay.

Comparable with the explant hDPSC, potential morphological changes of the enzymatically digested hDPSC were assessed by means of electron microscopy. With regard to cellular morphology and organelle content there were no clear differences between the different incubation periods on the one hand and the control situation and induction medium on the other hand. The cells were large and very active; they were very rich in organelle content (Fig S10A, B). In terms of organelle morphology, all cells seemed to have a dilated RER, which varied from a more circular shape to an irregular appearance (Fig. S10B, arrow). The mitochondria were characterized by a classical elongated shape (Fig. S10B, M). The cells also appeared to be containing a lot of vesicles (Fig. S10B, V) Regardless of the used medium, certain cells also displayed invaginated nuclei (Fig. S10C). Furthermore, in a great subset of cells actin fibers were visibly present, though mostly condensed in the periphery of the cells (Fig. S10D, arrows). One cell showed a structure which could potentially be a basal lamina (Fig. S10E, arrow). Secretion of collagen fibers by certain cells was also observed, although only in the inducing condition (Fig. S10F, C). There also appeared to be clear contact between the cells in certain cases (Fig. S10G). A clear difference between the control situation and the different induction media was the presence of electron-dense granules in almost all the cells of the inducing conditions, this was however only the case after 21 day of incubation (Fig. S10 H, I). In the control condition, only a small number of cells displayed electron-dense granules (Fig. S10J).




C.
1 µm
D.
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20 µm

Figure S10: The endothelial differentiation potential of enzymatically digested hDPSC after 10 or 21 days of incubation. hDPSC were incubated with several induction media and after 10 or 21 days, the potential morphological changes at the ultrastructural level were assessed by means of transmission electron microscopy. A. Cellular morphology. Scale bar = 20 µm. B. Cell organelles: dilated rough endoplasmic reticulum (arrow), vesicles (V) and mitochondria with an elongated appearance (M). Scale bar = 2 µm. C. Invaginated nucleus. Scale bar = 10 µl. D. Condensed peripheral actin fibers (arrows). Scale bar = 2 µm. E. Potential basal lamina (arrow). Scale bar = 500 nm. F. Collagen fibers (C). Scale bar = 2 µm. G. Cell-cell contact. Scale bar = 5 µm. H. Induction medium 1, 21 days: electron-dense granules. Scale bar = 20 µm. I. Induction medium 1, 21 days: electron-dense granules. Scale bar = 2 µm. J. Control condition, 21 days: electron-dense granules. Scale bar = 20 µm.

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Richting: **master in de biomedische wetenschappen-klinische moleculaire wetenschappen**
Jaar: **2011**

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