Dissertation

Endothelial differentiation potential of amnion-derived mesenchymal stromal cells

submitted by

Julia König, MSc

for the Academic Degree of

Doctor of Philosophy

(Ph.D.)

at the

Medical University of Graz

Institute of Cell Biology, Histology & Embryology

under the supervision of

Univ. Prof. Dr. Ingrid Lang-Olip

and

Univ. Prof. Dr. Berthold Huppertz

2011

Abstract

Mesenchymal stromal cells derived from the amnion (hAMSC) currently play an important role in stem cell research, as they are multipotent cells which can be isolated using non-invasive methods and are immunologically tolerated *in vivo*. The objective of this study was to evaluate their endothelial differentiation potential with regard to a possible therapeutic use in vascular diseases. hAMSC were isolated from human term placentas and cultured in DMEM (non-induced hAMSC) or endothelial cell medium EGM-2 (induced hAMSC). Induced hAMSC changed their fibroblast-like morphology towards an endothelial-like morphology, and were able to take up acetylated LDL and form endothelial-like networks in the Matrigel assay. However, they did not express the mature endothelial cell markers vWF and VE-cadherin. Gene expression analysis revealed that induced hAMSC significantly downregulated pro-angiogenic genes such as tenascin C, Tie-2, VEGF-A, CD146 and FGF-2, while they significantly upregulated anti-angiogenic genes such as serpinF1, sprouty1 and angioarrestin. Analysis of protein expression confirmed downregulation of FGF-2 and Tie-2 (27±8% and 13±1% of non-induced cells, respectively) and upregulation of the anti-angiogenic protein endostatin (226±4%).

Co-culture with hAMSC promoted formation of vessel-like structures by endothelial cells *in vitro*. In addition, conditioned media collected from hAMSC enhanced viability of endothelial cells and had a stabilizing effect on endothelial network formation as shown by LDH and Matrigel assay, respectively. This suggests a pericyte-like function of hAMSC and we could further demonstrate that hAMSC express the pericyte marker NG2 *in situ* and *in vitro*.

In summary, endothelial induced hAMSC acquired some angiogenic properties but resisted undergoing a complete differentiation into mature endothelial cells by upregulation of anti-angiogenic factors. Nevertheless, they had a survival-enhancing effect on endothelial cells which might be useful in a variety of cell therapy or tissue engineering approaches.

Zusammenfassung

Mesenchymale Stammzellen aus dem Amnion (hAMSC) spielen zurzeit eine wichtige Rolle in der Stammzellforschung, da sie ein hohes Differenzierungspotenzial aufweisen, nicht-invasiv isoliert werden können und beim Empfänger keine Immunreaktion hervorrufen. Das Ziel dieser Arbeit bestand darin herauszufinden, ob hAMSC in Endothelzellen differenzieren können, um so auf eine mögliche klinische Anwendung dieser Zellen im Bereich der Gefäßbiologie zu schließen.

hAMSC wurden von reifen Plazenten isoliert und entweder in DMEM (nicht-induzierte hAMSC) oder in Endothelzellmedium EGM-2 (induzierte hAMSC) kultiviert. Induzierte hAMSC veränderten ihre spindelförmige, fibroblastenähnliche Morphologie und nahmen ein endothelzellähnliches Aussehen an. Sie konnten im Gegensatz zu nicht-induzierten hAMSC acetyliertes LDL aufnehmen und Netzwerke auf Matrigel bilden, die denen von Endothelzellen ähnlich waren. Sie exprimierten jedoch keine Marker für reife Endothelzellen (vWF, VE-Cadherin). Genexpressionsanalysen zeigten, dass induzierte hAMSC pro-angiogene Gene wie Tenascin C, Tie-2, VEGF-A, CD146 und FGF-2 signifikant herrunterregulierten, während sie anti-angiogenge Gene wie SerpinF1, Sprouty1 und Angioarrestin hochregulierten. Diese Ergebnisse konnten auf Proteinebene bestätigt werden: FGF-2 und Tie-2 wurden herunterreguliert (27±8% bzw. 13±1% bezogen auf nicht-induzierte Zellen), das anti-angiogene Protein Endostatin wurde hochreguliert (226±4%).

In Kokultur mit Endothelzellen förderten hAMSC die Bildung von gefäßähnlichen Strukturen. Zusätzlich konnte mittels LDH-Messung bzw. Matrigelversuchen nachgewiesen werden, dass konditioniertes Medium von hAMSC die Vitalität von Endothelzellen erhöht und endotheliale Netzwerke stabilisiert. Dies lässt auf eine perizytenähnliche Funktion von hAMSC schließen, worauf auch hinweist, dass hAMSC den Perizytenmarker NG2 *in situ* and *in vitro* exprimieren.

Zusammenfassend konnte gezeigt werden, dass hAMSC durch die endotheliale Differenzierung einige endotheliale Eigenschaften annehmen, einer vollständigen Differenzierung in reife Endothelzellen jedoch standhalten, indem sie anti-angiogene Faktoren hochregulieren. Dennoch wirken sie vitalitätssteigernd und stabilisierend auf Endothelzellen, was in der Zelltherapie oder der Geweberegeneration von Nutzen sein könnte.

Index

1 Introduction

The placenta begins to develop very early during pregnancy, namely upon implantation of the blastocyst into the maternal endometrium, and continues growing until delivery. This suggests that even at term, the placenta might harbor stem cell-like cells with the potential to differentiate towards other cell types. In addition, these cells might have special immunomodulatory properties, as the placenta plays an important role in the maintenance of immune tolerance during pregnancy. Therefore, the placenta has attracted a lot of attention in the field of regenerative medicine where immunologically tolerated cells with stem cell properties are needed for the repair of dysfunctional tissues and an optimal cell source has still not been found.

Stem cells are generally defined by the ability of self-renewal through replication resulting in two identical stem cells, and the ability to differentiate into more specialized cells under appropriate physiological or experimental conditions. Depending on their origin, they are denominated as embryonic, postnatal/adult or induced pluripotent stem cells.

1.1 Embryonic stem cells

Embryonic stem cells have an unlimited self-renewing capacity and a pluripotent differentiation potential towards every cell of the three germ layers (endoderm, mesoderm, ectoderm). In 1998, Thomson et al. first succeeded in establishing a human embryonic stem cell line (Thomson et al., 1998). These cell lines can be used as model systems to investigate cellular differentiation processes and have raised new hope for treatments of degenerative diseases such as Alzheimer's disease, multiple sclerosis, or diabetes (Thomson et al., 1998, Reubinoff et al., 2000). However, a medical application of embryonic stem cells is still limited due to the risk of uncontrollable teratoma formation after *in vivo* transplantation and the danger of immune responses in the recipient (Fong et al., 2010). In addition, as the isolation of the cells from the inner cell mass of the blastocyst requires the destruction of the early embryo, the isolation and use of human embryonic stem cells are ethically controversial.

1.2 Induced pluripotent stem cells

An ethical controversy can be avoided by the use of induced pluripotent stem cells which are adult cells that have been genetically reprogrammed to a pluripotent, embryonic stem cell-like state. They were first generated in 2006 by Yamanaka's group by introducing four transcription factors (Klf4, Sox2, Oct4, and c-Myc) into mouse fibroblasts through retroviral transduction (Takahashi et al., 2006). Only one year later, induced pluripotent stem cells were obtained from adult human dermal (Takahashi et al., 2007) and foreskin fibroblasts (Yu et al., 2007). However, a clinical use of these cells is also not in sight yet due to difficulties such as poor efficiency, teratoma formation, and finding an alternative to viral transfection (Ben-David et al., 2011).

1.3 Adult/postnatal stem cells

Adult or postnatal stem cells are present in various tissues of adults and are thought to be part of the natural tissue repair system. Advantages of postnatal stem cells with regard to a clinical use are that they do not need to be genetically manipulated and that they can be used autologously. They are present in tissues with high regeneration potentials such as skin or intestinal epithelium (Alonso et al., 2003, Chia et al., 2010), but also in tissues with a low cell turnover such as the brain (Gage, 2000). Here they are responsible for the regeneration and supply of new cells as a substitute for damaged cells.

The bone marrow is the so far most extensively investigated source of adult stem cells. It harbors the hematopoietic stem cells (HSC) and the mesenchymal stem or stromal cells (MSC). HSC are responsible for a lifelong production of blood cells, and one single HSC is able to generate all cell types of the blood system. They are currently the only cell type routinely used for the treatment of patients, where they are applied as transplants to treat hematologic and non-hematologic malignancies (Copelan, 2006).

1.3.1 Mesenchymal stem or stromal cells (MSC)

MSC have first been described by Friedenstein et al. as plastic adherent, colony forming unit-fibroblasts (CFU-f) from the bone marrow (Friedenstein et al., 1976). Due to their capacity to differentiate into mesenchymal cells such as osteoblasts, adipocytes and chondroblasts they were later denominated as MSC (Caplan, 1991).

MSC are promising candidates for tissue engineering and cell-based therapies not only because of their multipotent differentiation potential but also due to their low immunogenicity which allows an allogeneic application (Pittenger et al., 1999). A large number of clinical trials show that ex vivo expanded MSC can be safely administered without immune reactions in the recipient (Horwitz et al., 2002, Le Blanc et al., 2008). MSC have been successfully applied to improve the engraftment of HSC after transplantation (Koc et al., 2000), and for the treatment of graft-versus-host disease (Le Blanc et al., 2004) and osteogenesis imperfecta (Horwitz et al., 1999) without being tumorigenic.

The occurrence of MSC is not restricted to bone marrow, and their existence has been demonstrated in other adult tissues such as adipose tissue (Gronthos et al., 2001), skin (Manini et al., 2011) or peripheral blood (Villaron et al., 2004). However, depending on the age and disease stage of the donors, proliferation and differentiation capacities of these cells may be impaired (Stenderup et al., 2003, Stolzing et al., 2006). Therefore, extracorporal fetal or postnatal tissues are an attractive alternative source, and indeed MSC have been isolated from umbilical cord and cord blood, placenta and fetal membranes (amnion, chorion laeve) (Erices et al., 2000, Barlow et al., 2008, Parolini et al., 2008, Hsieh et al., 2010, Kita et al., 2010, Parolini et al., 2010).

1.4 The placenta and the fetal membranes

The placenta

The placenta is a fetal organ responsible for the exchange of nutrients, gas and waste between mother and fetus. In addition, it plays an important role in the fetomaternal tolerance and secretes specific pregnancy-related hormones such as human chorionic gonadotropin. The human term placenta has a discoidal shape with an average diameter of 22 cm, a thickness of 2.5 cm and a weight of about 470 g. It is composed of the chorionic plate and the basal plate enclosing the intervillous space (Figure 1). The

chorionic plate faces the amnionic cavity and is covered by the amnion. The fetal vessels originating from the umbilical cord branch radially into the chorionic plate from where they vertically insert into the villous tree. These tree-like arranged vascularized villi project into the intervillous space where they are in contact with the maternal blood and enable the fetal-maternal exchange. Some villi terminate freely in the intervillous space while others are anchored to the basal plate.

The trophoblastic surface of the villi is composed of an outer continuous layer of multinucleated syncytiotrophoblast which is replenished by the underlying cytotrophoblast. The stroma of the villi contains the fetal vessels, which are embedded between connective tissue cells, macrophages and connective tissue fibers. The basal plate makes up the bottom of the intervillous space and represents the maternal, uterine part of the placenta. It is composed of a mixture of extravillous trophoblast cells, various endometrial stromal cells, decidual cells, uteroplacental vessels, and endometrial glands (Benirschke et al., 2006).

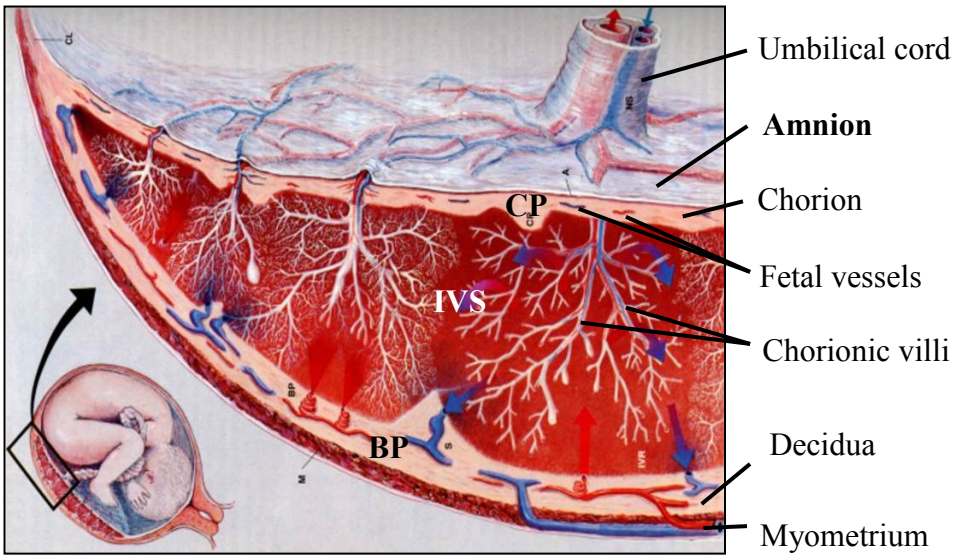

Figure 1: Structure of the mature human placenta. Modified from (Kaufmann et al., 1992). BP: basal plate, CP: chorionic plate. IVS: intervillous space.

The amnionic membrane

The amnionic membrane forms the inner avascular layer of the fetal membranes (Figure 2), which cover the placenta and extend from the edges of the chorionic plate to enclose the fetus and the amnionic fluid in the amnionic cavity. At term, they are about 1000-1200 cm^2 in area, of which 30% cover the chorionic plate and 70% form the

reflected membranes that are in contact with the decidua (Myatt et al., 2010). The amnion is composed of an epithelial cell layer (derived from the fetal ectoderm) which faces the amnionic fluid and forms a continuous single layer on top of a basal membrane. It is separated from the underlying mesenchyme (derived from the extraembryonic mesoderm) by an acellular compact layer. The amnionic mesenchyme consists of a more or less dense network of fibroblasts that are dispersed in a collagen matrix, and a few fetal macrophages (Benirschke et al., 2006). The amnion is only passively attached to the chorionic plate or the chorion laeve (reflected membranes). In contrast to the amnion, the chorionic plate contains fetal vessels (Figure 2B, arrows).
During pregnancy, the fetal membranes presumably have barrier and signaling functions between the maternal and fetal compartments (Myatt et al., 2010), with the amnion providing most of the tensile strength against the risk of premature rupture (Rowe et al., 1997).

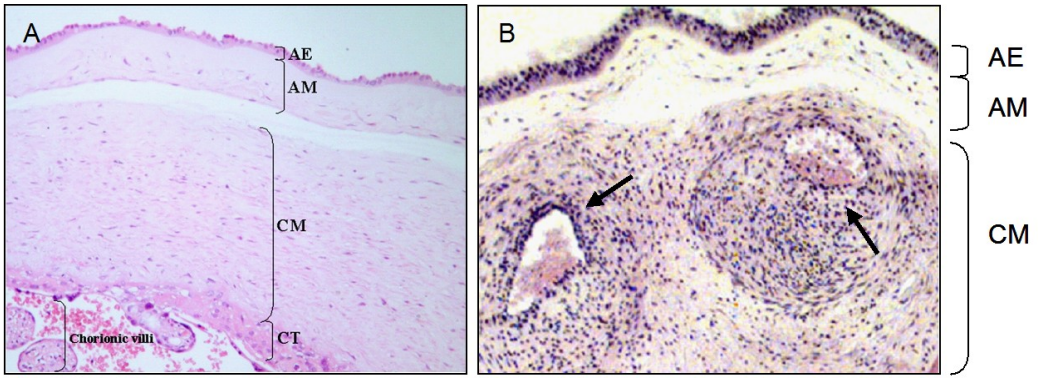

Figure 2: Cross-sectional H&E staining of amnionic membranes. (A) shows the typical structure of the amnion covering the chorionic plate. The amnion epithelium lies on top of the amnion mesenchyme which consists of an upper acellular and a lower layer with dispersed fibroblasts. (Parolini et al., 2008). (B) shows blood vessels (arrows) in the chorionic mesenchyme. AE: amnionic epithelium, AM: amnionic mesenchyme, CM: chorionic mesenchyme, CT: chorionic trophoplast.

1.5 The amnionic membrane as a source of MSC

The amnion is an especially promising source of cells for therapeutic use, as its feasibility in clinical applications has already been confirmed. Its first documented clinical use goes back to 1910, where it was applied as a surgical material in skin transplantation (Davis JW., 1910). Since then, it has been used in a variety of clinical settings such as treatment of chemical burns, skin ulcers, and ophthalmology. Its

beneficial effects are awarded to its anti-inflammatory, immunomodulatory and scar formation-reducing properties, amongst others (Dua et al., 2004).

MSC isolated from the amnionic membrane (hAMSC) have phenotypic and functional similarities to bone marrow-derived MSC. They have been successfully differentiated towards cells of the classical mesodermal (osteogenic, adipogenic, chondrogenic), ectodermal (neurogenic) and endodermal (hepatogenic, pancreatic) lineages (Parolini et al., 2008). Similar to bone marrow derived MSC, they actively suppress T-lymphocyte proliferation (Wolbank et al., 2007, Magatti et al., 2008, Chang et al., 2010) and block differentiation and maturation of monocytes into dendritic cells *in vitro* (Magatti et al., 2009). After xenogeneic transplantation into neonatal swine and rats, hAMSC engraft without immunosuppression (Bailo et al., 2004). Recently it was shown that amnionic membrane application reduces liver fibrosis in a bile duct ligation rat model (Sant'anna et al., 2010) and improves cardiac function of ischemic rat hearts (Cargnoni et al., 2009a). In addition, isolated allo- and xenogeneic hAMSC could reduce bleomycin-induced lung fibrosis in a mouse model (Cargnoni et al., 2009b).

These studies suggest that hAMSC hold great promise for a potential use in cell therapy and tissue engineering. One of the major challenges in this field lies in the vascularization of engineered tissues and in finding a suitable cell population for the endothelialization of vascular grafts. MSC seem to be an interesting choice, however, their endothelial differentiation potential is still controversial. While some groups have reported the differentiation of MSC originating from bone marrow or adipose tissue into endothelial-like cells (Oswald et al., 2004, Liu et al., 2007), opposite results showed that MSC from bone marrow could not be differentiated towards the endothelial lineage (Zhang et al., 2007, Fan et al., 2011). As bone marrow and adipose tissue are highly vascularized, it is difficult to obtain cultures free of primary endothelial cells. It might be possible that the isolated MSC populations already contained some endothelial or endothelial progenitor cells before endothelial induction *in vitro*. Thus, instead of inducing endothelial differentiation of MSC, the chosen angiogenic culture conditions might have led to a selective proliferation of already existing endothelial cells. Here, the amnion shows an additional advantage: Being avascular, it allows an easy isolation of MSC cultures free of endothelial cells.

1.6 Objectives / Aims of the study

Based on the current controversial reports, the objective of this study was to investigate the endothelial differentiation potential of hAMSC after excluding the presence of endothelial cells. In addition to applying phenotypic characterization and functional studies, the effect of angiogenic culture conditions on gene and protein expression of hAMSC was evaluated using microarray analysis and angiogenic protein arrays. Furthermore, direct and paracrine effects of hAMSC on the viability and network formation of endothelial cells were examined.

2 Materials and methods

2.1 Isolation and culture of hAMSC

Human term placentas of normal pregnancies (range 38 to 42 weeks, n=48) were obtained after spontaneous delivery or caesarean section with informed consent. Approval of the Ethical Committee of the Medical University of Graz was granted (No. 21-079 ex 09/10).

Isolation of hAMSC was performed according to the protocol of Soncini et al. (Soncini et al., 2007). The amnion was manually separated from the chorion (Figure 3) and washed with sterile 0.9% saline (Fresenius Kabi, Bad Homburg, Germany) supplemented with 150 IU/ml penicillin, 150 µg/ml streptomycin (both from PAA Laboratories, Pasching, Austria) and 0.4 µg/ml amphotericin B (Gibco, Invitrogen, Paisley, UK).

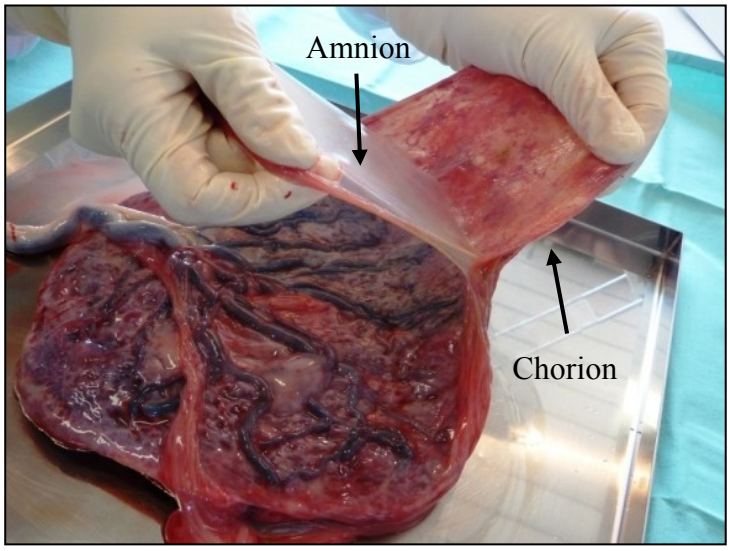

Figure 3: Manual separation of the amnion from the underlying chorion.

The amnion was cut into small pieces and incubated in 2.5 U/ml dispase (BD Biosciences, Bedford, USA) at 37°C for 9 min. Subsequently, the amnion was transferred to DMEM low glucose (Gibco, Invitrogen) supplemented with 15% FBS (FBS Gold, Gibco, Invitrogen), 100 IU/ml penicillin and 100 µg/ml streptomycin for 10 min. Next, the amnion was incubated with 1.0 mg/ml Collagenase A and 0.01 mg/ml DNase (both from Roche, Penzberg, Germany) for 2 h. After centrifugation for 3 min at 150 x g, the supernatant was poured over a 100 µm cell strainer (BD Biosciences) and

centrifuged for 10 min at 300 x g. The cell pellet was washed in PBS (Gibco, Invitrogen) and resuspended in either DMEM supplemented with 15% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin (non-induced hAMSC) or EGM-2 (Lonza, Walkersville, USA) for endothelial induction (induced hAMSC). EGM-2 contains 2% FCS, epidermal growth factor (EGF), hydrocortisone, vascular endothelial growth factor (VEGF), fibroblast-like growth factor-2 (FGF-2), insulin-like growth factor 1 (IGF-1), ascorbic acid and heparin. Aliquots of the cell suspensions were spun down on slides immediately after isolation (cytospins). Remaining cells were grown on culture flasks coated with 1% gelatin (Sigma-Aldrich, St. Louis, MO, USA) and harvested with accutase (PAA Laboratories). Medium was changed every 2 to 3 d.
For hypoxia studies, hAMSC were cultured in 2% oxygen using a hypoxia cell culture system (Xvivo Model G300C, Biospherix, Redfield, NY, USA).

2.2 Isolation and culture of human amnionic epithelial cells (hAEC)

Isolation of hAEC from human term placenta was performed according to Bilic et al. (Bilic et al., 2004). The amnionic membrane was prepared as described in the protocol for isolation of hAMSC. Then, enzymatic digestion was performed with 0.25% trypsin (PAA Laboratories) for 15 min at 37°C and the resulting suspension was centrifuged for 5 min at 150 x g. The supernatant was poured over a 100 µm cell strainer and then again centrifuged for 10 min at 300 x g. The cell pellet was washed in PBS and resuspended in DMEM supplemented with 15% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin. The treatment with trypsin was repeated four times and cell suspensions were either combined or seeded separately in culture flasks after discarding the first supernatant due to a possible contamination with cell debris and blood. hAEC were cultured in DMEM supplemented with 15% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin.

2.3 Isolation and culture of placental endothelial cells (PlEC)

Endothelial cells from normal term human placentas were isolated as published earlier (Lang et al., 2008). Briefly, after removal of the amnion, arterial chorionic blood vessels

at the apical surface of the chorionic plate were resected. Vessels were washed with Hank's balanced salt solution (HBSS, Gibco, UK) to remove residual blood. Endothelial cells were isolated by perfusion of vessels with HBSS containing 0.1 U/ml collagenase, 0.8 U/ml dispase (both from Roche), supplemented with 300 IU/ml penicillin and 300 µg/ml streptomycin, pre-warmed to 37°C. The perfusion time was limited to 7 min to avoid contamination with non-endothelial cells. The cell suspension was centrifuged (200 x g for 5 min), the pellet was resuspended with EGM-MV medium (Lonza) and the cells were plated on culture plates pre-coated with 1% gelatin. The endothelial identity was confirmed by positive staining for the classical endothelial marker von Willebrand factor (vWF, immunoglobulin fraction, rabbit anti-human, 0.7 µg/ml, Dako, Glostrup, Denmark) and absence of markers against fibroblasts (CD90, clone ASO2, 0.1 µg/ml, mouse IgG_1, Dianova, Hamburg, Germany) and smooth muscle cells (smooth muscle actin, clone 1A4, 0.2 µg/ml, mouse IgG_{2a} and desmin, clone D33, 0.4 µg/ml, mouse IgG_1, both from Dako).

2.4 Scanning electron microscopy

hAMSC were grown on gelatin-coated coverslips and fixed in a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 15 min at room temperature. After washing in cacodylate buffer (15 min), the samples were postfixed in 2% OsO4 for 15 min and dehydrated in a graded series of ethanol followed by acetone 100%. After critical point drying the vessels were gold sputtered and viewed under a Zeiss DSM950.

2.5 Flow cytometry analysis

Flow cytometry analyses were carried out at the Flow Cytometry Core Facility at the Center for Medical Research (ZMF) and at the Institute of Cell Biology, Histology and Embryology of the Medical University of Graz. The surface marker expression of hAMSC was analyzed with a FACSLSRII® instrument equipped with 355 nm and 405 nm UV lasers, a 488 nm argon ion laser and a 635 nm red diode laser (Becton Dickinson, Franklin Lakes, NJ, USA). hAMSC were washed and labeled for 30 min at

4°C at concentrations according to individual titration with monoclonal antibodies from Table 1. Appropriate isotype-matched antibodies were used as negative controls (BD). Data from 10,000 viable cells were acquired. List mode files were analyzed with FCS Express Software (BD).

Table 1: Antibodies used for flow cytometry

Antigen	Conjugation	Clone	Company	Dilution
CD14	FITC	MOP9	BD	1:100
CD34	APC	8G12	BD	1:100
CD44	PE	515	BD	1:33
CD45	PE-Cy7	HI30	BD	1:100
CD73	PE	AD2	BD	1:20
CD90	APC	5E10	BD	1:100
CD105	PE	SN6	Caltag*)	1:33
HLA-DR	PerCP	L243 (G46-6)	BD	1:100

*) Caltag Laboratories, Burlingame, CA, USA

2.6 Immunohistochemistry/Immunocytochemistry

Cryosections (5 µm) of human term placental samples were mounted on microslides (Assistent, Karl Hecht AG, Sondheim, Germany). hAMSC were grown on gelatin-coated glass chamber slides (Lab-Tek II, Nalgene Nunc International, Naperville, IL, USA) and washed with PBS before harvest. Cryosections and cells on cytospins and chamber slides were air-dried for at least 4 h and stored frozen. Prior to immunostaining, tissue and cells were fixed in acetone for 4 min. Slides were immunolabeled using the UltraVision LP Detection System (Thermo Scientific, Fremont, CA, USA) according to the manufacturer's instructions. The antibodies (Table 2) were diluted in antibody diluent (Dako) and applied for 30 min at room temperature. IgG controls and normal rabbit immunoglobulin fraction control (Dako) for vWF were used in the same concentrations as the respective antibodies. After three washing steps in PBS, slides were incubated with primary antibody enhancer for 10 min, followed by HRP-polymer for 15 min. The slides were washed again three

times in PBS, and immunolabeling was visualized by a 5 min exposure to 3-amino-9-ethylcarbacole (AEC, all from UltraVision kit, Thermo Scientific). The slides were counterstained with Mayer's hemalum (Merck, Darmstadt, Germany), washed in distilled water and mounted with Kaiser's glycerol gelatin (Merck).

Table 2: Antibodies used for immunohistochemistry

Antigen	Host/Isotype	Clone	Company	Concentration
CD14	mouse IgG_{2a}	UCHM-1	Chemicon	1.0 µg/ml
CD34	mouse IgG_1	QBEnd-10	Dako	3.7 µg/ml
CD44	mouse IgG_1	DF1485	Dako	0.4 µg/ml
CD45	mouse IgG_1	T29/33	Dako	5.0 µg/ml
CD73	mouse IgG_1	AD2	BD Biosciences	1.7 µg/ml
CD90	mouse IgG_1	AS02	Dianova, Germany	0.03 µg/ml
CD105	mouse IgG_1	SN6	AbD Serotec, UK	0.7 µg/ml
CD146	rabbit	EPR3208	Millipore	1:1500 [#])
Cytokeratin-7	mouse IgG_1	OV-TL 12/30	Neomarkers, USA	0.2 µg/ml
HLA-DR	mouse IgG_1	MEM-12	ABR, CO, USA	0.2 µg/ml
NG2	mouse IgG_{2a}	9.2.27	BD Biosciences	1.0 µg/ml
PDGFR-ß	mouse IgG_{2b}	28/CD140b	BD Biosciences	0.3 µg/ml
VE-cadherin	mouse IgG_1	F-8	Santa Cruz, USA	0.3 µg/ml
VEGFR-2	mouse IgG_1	FLT-19	Sigma-Aldrich	10 µg/ml
Vimentin Ab-2	mouse IgG_1	V9	Neomarkers, USA	0.2 µg/ml
vWF	rabbit	polyclonal	Dako	0.7 µg/ml
IgG_1 control	mouse IgG_1	MPOC-21	BD Biosciences	*)
IgG_{2a} control	mouse IgG_{2a}	DAK-GO5	Dako	*)
IgG_{2b} control	mouse IgG_{2b}	DAK-GO9	Dako	*)
Ig control	rabbit	polyclonal	Dako	0.7 µg/ml

[#] no concentration available; *applied in the same concentration as the respective antibodies

2.7 Immunofluorescence

Cryosections, cytospins and chamber slides were prepared as described in the previous paragraph. Primary antibodies and the respective IgG controls (see Table 3) were applied for 30 min at room temperature. After three washing steps in PBS, slides were incubated with fluorescence-labeled secondary antibodies Cy2 (donkey anti rabbit, 1:100) and Cy3 (goat anti mouse, 1:100, both from Jackson ImmunoResearch, PA, USA) for 30 min at room temperature. Slides were counterstained with DAPI (4',6-diamidino-2-phenylindole, 2.5 µg/ml, Invitrogen, Eugene, OR, USA) for 5 min at room temperature. Slides were rinsed in PBS, mounted with ProLong® Gold antifade reagent (Invitrogen) and analyzed by fluorescent microscopy using an Axiophot microscope connected to an AxioCam HRc digital camera (Zeiss, Germany) or a Leica DM6000B microscope connected to an Olympus DP72 digital camera (Olympus, Austria). For the quantification of vimentin and cytokeratin-7 positive cells, 5 randomly selected microscopic fields of each time point (n=3) were analyzed using newCAST software (Visiopharm, Hoersholm, Denmark).

Table 3: Primary antibodies used for immunofluorescence

Antigen	Host/Isotype	Clone	Company	Concentration
Cytokeratin-7	rabbit	KRT7	Acris Antibodies	5.0 µg/ml
Vimentin	mouse IgG_1	Ab-2 V9	Neomarkers	3.7 µg/ml
NG-2	mouse IgG_{2a}	9.2.27	BD Biosciences	1.0 µg/ml
vWF	rabbit	polyclonal	Dako	0.7 µg/ml
IgG_1 control	mouse IgG_1	MPOC-21	BD Biosciences	3.7 µg/ml
IgG_{2a} control	mouse IgG_{2a}	DAK-GO5	Dako	1.0 µg/ml
Ig control	rabbit	polyclonal	Dako	*)

*) applied in the same concentration as the respective antibodies

2.8 Osteogenic and adipogenic differentiation

To induce osteogenic differentiation, cells were cultured on 2-well-chamber slides in DMEM/F12 (Gibco, Invitrogen) supplemented with 0.1 µM dexamethason, 100 µM L-Ascorbic acid 2-phosphate, 10 mM glycerol-2-phosphate, and ITS (ITS+1, insulin-transferrin-sodium selenite liquid media supplement, all from Sigma). Control cells were grown in DMEM/F12 with 10% FBS. After two and three weeks, cells were assessed for alkaline phosphatase activity with a leukocyte alkaline phosphatase kit (Sigma) following the manufacturer's instructions. In addition, calcium deposits were stained for with Alizarin Red S (Sigma) after fixation in 100% ethanol. Alizarin Red was prepared according to the manufacturer's instructions (0.01 g/ml in distilled water containing 2% ethanol).

For adipogenic induction, cells were grown on 2-well-chamber slides in DMEM/F12 supplemented with 10% FBS, 1 µM dexamethason, 200 µM indomethacin, 500 µM 3-isobutyl-1-methylxanthine (IBMX), and 10 µg/ml insulin (from bovine pancreas, all from Sigma). Control cells were cultured in DMEM/F12 with 10% FBS. To visualize lipid droplets, cells were fixed in 60% 2-propanol and stained with Oil Red O (stock: 0.5% Oil Red O in 2-propanol, 3:2 dilution in distilled water) for 10 min.

Sclerotic arteries (ethical approval: no. 19-293 ex 07/08) were used as positive controls for Alizarin Red and Oil Red O stainings.

2.9 Cumulative population doublings of hAMSC

hAMSC were either cultured under standard conditions in DMEM supplemented with 15% FBS on gelatin coated plates or under different endothelial induction conditions, consisting of culture in EGM-2 in the absence or presence of additional 50 ng/ml VEGF ($VEGF_{165}$, ReliaTech, Wolfenbuettel, Germany), and on gelatin or fibronectin (1 µg/cm^2, R&D Systems, Minneapolis, USA) coated plates. Cells were seeded in triplicates with a density of 10^4 cells/well in 6-well-plates and harvested after 5 d. Cells were counted by automatic cell counting (Casy® Model TT, Schärfe Systems, Reutlingen, Germany), reseeded in a density of 10^4 cells/well and again harvested after 4 d. Cumulative population doublings were calculated according to the formula $(\ln N - \ln N_0) / \ln 2$, with N = amount of harvested cells and N_0 = amount of seeded cells.

For the determination of the cumulative population doublings over several passages, cells were harvested at 90% confluence.

2.10 DiI-Ac-LDL-uptake assay

hAMSC were seeded on gelatin-coated glass chamber slides and cultured in DMEM with 15% FBS (non-induced hAMSC) or in EGM-2 (induced hAMSC) for at least 7 d. Subsequently, cells were incubated with 10 µg/ml acetylated low-density lipoprotein labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindo-carbocyanine perchlorate (DiI-Ac-LDL, BTI Biomedical Technologies, Stoughton, MA, USA) for 4 h according to the manufacturer's instructions. Cells were fixed with 4% paraformaldehyde, stained with DAPI and observed with a Leica DM6000B fluorescent microscope (Leica, Wetzlar, Germany) connected to an Olympus DP72 digital camera (Olympus, Tokyo, Japan). Placental endothelial cells served as positive controls.

2.11 Semi-quantitative RT-PCR

hAMSC were isolated from five different placentas and aliquots were cultured either in DMEM + 15% FCS (non-induced hAMSC) or EGM-2 (induced hAMSC) on gelatin-coated culture flasks until cells reached 90% confluence (about 5-8 d). Then cells were reseeded and cultured in the respective media for 7 and 14 d before RNA isolation. Total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA from placental endothelial cells was used as control. The integrity of each RNA sample was determined using an Agilent 2100 Bioanalyzer (Agilent, Foster City, CA, USA) and only RNA samples with integrity values of 9.5-10 were used. Primers (Table 4) for vWF, VE-cadherin and VEGFR-2 were purchased from Invitrogen, primers for the internal control ribosomal protein L30 (RPL30) were purchased from MWG Biotech AG (Ebersberg, Germany). Total RNA (200 ng) was used for the one-step RT-PCR kit (Qiagen, Germantown, USA) which was performed according to the manufacturer's instructions.

Table 4: Primers used for RT-PCR

Gene	Forward primer	Reverse primer
vWF (NM_000552)	TTTGATGGCATCCAGAACAA	AGTCCCCAATGGACTCACAG
VE-cadherin (NM_001795)	CCTACCAGCCCAAAGTGTGT	GACTTGGCATCCCATTGTCT
VEGFR-2 (NM_002253)	TGATCGGAAATGACACTGGA	TGCTTCACAGAAGACCATGC
RPL30 (NM_000989)	CCTAAGGCAGGAAGATGGTG	AGTCTGTTCTGGCATGCTT

The following numbers of cycles were applied in a thermo cycler Gene Amp® PCR System 9700 (Applied Biosystems) with an annealing temperature of 60°C: 32 cycles for vWF, 28 cycles for VE-cadherin, 28 cycles for VEGFR-2, and 24 cycles for L30. Samples were loaded on a 1.5% agarose gel and band intensity was detected by Multimage III (Cell Bioscience, Santa Clara, USA). Gene expression was quantified with RPL30 using AlphaView software (Cell Bioscience).

2.12 Matrigel assay

hAMSC were cultured under standard (non-induced) or angiogenic (induced) conditions for at least 10 d. Then cells were seeded in a 96-well plate pre-coated with 40 µl Matrigel (BD Biosciences) according to the manufacturer's instructions at a density of 10^4 cells/well in 100 µl EGM-2. Cells were observed using a Cell-IQ Analyzer 2004-01 (Chip-Man Technologies, Tampere, Finland). Placental endothelial cells served as positive controls.

2.13 Immunofluorescence staining of Matrigel networks

For immunofluorescence staining of networks, induced hAMSC and placental endothelial cells were seeded in 4-well chamber slides coated with 250 µl Matrigel per well ($7x10^4$ cells/well in 500 µl EGM-2). After 24 h, cells were fixed with DMSO:Methanol (20:80) at 4°C overnight. Then slides were washed with PBS and treated with Triton X 100 (0.5% in PBS, 7 min at 4°C). After a 90 min blocking step with 3% BSA in PBS, cells were washed with Tween 20 (3% BSA in PBS) for 5 min. The primary antibody against vWF (Dako, 2.9 µg/ml) was applied overnight at 4°C.

Cells were washed twice in PBS, once in PBS with 3% BSA and once in PBS with 3% BSA and 0.1% Tween 20, followed by incubation with the secondary antibody (Alexa488, goat anti rabbit, 1:200) for 1 h at 37°C. Slides were again washed in PBS, mounted with ProLong® Gold antifade reagent (Invitrogen) and analyzed by fluorescent microscopy using an Axiophot microscope connected to an AxioCam HRc digital camera (Zeiss, Germany).

2.14 RNA isolation and design of microarray experiments

hAMSC were isolated from three different placentas and aliquots were cultured either in DMEM + 15% FCS (non-induced hAMSC) or EGM-2 (induced hAMSC) on gelatin-coated culture flasks until cells reached 90% confluence (about 5-8 d). Then cells were reseeded and cultured in the respective media for 14 d before RNA isolation. Total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany). The integrity of each RNA sample was determined using an Agilent 2100 Bioanalyzer (Agilent, Foster City, CA, USA) and only RNA samples with integrity values of 9.5-10 were used for hybridization.

2.15 Hybridization and data analysis of microarrays

Total RNA was labeled using the Affymetrix GeneChip® Whole Transcript Sense Target Labeling Assay and hybridized to GeneChip Human 1.0 ST arrays as described by the manufacturer (Affymetrix, Santa Clara, CA, USA). Hybridizations were carried out at the Molecular Biology Core Facility at the Center of Medical Research at the Medical University of Graz. Briefly, 100 ng of total RNA were reverse transcribed to cDNA using random hexamers tagged with a T7 promoter sequence. Double stranded cDNA was subsequently used as a template in an *in vitro* transcription reaction followed by cDNA synthesis, fragmentation and labeling through a terminal deoxynucleotidyl transferase. The hybridization cocktail was incubated overnight at 45°C while rotating in a hybridization oven. After 16 h of hybridization, arrays were washed and stained in an Affymetrix GeneChip fluidics station 450, according to the Affymetrix-recommended protocol. Arrays were scanned on an Affymetrix GeneChip scanner. CEL

files were imported into Partek Genomic Suite v6.4 software (Partek Inc, St Louis, MO) and robust multi-chip average normalized (including background correction, quantile normalization across all arrays, median polished summarization based on log transformed expression values). For statistical analysis a paired-sample *t*-test was performed between the treatment groups. Differentially expressed genes were selected by $p < 0.005$ (heat map, Tables 7+8) or $p < 0.05$ (Venn diagram) and a fold change ≥ 2. Expression of genes above background was determined by a signal intensity level >5 after background correction.

The according data has been deposited in NCBI's Gene Expression Omnibus (GEO) (Edgar et al., 2002) and is accessible through GEO Series accession number GSE28385 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE28385).

2.16 Protein isolation and angiogenic protein array analysis

hAMSC were isolated from five different placentas and either cultured in DMEM with 15% FBS (non-induced hAMSC) or in EGM-2 (induced hAMSC) on gelatin-coated culture flasks until cells reached 90% confluence (about 5-8 d). Then cells were reseeded and cultured in the respective media for 14 d. Before protein isolation, cells were washed twice in PBS and lysed with RIPA-buffer (Sigma-Aldrich, St. Louis, MO, USA) containing 4% complete protease inhibitor cocktail (Roche, Mannheim, Germany) for 5 min. Lysates were clarified by centrifugation at 300 x g for 10 min at 4°C. Supernatant was used immediately or stored at -80°C for further analysis. Total protein concentration was determined by Lowry protein assay. Protein of 5 hAMSC isolations was pooled and a total of 250 µg was applied to the Human Angiogenesis Antibody Array C1000 (RayBiotech, Norcross, GA, USA). This array contains 43 different angiogenic proteins spotted in duplicates onto two membranes. Membranes were processed according to the manufacturer's instructions. Chemiluminescent imaging was performed using the FluorChemQ system. Signal densities were analyzed with AlphaView software version 2.0.1.1 (both from AlphaInnotech, Cell Biosciences, Santa Clara, CA, USA) and ratios of the respective protein and internal standard densities were determined. Protein expressions by induced hAMSC are presented as percentages of the expression of non-induced cells (set to 100%).

2.17 Co-culture of hAMSC and placental endothelial cells

For co-culture assays on Matrigel, endothelial cells were allowed to form networks for 5 h. Non-induced and induced hAMSC were labeled with CellTrackerTM Green CMFDA (1:1000, Invitrogen) for 30 min at 37°C and added to the endothelial networks in different ratios (2:1, 3:1, 4:1 – EC:hAMSC). Alternatively, green-fluorescent labeled hAMSC were applied to the Matrigel together with the endothelial cells from the beginning. Cells were observed using Cell-IQ Analyzer 2004-01 (Chip-Man Technologies).

For co-culture assays on gelatin-coated chamber slides, non-induced or induced hAMSC were co-cultured with endothelial cells in EGM-MV or EGM-2 in the absence or presence of additional 50 ng/ml VEGF for 7 to 14 d. Again, different ratios were applied (1:5, 1:1, 5:1 – EC:hAMSC). Upon harvest, cells were visualized using immunocytochemistry or immunofluoresecence.

2.18 Preparation and application of hAMSC-conditioned media (CdM)

To prepare hAMSC-CdM, confluent non-induced and induced hAMSC were washed with PBS and then incubated with EGM-2 for 48 h. Control medium (EGM-2) was prepared in parallel in culture flasks without cells. Upon harvest, hAMSC-CdM and control medium were centrifuged at 300 x g for 10 min and then stored at -80°C.

For determining the effect of hAMSC-CdM on endothelial cell viability, LDH activity was analyzed in supernatants of placental endothelial cells that were cultured in 96-well plates for 96 h either in CdM or in the respective control medium. LDH activity was measured using a LDH Cytotoxicity Detection Kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. Experiments were performed with non-induced and induced hAMSC from 2 and 4 different isolations, respectively, using triplicates per experiment. Statistical analysis was performed using paired Student's *t*-test. Data were expressed as mean ± SD. p values of <0.001 were considered statistically significant.

To test the effect of hAMSC-CdM on network formation of endothelial cells, placental endothelial cells were resuspended in hAMSC-CdM or in control medium EGM-2 and

cultured on Growth Factor Reduced Matrigel (BD Biosciences) according to the manufacturer's instructions. Cells were observed using Cell-IQ Analyzer 2004-01 (Chip-Man Technologies).

3 Results

3.1 Characterization of hAMSC

3.1.1 Isolation of hAMSC

Treatment of the amnionic membrane with collagenase A/DNase releases hAMSC without disturbing the epithelial layer (Figure 4).

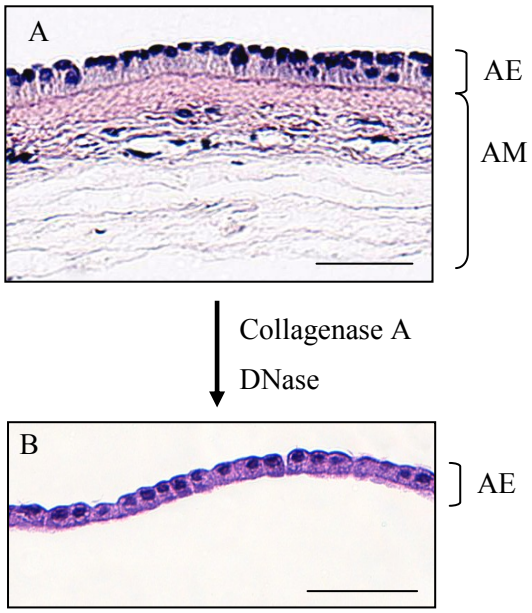

Figure 4: Amnion before (A) and after (B) treatment with collagenase A/DNase. AE: amnionic epithelium, AM: amnionic mesenchyme. Scale bar: 50µm.

3.1.2 Morphology of hAMSC

After a few days in culture, hAMSC show a typical mesenchymal morphology (Figure 5A) which clearly distinguishes them from amnionic epithelial cells (hAEC, Figure 5B). hAEC can be released by digestion with trypsin and grow in cobblestone-like colonies.

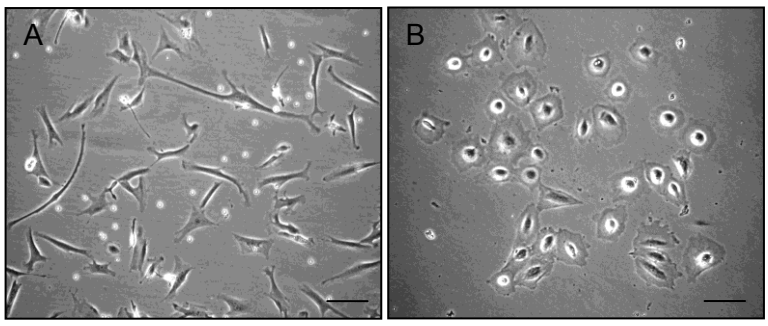

Figure 5: Morphology of hAMSC (A) and hAEC (B) after isolation. Scale bar 100 µm.

Morphological analyses of hAMSC after passage 1 by phase-contrast microscopy (Figure 6A) and scanning electron microscopy (Figure 6B,C) show the mesenchymal phenotype of cultured hAMSC: The cells have a smooth surface with a varying density of microvilli and grow in loose arrangements. They are connected by thin cytoplasmic projections (arrows) and filament-rich pseudopodial-like extensions (arrowheads). The central zone contains the nucleus (n) and most of the organelles. The peripheral margins are characterized by low cytoplasmic density.

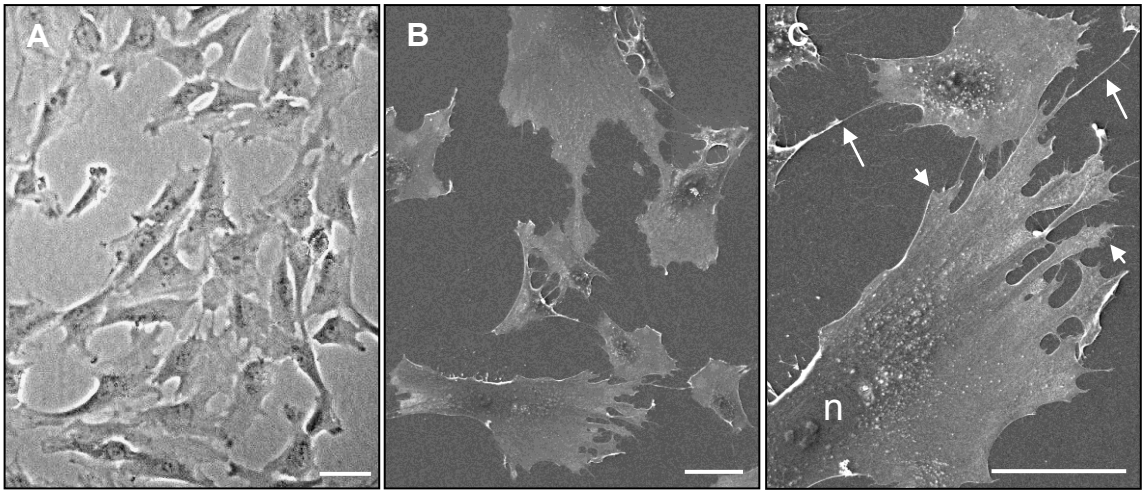

Figure 6: Morphology of isolated non-induced hAMSC. Presented as phase-contrast micrograph (A) and scanning electron micrographs (B,C). Arrows in C show thin cytoplasmic projections, arrowheads point to filament-rich pseudopodial-like extensions. n: nucleus. Scale bar: 50µm.

3.1.3 Expression of vimentin and cytokeratin-7

To confirm the absence of epithelial cells in culture, immunostaining with the mesenchymal marker vimentin and the epithelial marker cytokeratin-7 was performed.

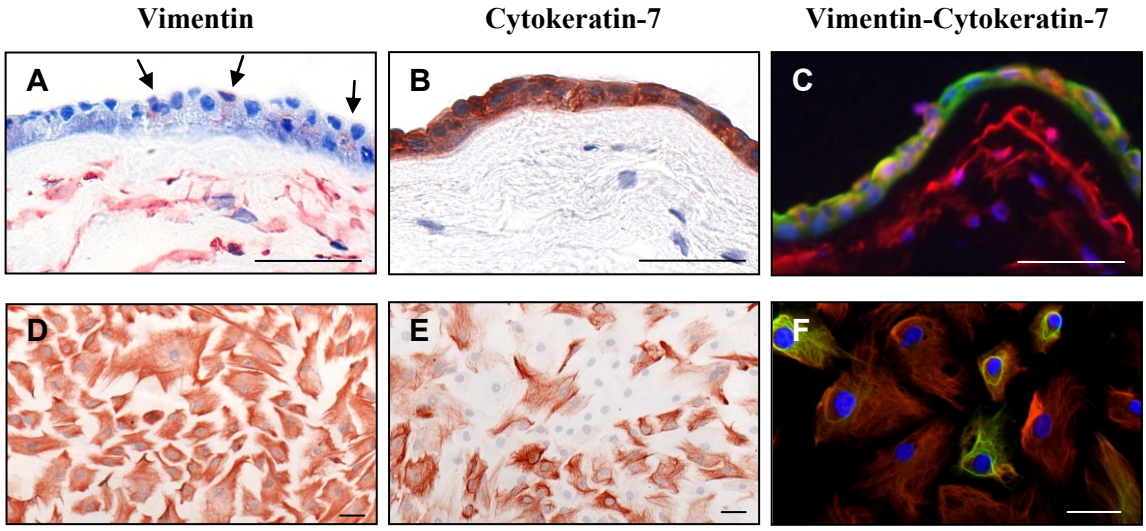

Figure 7: Expression of vimentin and cytokeratin-7. A-C: Amnion *in situ.* Arrows in A point to vimentin-positive epithelial cells. D-F: hAMSC *in vitro* after passage 1. Double fluorescence (C,F) shows expression of vimentin (*red*) and cytokeratin-7 (*green*) at the single cell level. Scale bar: 50µm.

In situ, vimentin not only stains amnionic mesenchymal cells but also some epithelial cells (Figure 7A, arrows), whereas cytokeratin-7 is specifically expressed by the amnionic epithelium (Figure 7B). Figure 7C shows the co-expression of vimentin and cytokeratin-7 on amnionic epithelial cells *in situ*.

After the first passage, hAMSC are concordantly positive for vimentin (Figure 7D) and the number of cells that additionally express cytokeratin-7 strongly increases to about 50% (Figure 7E). The fluorescence staining (Figure 7F) shows the co-expression of vimentin and cytokeratin-7 on the single cell level.

Quantitative analysis (Figure 8) shows that after 1 d in culture, 91.9% (±6.6%) of the isolated cells express only vimentin and not cytokeratin-7, while 1.8% (±1.4%) express only cytokeratin-7, and 5.0% (±4.0%) co-express cytokeratin-7 and vimentin. After 5 d in culture, the amount of double positive cells slightly increases to 7.1% (±2.9%), while exclusive expression of cytokeratin-7 or vimentin remains the same (1.1% ±1.5% and 91.7% ±1.3%, respectively). Following the first passage, 100% of the cells are positive

for vimentin, and 54.1 ± 8.8% additionally co-express cytokeratin-7. Cells positive for only cytokeratin-7 cells cannot be detected anymore.

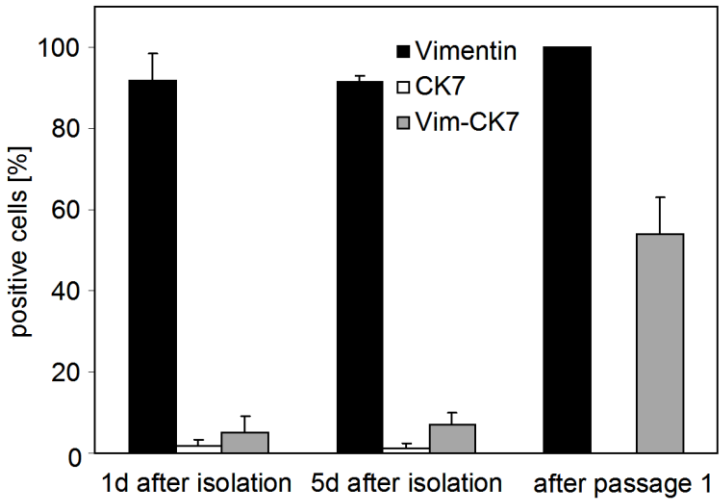

Figure 8: Expression of vimentin and cytokeratin-7 of isolated cells at different time points. Percentage of positive cells was determined 1 d and 5 d after isolation and 3 d after passage 1 (8-11 d in total). *n* = 3. Vim: vimentin, CK7: cytokeratin-7, Vim-CK7: co-expression of vimentin and cytokeratin-7.

3.1.4 Expression of common MSC markers

Characterization of the hAMSC *in situ* and *in vitro* by immunohistochemistry, revealed the expression of the common MSC markers CD73, CD105, CD90, and CD44 but also of CD14, CD45, HLA-DR, and CD34.

Table 5: Expression of common MSC markers

Marker	Description	Positive cells	
		Amnion *in situ*	hAMSC *in vitro*
CD73	Ecto-5´-nucleotidase	<100%	100%
CD105	Endoglin	100%	100%
CD90	Thy-1	100%	100%
CD44	Receptor for hyaluronic acid	100%	100%
CD14	on monocytes/macrophages	50%	0-10%
CD45	on hematopoietic cells	50%	0-10%
HLA-DR	MHC-II, on antigen presenting cells	50%	0-10%
CD34	on HSC and endothelial cells	20%	0%

HSC: hematopoietic stem cells

Especially *in situ*, the amnionic mesenchyme contains many hematopoietic cells. However, only a small subpopulation (0-10%) of these cells adheres to the tissue culture flasks (Table 5, Figure 9). After 2-3 weeks in culture, their amount decreases to clearly less than 1% of the total cells (data not shown). CD34-positive cells could not be detected *in vitro* at all (Table 5).

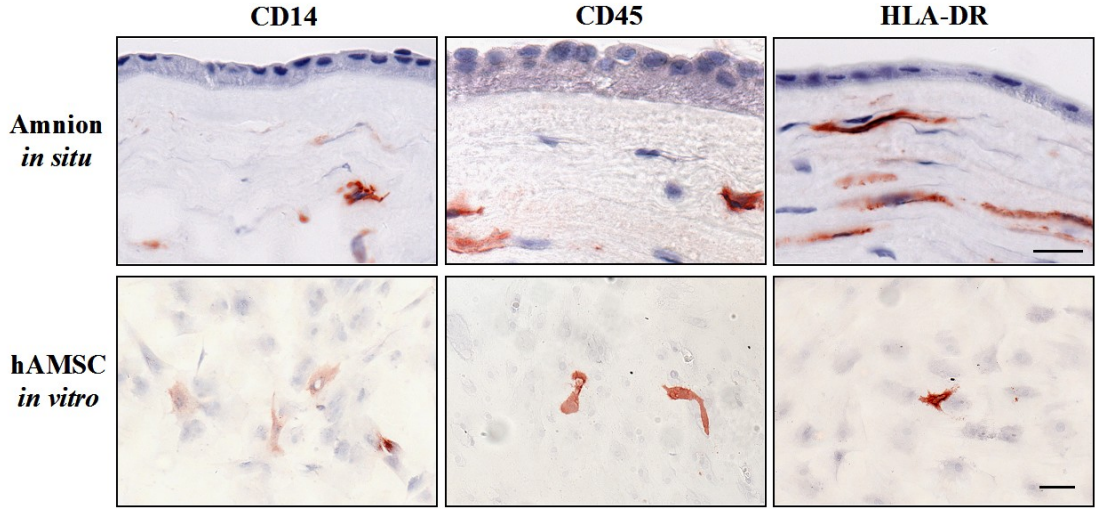

Figure 9: Expression of hematopoietic markers. Scale bar *in situ*: 20µm, *in vitro*: 100µm.

In addition, cultured cells were analyzed using flow cytometry. hAMSC were positive for CD90, CD73, CD105, and CD44 while being negative (or only slightly positive) for CD14, CD45, HLA-DR, and CD34 (Figure 10, Table 6).

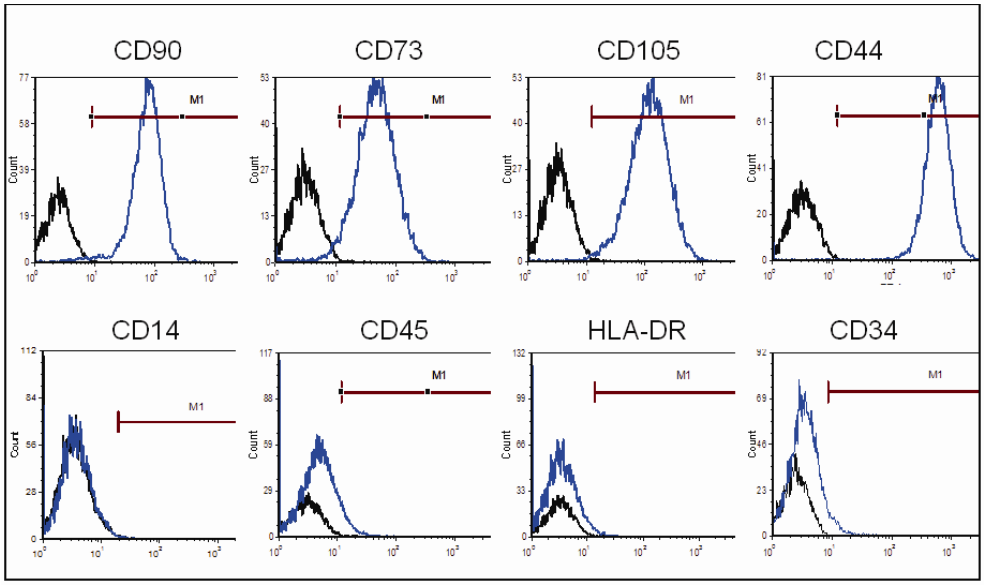

Figure 10: Immunophenotypic characterization of hAMSC by flow cytometry. hAMSC express CD90, CD73, CD105, and CD44 and show low or no expression of CD14, CD45, HLA-DR, and CD34. Analyses were carried out at the Flow Cytometry Core Facility at the Center for Medical Research (ZMF) of the Medical University of Graz.

Table 6: Expression of common MSC markers by flow cytometry (% positive cells)

#	CD90	CD73	CD105	CD44	CD14	CD45	HLA-DR	CD34
1	97,7	95,5	98,8	99,0	0,5	9,3	2,3	6,4
2	100,0	96,7	98,8	99,9	0,0	0,0	0,8	2,1
3	100,0	94,8	98,7	100,0	1,4	7,0	0,8	1,0

Representative isolations # 1-3.

3.1.5 Osteogenic and adipogenic differentiation potential

hAMSC barely differentiated towards the osteogenic lineage as shown by almost negative staining with Alizarin Red which detects calcium deposits (Figure 11A). Adipogenic induction led to accumulation of lipid droplets detected with Oil Red O staining (Figure 11D). hAMSC cultured in DMEM/F12 + 15% FBS served as undifferentiated negative controls (Figure 11B,E) and sclerotic arteries as positive controls (Figure 11C,F) for the respective staining.

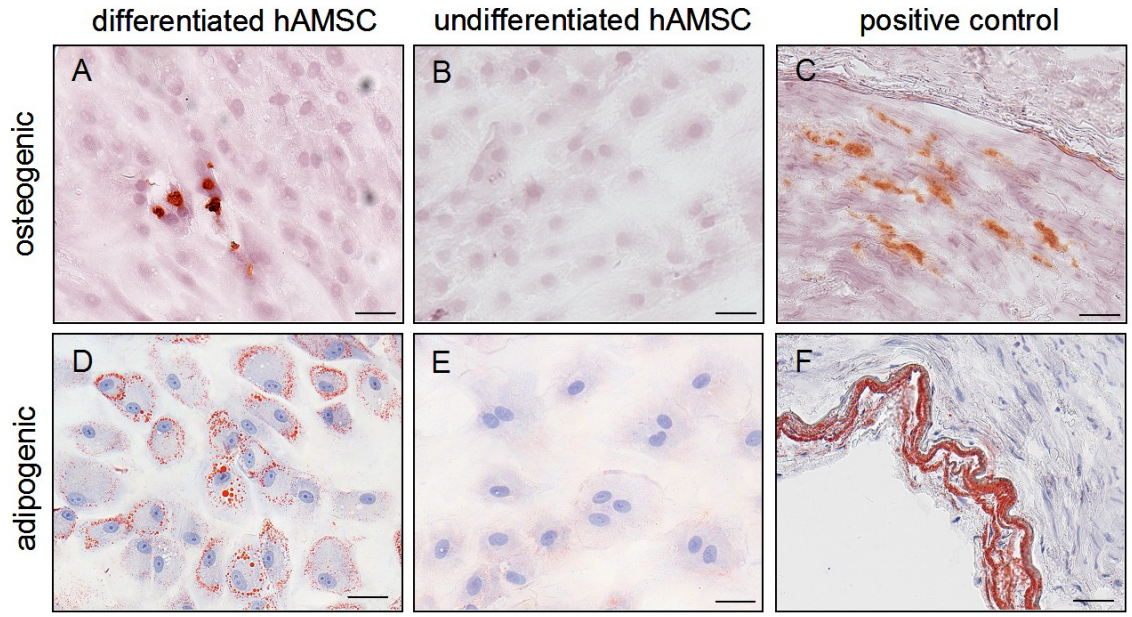

Figure 11: Osteogenic and adipogenic differentiation potential of hAMSC. Cells cultured under osteogenic (A) or adipogenic (D) culture conditions were stained for calcium deposits (A: Alizarin Red staining) or lipid droplets (D: Oil Red O staining), respectively. hAMSC cultured in DMEM + 15% FBS served as undifferentiated, negative controls and were stained accordingly (B: Alizarin Red, E: Oil Red O). Sclerotic arteries served as positive controls (C: Alizarin Red, F: Oil Red O). Scale bar 50 µm.

3.1.6 Expression of endothelial markers by hAMSC *in situ* and *in vitro*

hAMSC did not express the mature endothelial cell markers vWF and VE-cadherin *in situ* (tissue sections), directly after isolation (cytospins), or *in vitro* (cells in culture), which confirms the amnion to be an avascular tissue. However, a subpopulation of hAMSC expressed the endothelial precursor cell marker VEGFR-2. Placental endothelial cells served as positive controls (Figure 12).

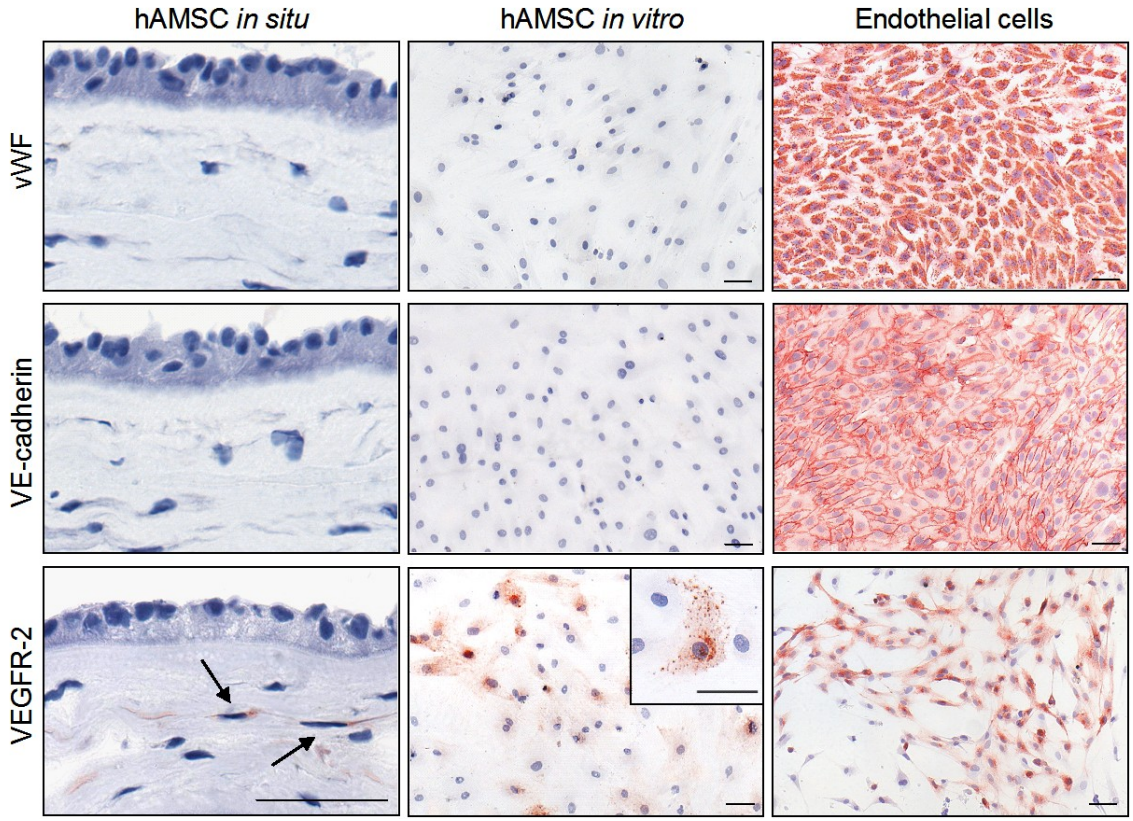

Figure 12: Expression of endothelial markers. hAMSC do not express vWF or VE-cadherin *in situ* (tissue sections) and *in vitro* (cells in culture). However, a subpopulation shows expression of VEGFR-2 *in situ* (see arrows) and *in vitro* (see insert for higher magnification). Placental endothelial cells served as positive controls. Scale bar: 50 µm.

3.2 Endothelial differentiation potential

3.2.1 Proliferation of hAMSC upon endothelial induction

We tested the effect of different endothelial culture conditions on the proliferation of hAMSC to evaluate optimal culture conditions. Non-induced hAMSC (cultured in DMEM + 15% FBS on gelatin) showed cumulative population doublings of 2.5 ± 0.1 after 5 d and 2.9 ± 0.8 after 9 d. Endothelial culture conditions highly increased the proliferation potential of hAMSC. The mean cumulative population doublings under endothelial conditions were 7.3 ± 0.2 and 13.1 ± 0.3 after 5 and 9 d, respectively. There were no distinct effects amongst the different endothelial conditions consisting of culture on fibronectin or gelatin and in the presence or absence of 50 ng/ml VEGF in EGM-2 (Figure 13).

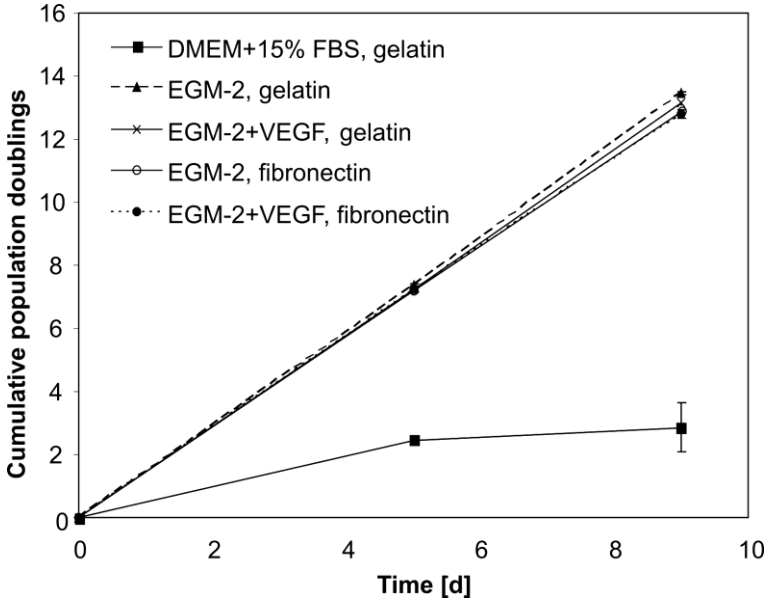

Figure 13: Cumulative population doublings of hAMSC. Cells were either cultured in DMEM + 15% FBS on gelatin or under different endothelial conditions (EGM-2 +/- 50 ng/ml VEGF on gelatin or fibronectin coating).

For further studies on endothelial induction, the use of gelatin-coating and EGM-2 medium without additional VEGF was chosen. Figure 14 shows that under these conditions the cumulative population doublings of hAMSC were clearly higher over several passages compared to non-induced hAMSC.

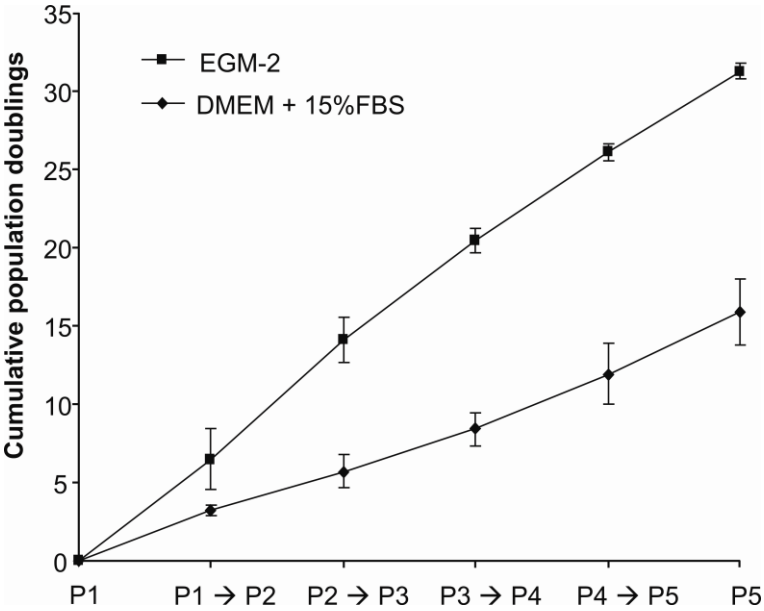

Figure 14: Cumulative population doublings of hAMSC over several passages. Cells were cultured under endothelial conditions (EGM-2) or in DMEM + 15% FBS from passage (P) 1 through 5 (both on gelatin coating). n=3.

3.2.2 Change of morphology and uptake of DiI-Ac-LDL

Non-induced hAMSC showed a fibroblast-like morphology (Figure 15A). After culturing the cells in EGM-2 for a minimum of 5 d, they changed their morphology to a cobblestone-like phenotype (Figure 15B), similar to placental endothelial cells (Figure 15C).

The uptake of DiI-Ac-LDL is specific for endothelial cells and macrophages and occurs via a scavenger receptor. Non-induced hAMSC did not take up DiI-Ac-LDL (Figure 15D). After induction with EGM-2, hAMSC internalized DiI-Ac-LDL with varying intensity (Figure 15E). Placental endothelial cells served as positive control (Figure 15F).

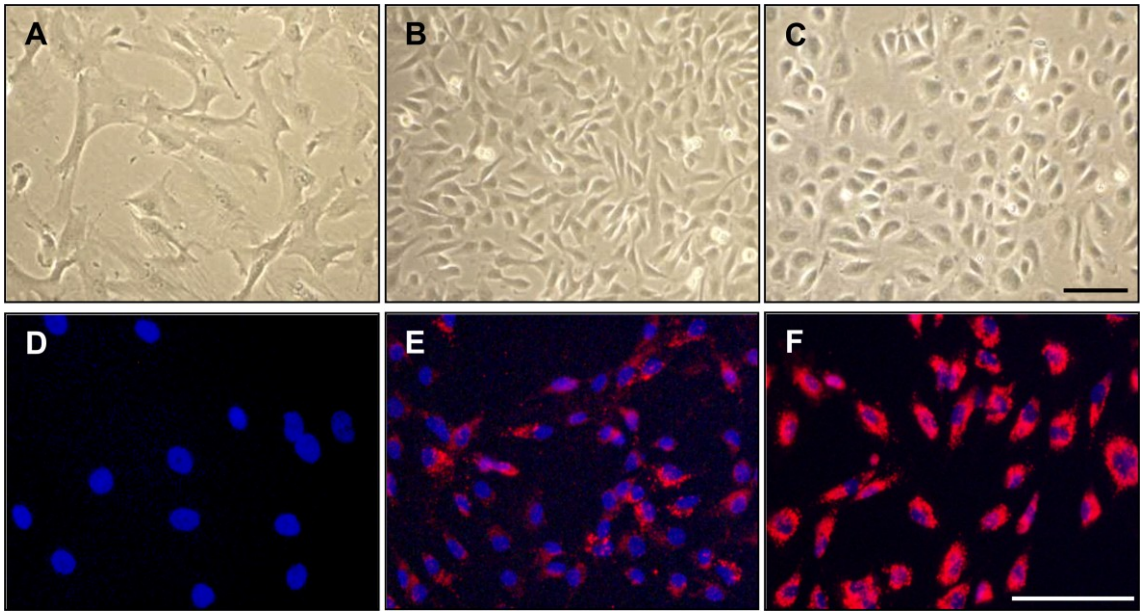

Figure 15: Morphology and DiI-Ac-LDL uptake by non-induced and induced hAMSC compared to placental endothelial cells. Non-induced hAMSC show a fibroblast-like morphology (A). After endothelial induction for a minimum of 5 d, they change their morphology to a cobblestone-like phenotype (B), similar to placental endothelial cells (C). Non-induced hAMSC do not take up DiI-Ac-LDL (D). Induced hAMSC show uptake of DiI-Ac-LDL with varying intensity (E). Placental endothelial cells served as positive control (F). Scale bar: 100 µm.

3.2.3 Expression of endothelial markers upon endothelial induction

Endothelial culture conditions did not result in the appearance of vWF or VE-cadherin positive cells, even when EGM-2 was supplemented with concentrations of VEGF of up to 100 ng/ml and cells were grown on fibronectin. Furthermore, neither extension of the induction period to three weeks nor culturing the cells under low oxygen concentrations (2%) could induce expression of vWF or VE-cadherin proteins. VEGFR-2 expression did not increase noticeably (Figure 16).

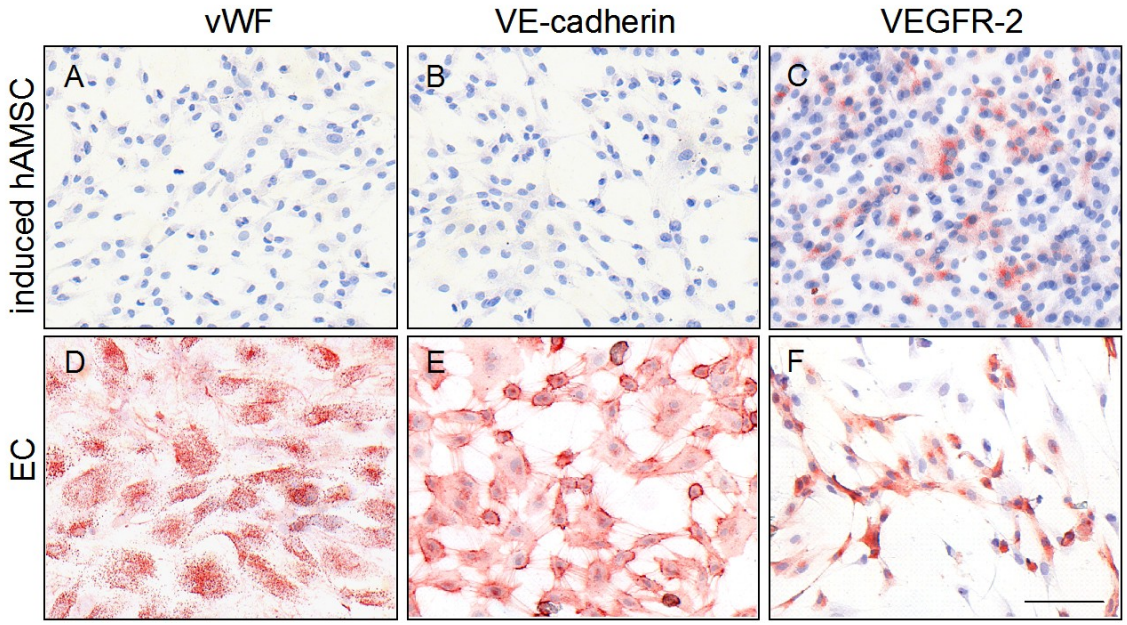

Figure 16: Expression of endothelial proteins by induced hAMSC. Immunocytochemical staining shows absent protein expression of vWF (A) and VE-cadherin (B) and expression of VEGFR-2 (C) by induced hAMSC. Placental endothelial cells (EC) served as positive controls (D-F). Scale bar 100 µm.

RT-PCR data (Figure 17) shows that after 14 d of culture, non-induced hAMSC express low levels of vWF mRNA. In 4 of 5 cases endothelial induction led to a down-regulation of vWF mRNA, while in one case, no change occured (isolation no. 4). Neither non-induced nor induced hAMSC expressed VE-cadherin mRNA. Expression of VEGFR-2 did not change considerably under endothelial induction after 14 d.

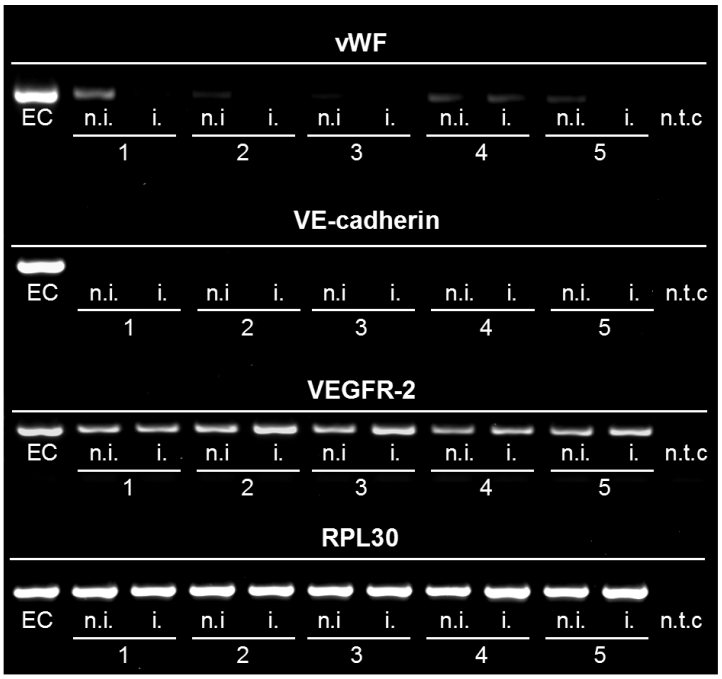

Figure 17: Expression of endothelial genes by hAMSC. RT-PCR shows mRNA expression of vWF, VE-cadherin, VEGFR-2, and RPL30 (ribosomal protein L30) by endothelial cells (EC) and non-induced (n.i.) and induced (i.) hAMSC from isolations 1-5. hAMSC were cultured in DMEM (n.i.) or EGM-2 (i.) for 14 d after passage 1. n.t.c: non-template control.

Semiquantitative analysis of mRNA expression after 7 and 14 d (Figure 18) shows that induced hAMSC express significantly less vWF mRNA than non-induced hAMSC after 7 d. After 14 d, no vWF mRNA expression can be detected anymore by induced hAMSC, while non-induced hAMSC still express small amounts.

VEGFR-2 expression after 7 d by non-induced hAMSC is significantly higher than by induced hAMSC. After 14 d, VEGFR-2 expression by non-induced hAMSC decreases significantly, while the increase of VEGFR-2 mRNA expression by induced hAMSC from 7 d to 14 d is not significant.

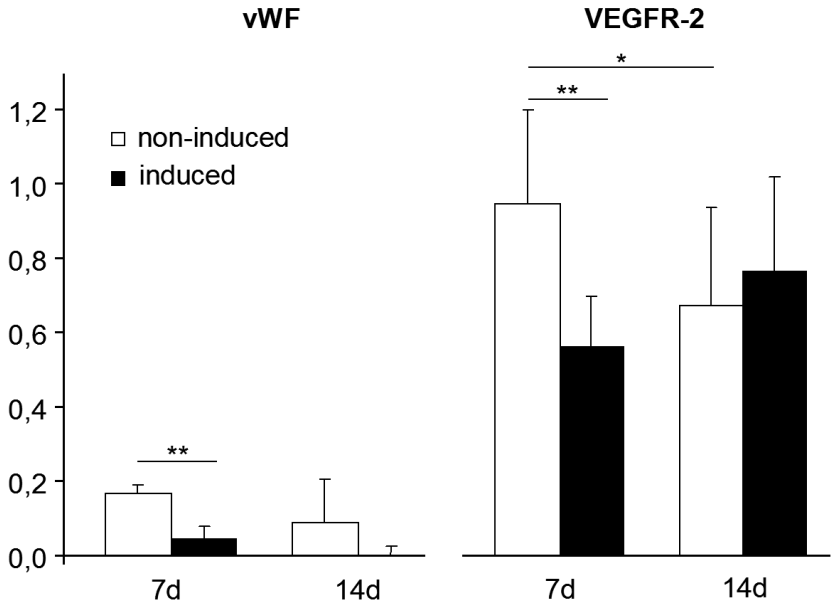

Figure 18: Semiquantitative analysis of vWF and VEGFR-2 mRNA expression by hAMSC. hAMSC were cultured in DMEM (non-induced, white bars) or EGM-2 (induced, black bars) for 7 or 14 d after passage 1. mRNA expressions are normalized to RPL30 (ribosomal protein L30, internal control) and presented as mean ± SD (7 d: n = 4, 14 d: n = 5). ** p <0.05, * p <0.01.

3.2.4 Gene expression changes upon endothelial induction

To identify the effect of endothelial culture conditions on gene expression changes of hAMSC, a microarray was performed. Of the 28,870 transcripts analyzed, 16,761 genes were expressed (signal level >5 after background correction). After filtering with p<0.005, 200 genes were found to be differentially regulated with a fold change >2, of which 92 were upregulated and 108 downregulated upon endothelial induction (Figure 19A). Genes were ranked by fold change (FC) and screened for angiogenic functions. The first 25 genes that were up- and downregulated under angiogenic conditions are shown in Table 7 and 8. Selected genes of interest are displayed in a Venn diagram (Figure 19B). The according data has been deposited in NCBI's Gene Expression Omnibus (GEO) and is accessible through GEO Series accession number GSE28385 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE28385).

Interestingly, induced hAMSC significantly downregulated typical pro-angiogenic genes such as tenascin C (FC -27.9), Tie-2 (-16.8), VEGF-A (-5.7), CD146 (-3.8) and FGF-2 (-2.2, p <0.05), while they upregulated genes with anti-angiogenic functions

such as serpin peptidase inhibitor F1 (serpin F1, FC 31.4, p <0.01), the FGF-2 signaling antagonist sprouty1 (FC 10.6) and angioarrestin (angiopoietin-like 1, 8.2). The only factor with a possible pro-angiogenic function we found to be upregulated was platelet-derived growth factor-D (PDGF-D, 26.6).

The genes of the MSC markers CD90, CD73, and CD105 were expressed equally by non-induced and induced hAMSC (FC >-2 and <2, signal level >5). In addition, both expressed VEGF-B and -C, placental growth factor (PlGF), VEGFR-1 & 2, angiopoietin-1, neuropilin 1 & 2, tissue inhibitors of metalloproteinases 1 & 2 (TIMP1 & 2), and the pericyte markers NG2 and platelet-derived growth factor receptor-ß (PDGFR-ß). The expression of vWF was below background.

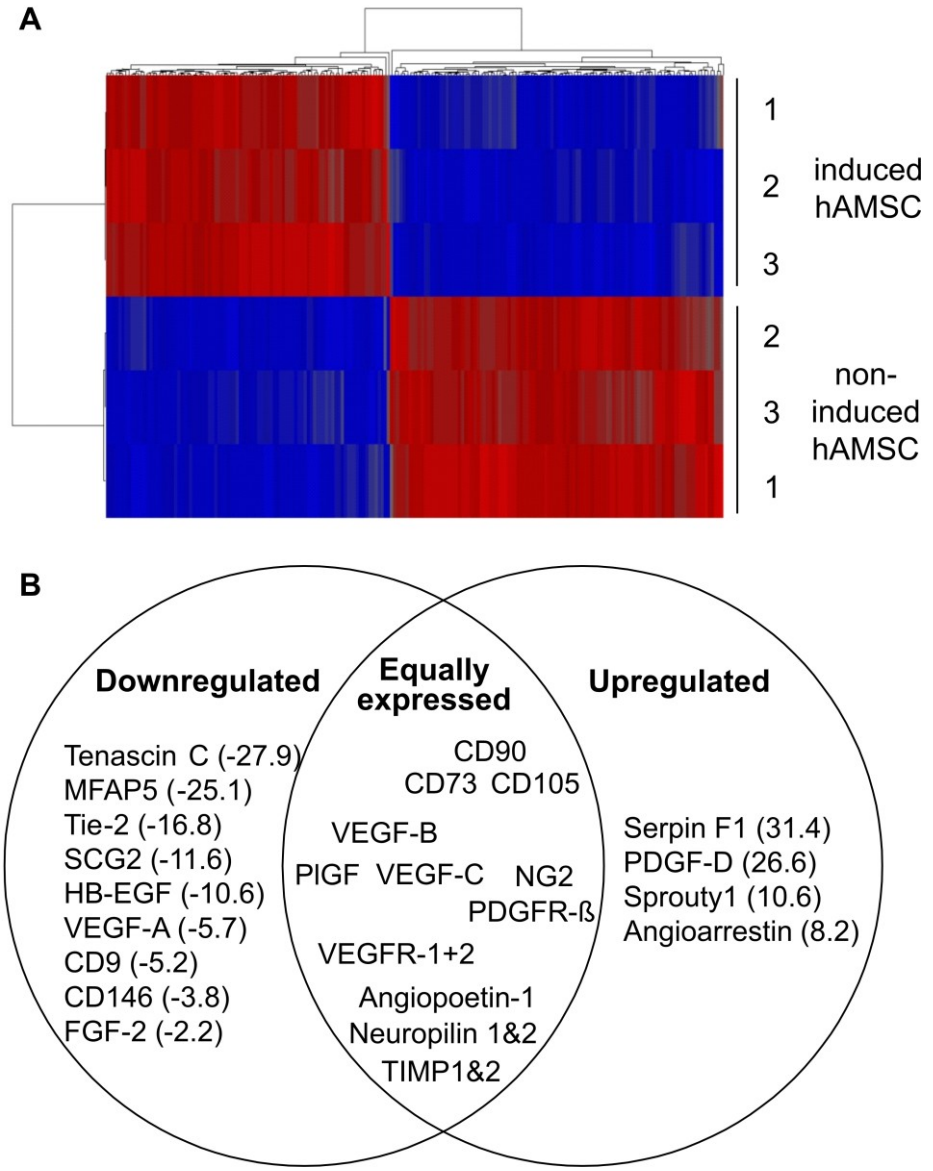

Figure 19: Gene expression changes upon endothelial induction. A: Heat map showing the differential gene expression of induced vs. non-induced hAMSC (fold change >2, p<0.005). Red color stands for high and blue for low signal intensity. Cell preparations were obtained from three placentas (1, 2, 3). B: Venn diagram showing the up- and downregulation of angiogenesis-associated genes after endothelial induction. Genes listed in the overlapping area (equally expressed) are expressed with a fold change >-2 and <2 and a signal level >5 after background correction. Up- and downregulated genes are expressed with a fold change >2 (upregulated) or <-2 (downregulated). $p<0.05$. The according data has been deposited in NCBI's Gene Expression Omnibus and is accessible through GEO Series accession number GSE28385 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE28385).

Table 7: Upregulated genes under angiogenic conditions

Gene	Symbol	FC	p-value	Known angiogenic function
Dermatopontin (TRAMP)	DPT	78.1	0.0010	-
H19, imprinted maternally expressed transcript (non-protein)	H19	62.4	0.0036	-
Prolactin receptor	PRLR	53.1	0.0033	-
Delta-like 1 homolog (Drosophila)	DLK1	50.2	0.0048	-
FK506 binding protein 5	FKBP5	36.1	0.0011	-
Absent in melanoma 1	AIM1	30.8	0.0010	-
Monoamine oxidase A	MAOA	29.9	0.0004	-
Adenomatosis polyposis coli downregulated 1	APCDD1	27.3	0.0044	-
Platelet derived growth factor D	**PDGFD**	**26.6**	**0.0047**	**pro-angiogenic** (Li et al., 2003)
Signal peptide, CUB domain, EGF-like 2	SCUBE2	17.7	0.0035	-
Interleukin 1 receptor, type I	IL1R1	16.4	0.0025	-
LIM domain only 3 (rhombotin-like 2)	LMO3	16.1	0.0009	-
Interleukin-1 receptor-associated kinase 3	IRAK3	15.8	0.0046	-
Serum amyloid A1	SAA1	14.4	0.0028	-
Laminin, gamma 3	LAMC3	11.1	0.0025	-
Family with sequence similarity 180, member A	FAM180A	10.6	0.0027	-
Sprouty 1	**SPRY1**	**10.6**	**0.0019**	**anti-angiogenic** (Lee et al., 2010)
Interferon-induced protein 44-like	IF144L	10.3	0.0017	-
Zinc finger and BTB domain containing 16	ZBZB16	10.1	0.0031	-
Nidogen 1	nidogen1	10.0	0.0003	-
BTB (POZ) domain containing 3	BTBD3	9.2	0.0008	-
Phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	PIK3R1	8.9	0.0016	-
Prostaglandin E receptor 2 (subtype EP2)	PTGER2	8.7	0.0039	-
Interleukin 1 receptor, type II	IL1R2	8.6	0.0012	-
Angioarrestin/Angiopoietin-like 1	**ANGPTL1**	**8.2**	**0.0040**	**anti-angiogenic** (O'Reilly et al., 1994)

Table 8: Downregulated genes under angiogenic conditions

Gene	Symbol	FC	p-value	Known angiogenic function
Carboxypeptidase A4	CPA4	-34.3	0.0029	-
Tenascin C	**TNC**	**-27.9**	**0.0029**	**pro-angiogenic** (Schenk et al., 1999)
Claudin 11	CLDN11	-25.2	0.0028	-
Microfibrillar associated protein 5	**MFAP5**	**-25.1**	**0.0044**	**pro-angiogenic** (Albig et al., 2008)
Kallmann syndrome 1 sequence	KAL1	-19.9	0.0021	-
KIAA1199	KIAA1199	-18.2	0.0043	-
Tie-2/TEK tyrosine kinase, endothelial	**TEK**	**-16.8**	**0.0048**	**pro-angiogenic** (Huang et al., 2010)
Adenylate cyclase-associated protein, 2 (yeast)	CAP2	-13.3	0.0046	-
Retinol dehydrogenase 10 (all-trans)	RDH10	-12.9	0.0039	-
Bone marrow stromal cell antigen 1	BST1	-12.7	0.0001	-
V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	KIT	-11.6	0.0006	-
Secretogranin II (chromogranin C)	**SCG2**	**-11.6**	**0.0020**	**pro-angiogenic** (Kirchmair et al., 2004)
Heparin-binding EGF-like growth factor	**HBEGF**	**-10.6**	**0.0011**	**pro-angiogenic** (Abramovitch et al., 1998)
Lysophosphatidylcholine acyltransferase 2	LPCAT2	-8.8	0.0022	-
ATPase type 13A3	ATP13A3	-7.3	0.0001	-
NCK-associated protein 5	NCKAP5	-7.0	0.0040	-
Cysteine rich transmembrane BMP regulator 1 (chordin-like)	CRIM1	-6.9	0.0032	-
Sorting nexin 25	SNX25	-6.9	0.0001	-
Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	ITGA2	-6.8	0.0026	-
Glutamate receptor, ionotropic, kainate 2	GRIK2	-6.5	0.0018	-
LOC652811 // similar to adlican	LOC652811	-5.8	0.0040	-
Keratin 19	KRT19	-5.7	0.0024	-
Vascular endothelial growth factor A	**VEGFA**	**-5.7**	**0.0043**	**pro-angiogenic** (Ferrara et al., 2003)
CD9 molecule	**CD9**	**-5.2**	**0.0000**	**pro-angiogenic** (Klein-Soyer et al., 2000)
Prostaglandin F2 receptor negative regulator	PTGFRN	-5.2	0.0045	-

3.2.5 Expression of angiogenic proteins by hAMSC

hAMSC were analyzed for the presence of angiogenic proteins using the Human Angiogenesis Antibody Array C1000 (Figure 20). Non-induced cells expressed high levels of the pro-angiogenic factor FGF-2. Endothelial induction decreased its expression to 27 ± 8% compared to non-induced cells. Additional angiogenic factors which were downregulated include interleukin-8 (IL-8, 21 ± 5%), matrix metalloprotease-1 (MMP-1, 31 ± 1%), Tie-2 (13 ± 1%), and uPAR (urokinase-type plasminogen activator receptor, 23 ± 1%). On the contrary, the expression of the anti-angiogenic protein endostatin increased to 226 ± 4% as a result of angiogenic induction. In addition, epidermal growth factor (EGF) was upregulated to 236 ± 99%.

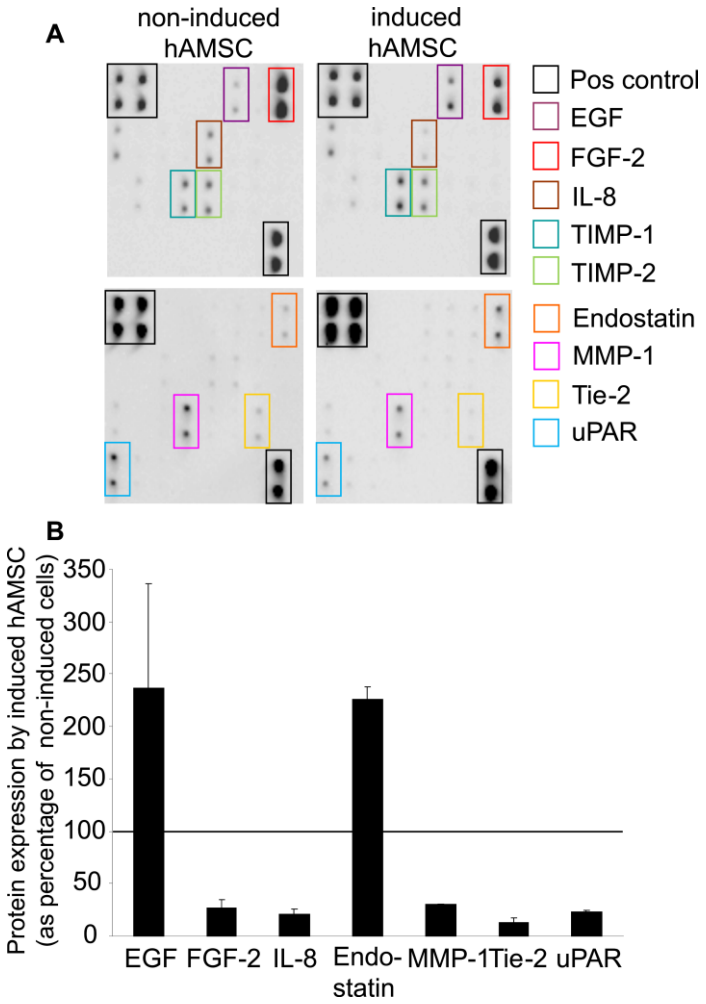

Figure 20: Expression of angiogenic proteins. A: Human Angiogenesis Antibody Array. Protein of non-induced and induced hAMSC from 5 different placentas was pooled and incubated with two membranes, onto which 43 angiogenic proteins are blotted in duplicates. The rectangles highlight proteins of interest. B: Arrays were analyzed by densitometry and normalized to the internal positive control. Protein expressions by induced hAMSC are shown as percentages of protein expression by non-induced cells (set to 100%).

3.2.6 Angiogenesis assay – network formation

The Matrigel assay is a commonly used method to evaluate network formation by endothelial cells and was applied to investigate if induced hAMSC were also able to form networks.

After culture under standard (non-induced) or endothelial (induced) conditions for at least 10 d, hAMSC were seeded on Matrigel in EGM-2. Non-induced hAMSC formed network-like structures within 6 h, however, these networks disintegrated fast (Figure 21A-D). Induced hAMSC (Figure 21E-H) formed networks similar to placental endothelial cells (Figure 21I-L). While the endothelial networks remained static, networks of induced hAMSC became more wide-spread over time. In addition, branches formed by placental endothelial cells showed signs of degeneration (dark cell clusters in Figure 21K-L) already after 24 h, while networks formed by induced hAMSC remained viable for about 48 h. However, cells within these networks remained negative for vWF (Figure 21M-N). Placental endothelial cells served as positive control (Figure 21O-P).

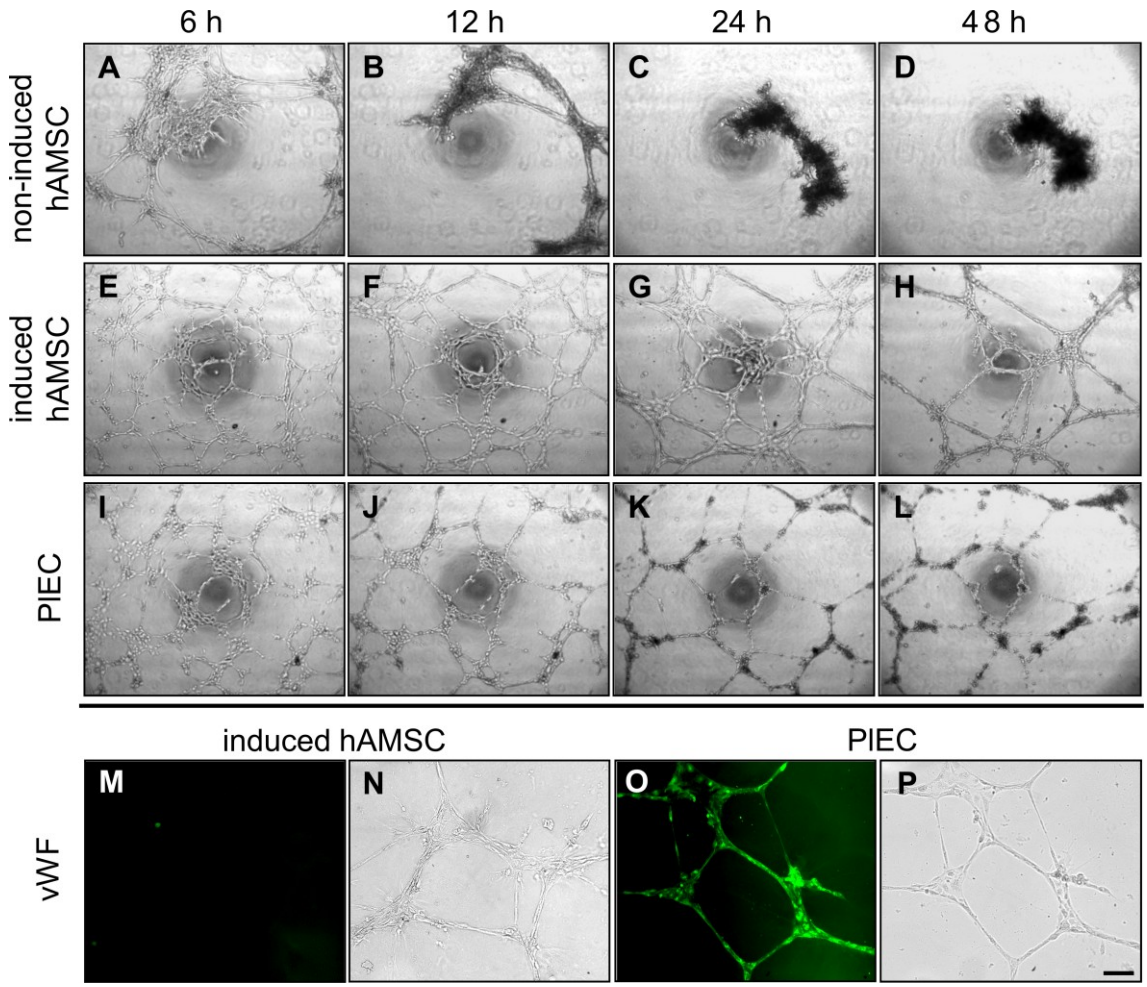

Figure 21: Network formation on Matrigel. hAMSC were cultured under standard (non-induced) or endothelial (induced) conditions for at least 10 d. Then they were seeded on Matrigel in EGM-2. Pictures were taken at different time points (6 h, 12 h, 24 h, 48 h). Placental endothelial cells (PlEC) served as positive control. Networks of induced hAMSC cultured on Matrigel for 24 h do not express vWF (M: fluorescence, N: phase contrast). PlEC served as positive control (O: fluorescence, P: phase contrast). Scale bar: 100 µm.

3.3 Effect of hAMSC on placental endothelial cells

3.3.1 Co-culture of hAMSC and endothelial cells on Matrigel

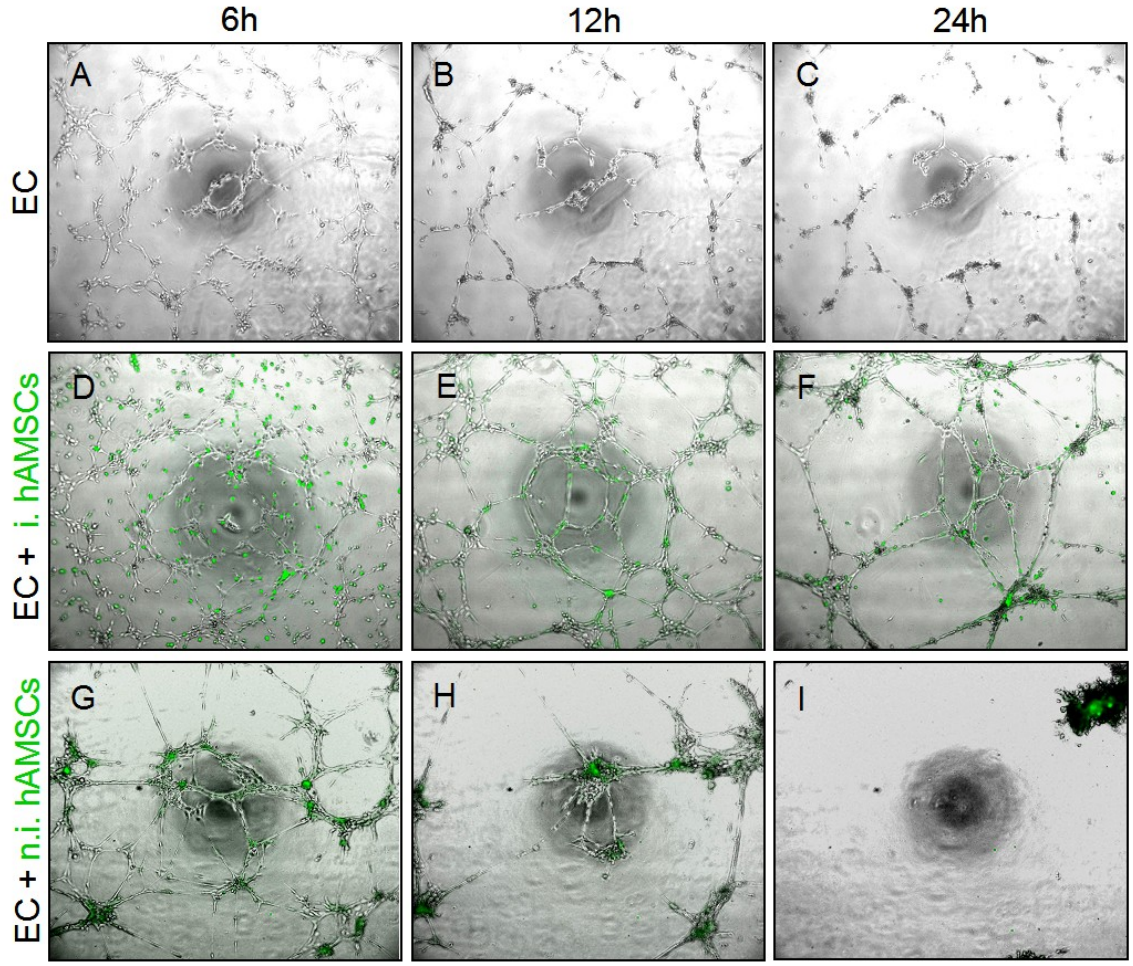

Figure 22: Placental endothelial cells (EC) in co-culture with hAMSC on Matrigel. EC were seeded on Matrigel and allowed to form networks (A-C). After 5 h, *green* fluorescent labeled induced hAMSC (i. hAMSC, D-F) or non-induced (n.i. hAMSC, G-I) were added in a ratio of 3:1 (EC : hAMSC). Pictures were taken at different time points (6 h, 12 h, 24 h).

In specific cases, endothelial cells showed poor network formation on Matrigel (Figure 22A-C). In these cases, cells underwent cell death already after 12 h (Figure 22B) and networks had disintegrated after 24 h (Figure 22C). Green-fluorescent labeled induced hAMSC added to the endothelial networks after 5 h in a ratio of 3:1 (EC : hAMSC) rapidly incorporated into pre-existing networks and stabilized them (Figure 22D-F). Non-induced hAMSC also migrated to networks of endothelial cells, however, these networks disintegrated fast (Figure 22G-I). Equivalent results were obtained when hAMSC were co-seeded with endothelial cells on Matrigel from the

beginning instead of being added after 5 h and when they were added in different ratios (2:1 and 4:1 – EC : hAMSC, data not shown).

3.3.2 Co-culture of hAMSC and endothelial cells on gelatin-coated culture flasks

Co-culture of placental endothelial cells and induced hAMSC on normal gelatin-coated tissue culture flasks resulted in the formation of vessel-like structures by the endothelial cells (Figure 23A). Additional supplementation of the culture medium (EGM-MV) with 50 ng/ml VEGF during co-culture enhanced endothelial sprouting (Figure 23B). Co-culture of placental endothelial cells with non-induced hAMSC resulted in the formation of big clusters by endothelial cells without noticeable sprout formation (Figure 23C).

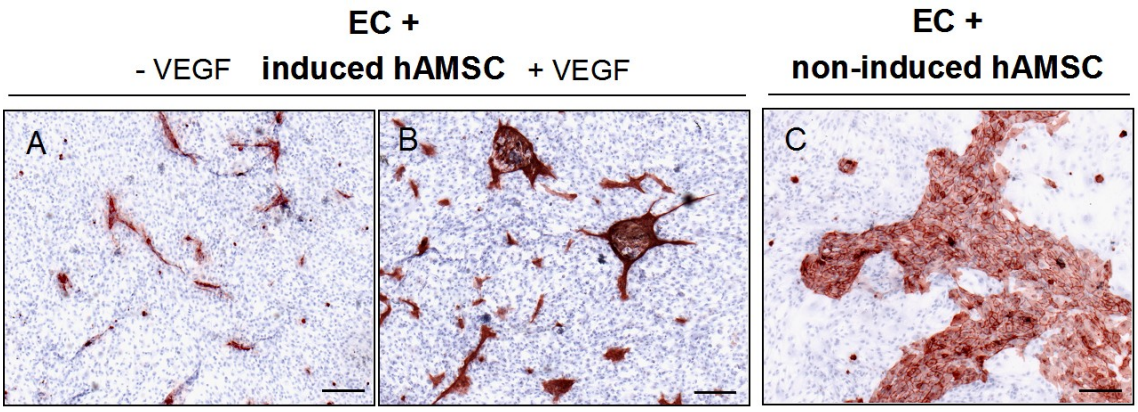

Figure 23: Co-culture of placental endothelial cells (EC) and hAMSC on gelatin. Induced (A,B) and non-induced (C) hAMSC were co-cultured with EC in the absence (A,C) or presence (B) of 50 ng/ml VEGF for 8 d. PlEC are stained in *red* with antibodies against vWF (A) or VE-cadherin (B,C). Scale bar: 200 µm.

3.3.3 Expression of pericyte markers *in situ* and *in vitro*

Different antibodies against pericyte markers were tested *in situ* (human term placenta) and *in vitro* (isolated cells). *In situ*, the antibody against NG2 was the most suitable to detect pericytes and other mural cells (Figure 24A-B). Interestingly, cells in the amnionic mesenchyme also expressed NG2 (Figure 24C).

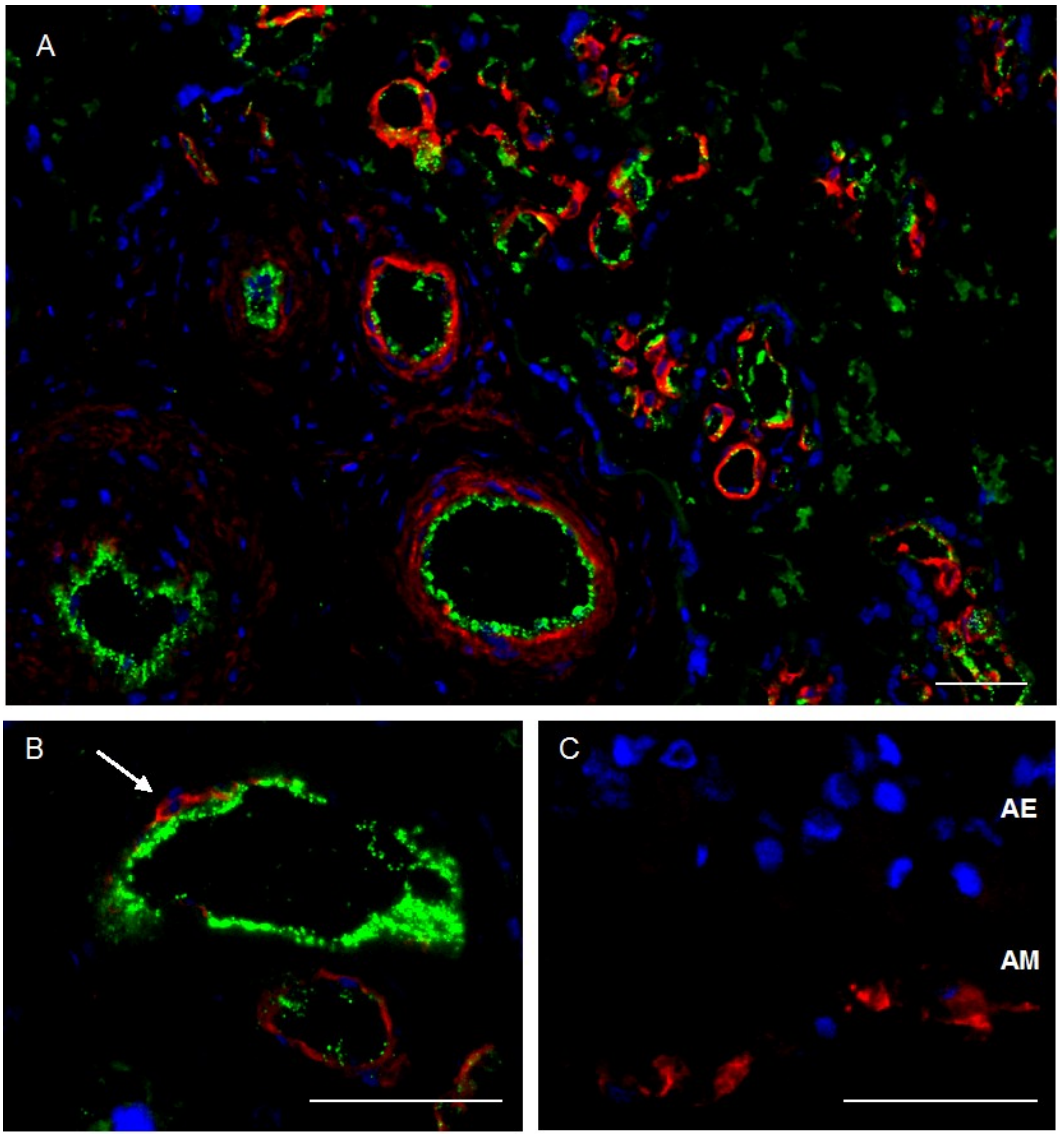

Figure 24: NG2 expression of human term placenta. A: NG2 (*red*) is expressed by pericytes and mural cells surrounding endothelial cells which are stained for with antibody against vWF in *green*. B: Arrow points to a pericyte (*red*) lining a small vessel (*green*). C: Cells in the amnionic mesenchyme (AM) also express NG2 (*red*). Nuclei were counterstained with DAPI in *blue*. Scale bar: 50 µm. AE: amnionic epithelium, AM: amnionic mesenchyme.

In vitro, NG2 is expressed by both non-induced and induced hAMSC, thus the pericyte-like phenotype of hAMSC is not changed by endothelial induction (Table 9). In co-culture with endothelial cells, induced hAMSC consistently express NG2, independent of their proximity to the endothelial vessel-like structures (Figure 25).

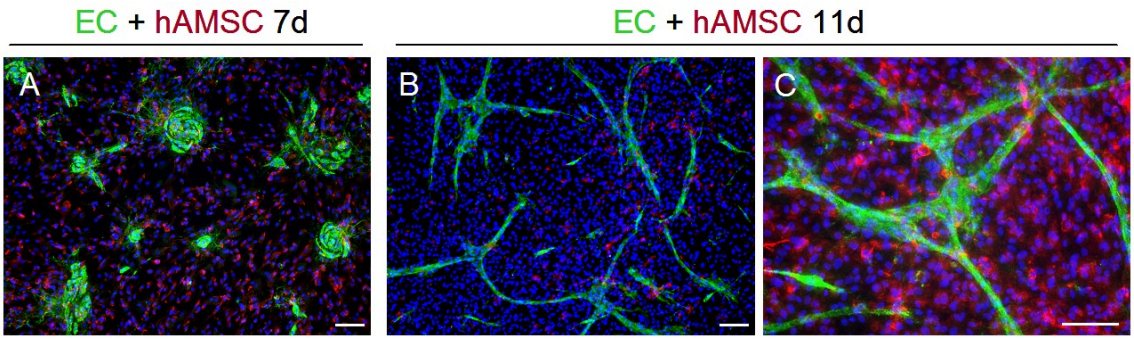

Figure 25: NG2 expression of induced hAMSC in co-culture with endothelial cells (EC) after 7 d (A) and 11 d (B,C) in a ratio of 80:20 (EC : hAMSC). NG2 (*red*) is expressed by hAMSC independent of their proximity to endothelial cells (EC), which are stained with antibody against vWF in *green*. Nuclei were counterstained with DAPI in *blue*. Scale bar: 100 µm.

In vitro, hAMSC expressed PDGFR-ß in addition to NG2 but were negative for CD146 (Table 9).

Table 9: Expression of pericyte markers *in vitro*

	hAMSC		EC
	non-induced	induced	
NG2	+	+	-
PDGFR-ß	-/+*	-/+*	-
CD146	-	-	+

EC: Endothelial cells, * ICC negative, IF positive.

3.3.4 Effect of hAMSC conditioned medium (CdM) on endothelial cells

To investigate if hAMSC influence endothelial network formation via direct cell-cell contact or via paracrine factors, the effect of CdM collected from hAMSC on endothelial cells was examined.

Lactate dehydrogenase (LDH) is an enzyme which is released into the culture medium by damaged cells (Decker et al., 1988). When placental endothelial cells were cultured in the presence of CdM collected from induced hAMSC, the activity of LDH in the culture supernatants was significantly reduced to 48.7 ± 14.9% of control, thus,

endothelial cell viability was clearly enhanced (Figure 26A). The addition of induced hAMSC-CdM to placental endothelial cells supported the formation of network-like structures in the Matrigel assay. While networks formed by endothelial cells in control medium had disintegrated after 72 h (Figure 26B), networks formed by cells cultured with induced hAMSC-CdM were still stable (Figure 26C). Under the same conditions, CdM collected from non-induced hAMSC neither enhanced endothelial cell viability (Figure 26D) nor supported endothelial network formation (Figure 26E-F).

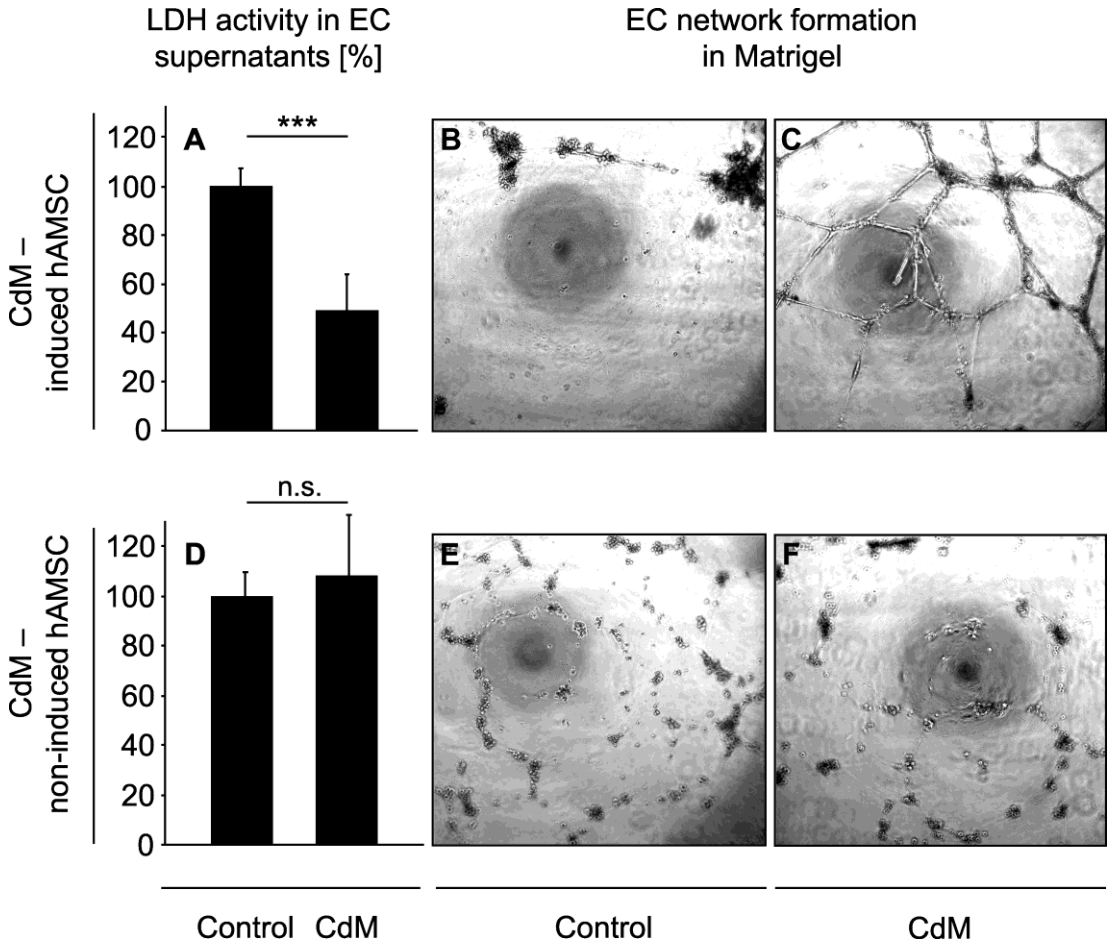

Figure 26: Effect of hAMSC-conditioned medium (CdM) on endothelial cells (EC). Induced hAMSC-CdM significantly reduces LDH activity in placental EC supernatants, shown as percentage of control after 96 h of culture (A). $n = 4$. *** $p < 0.001$. After 72 h on Matrigel, networks formed by placental EC in control medium have already disintegrated (B), while networks formed by cells cultured with CdM from induced hAMSC are still stable (C). Under the same conditions, hAMSC-CdM collected from non-induced hAMSC neither enhanced endothelial cell viability (D) nor supported endothelial network formation (E-F). $n = 2$. n.s.: not significant. Data presented in this figure was collected by Gregor Weiß.

4 Discussion

4.1 Characterization of hAMSC

hAMSC were carefully tested for the expression of epithelial and endothelial markers to exclude a possible contamination with these cell types. Further, to assure an MSC phenotype, hAMSC were characterized for the expression of common MSC markers in addition to evaluating their adipogenic and osteogenic differentiation potential.

4.1.1 Co-expression of vimentin and cytokeratin-7

Generally, cells originating from the epidermal layer are positive for cytokeratins but negative for vimentin, while mesoderm-derived cells are positive for cytokeratins but negative for vimentin. Analysis of hAMSC cultures led to the detection of cells expressing both vimentin and cytokeratin-7. Even though *in situ* hAMSC are negative for cytokeratin-7, a subpopulation of the isolated cells (about 50%) starts to express this epithelial marker in addition to vimentin after the first passage. Thus, we conclude that under culture, hAMSC adopt a hybrid mesenchymal-epithelial phenotype. This would be in line with ultrastructural investigations showing epithelial features of hAMSC (Pasquinelli et al., 2007).

A similar phenomenon has actually been demonstrated for hAEC, which co-express cytokeratin and vimentin already *in situ* (Cremer et al., 1981, Regauer et al., 1985) and adopt a mesenchymal-like phenotype during culture (Portmann-Lanz et al., 2006, Bilic et al., 2008, Stadler et al., 2008).

Therefore, the vimentin-cytokeratin-7 double-positive cells present in the cultures one day after isolation are probably either hAEC as a by-product of hAMSC isolation or hAMSC that already underwent mesenchymal-epithelial transition. Regardless of their identity, they barely proliferate until day 5. Only after the first subculture, the number of double-positive cells increases noticeably. Even though we cannot exclude the possibility that this happens due to an overgrowth of double-positive epithelial cells, this is very unlikely as hAEC have low growth rates *in vitro,* need high seeding densities to obtain proliferative cultures and undergo a growth retardation during the epithelial-mesenchymal transition (Portmann-Lanz et al., 2006, Bilic et al., 2008).

It is however plausible that the cultures contain a mixture of both: Mesenchymal cells that adopt epithelial properties and to a smaller amount epithelial cells that adopt mesenchymal properties. In order to avoid contamination of hAMSC cultures with hAEC it is essential to carefully examine them microscopically in the first days after isolation when they can still be distinguished by their morphology.

4.1.2 Expression of MSC markers

As hAMSC have been described to be phenotypically similar to MSC from other sources such as bone marrow, cultured cells and term placental cryosections were characterized for the expression of commonly known MSC markers. As a specific marker for these cells has not been found yet, the International Society of Cellular Therapy (ISCT) suggests a panel of surface markers to identify MSC (Dominici et al., 2006): MSC from whichever source should express CD73, CD105 and CD90, and at the same time, they should lack the expression of the hematopoietic marker CD45, the monocyte and macrophage marker CD14, and of HLA-DR, which is expressed by antigen presenting cells such as B-lymphocytes and macrophages. In addition, they should be negative for CD34 which is expressed by endothelial and hematopoietic progenitor cells. In accordance with these criteria, hAMSC used in our study expressed CD73, CD105, and CD90, both *in situ* as well as *in vitro*. In addition, they expressed CD44, a receptor for hyaluronic acid, which has also been described as a marker for MSC (Pittenger et al., 1999). Immunohistochemistry revealed the presence of monocyte/macrophage-like cells expressing CD14, CD45, and HLA-DR in the amnionic mesenchyme, however, only a small subpopulation was detected in hAMSC cultures. Magatti et al. could show that these cells are of fetal origin and that they were capable of providing co-stimulatory signals to primed T-cells, which indicates that there exists a stimulatory cell subpopulation within the amnion (Magatti et al., 2008). However, with regard to a potential clinical application it is important to note that many studies have shown that hAMSC do not provoke immune reactions neither *in vitro* (Bailo et al., 2004, Wolbank et al., 2007) nor *in vivo* (Bailo et al., 2004, Cargnoni et al., 2009b). In addition, the amount of these putative immunostimulatory cells decreases to clearly less than 1% after 2-3 weeks in culture.

4.1.3 Osteogenic and adipogenic differentiation potential

Several studies have described a multipotent differentiation potential of hAMSC towards different cell types (Parolini et al., 2008). In our hands, hAMSC were capable of differentiation towards the adipogenic but barely towards the osteogenic lineage. This is in line with a study from Stadler et al. showing that in 4 out of 4 cases hAMSC did not differentiate along the osteogenic lineage when standard osteogenic differentiation protocols were applied (Stadler et al., 2008). In their study, only a special osteogenic stimulatory kit (O-kit, Stem Cell Technologies) was able to induce osteogenic differentiation of hAMSC. Adipogenic differentiation, however, was successful in 2 out of 4 cases using standard protocols.
The discrepancies in the described differentiation potentials of hAMSC might be due to different isolation and induction protocols used.

4.1.4 Expression of endothelial markers

We could show that the amnionic mesenchyme is free of mature endothelial cells as documented by the absent staining with the classical endothelial markers vWF and VE-cadherin. Careful immunocytochemical analysis for these markers on freshly isolated cells was performed to exclude a contamination with endothelial cells before endothelial induction. All isolations in this study were free of endothelial cells.
Interestingly, a subpopulation of hAMSC *in situ* and *in vitro* is positive for VEGFR-2. Together with its ligand VEGF, VEGFR-2 plays an important role during early placental and embryonic vascular development. Its expression has been documented in vasculogenic and angiogenic precursor cells found in placental villi (Demir et al., 2004). Mice deficient in VEGFR-2 (VEGFR-$2^{-/-}$) died in utero as a result of an early defect in the development of hematopoietic and endothelial cells (Shalaby et al., 1995). Therefore, the presence of VEFGR-2 in the amnionic mesenchyme suggests an endothelial progenitor potential of hAMSC.

4.2 Endothelial induction and angiogenic properties of hAMSC

The main objective of this study was to investigate the effect of endothelial induction on hAMSC. In addition, direct and paracrine effects of hAMSC on endothelial cells were examined.

4.2.1 Endothelial differentiation potential

Upon endothelial induction with EGM-2 containing 2% FCS, EGF, hydrocortisone, VEGF, FGF-2 and IGF, hAMSC showed some endothelial-like characteristics: They changed their fibroblast-like to a more endothelial cell-like morphology and acquired the ability to take up Ac-LDL. In addition, *only* induced hAMSC were able to form long-lasting networks similar to endothelial cells in the Matrigel assay. Non-induced hAMSC cultured in DMEM with 15% FBS for at least ten days initially also formed network-like structures in Matrigel. This could be explained by the fact that before the application in the Matrigel assay, these cells were resuspended in EGM-2. Therefore, the network formation may be due to a short-term stimulatory effect of abundant growth factors present in both the Matrigel and in EGM-2. However, these networks were very unstable and disintegrated within 6 h.

Even though hAMSC were responsive to the angiogenic factors present in EGM-2, they did not differentiate into mature endothelial cells. Induced hAMSC still expressed the common MSC markers CD90, CD73, and CD105. None of the angiogenic conditions used by previous studies (Oswald et al., 2004, Liu et al., 2007) or addition of high concentrations of VEGF (100 ng/ml) and the use of fibronectin, an extracellular matrix protein known to promote endothelial differentiation (Wijelath et al., 2004) led to an expression of vWF or VE-cadherin on the protein level. Even cells forming the network-like structures in the Matrigel assay remained negative for vWF. Also endothelial induction under 2% oxygen did not promote endothelial differentiation, although low oxygen conditions are known to induce angiogenesis (Pugh et al., 2003) and have a pro-angiogenic effect on MSC (Hu et al., 2008).

Surprisingly, non-induced hAMSC showed a low expression of vWF mRNA, which was, however, downregulated upon endothelial induction. Gene expression of VEGFR-2 remained unchanged.

So far, only one other study has investigated the endothelial differentiation potential of hAMSC (Alviano et al., 2007). This study reported that culture in DMEM supplemented

with 2% FBS and 50 ng/ml VEGF led to a slightly increased expression of VEGFR-2 as well as appearance of vWF-positive cells. The discrepancies between the results of this previous study and our data might be due to differences in cell isolation protocols. In contrast to our findings, in this former study also non-induced hAMSC formed stable networks in Matrigel. Thus, maybe a subset of endothelial-like cells was present within the primary isolated cell population, possibly caused by an incomplete separation of the amnion from the underlying vascularized chorion. This hypothesis is supported by our Matrigel data which clearly showed that non-induced hAMSC cultures devoid of endothelial cells did not generate stable networks.

4.2.2 Gene and protein expression array data

Our microarray analysis did not reveal upregulation of endothelial-specific genes in angiogenic-induced hAMSC. Under standard conditions, hAMSC express a variety of pro-angiogenic genes. Tenascin C, a large extracellular glycoprotein, has been shown to promote endothelial cell elongation and sprouting in bovine aortic endothelial cells and human umbilical vein endothelial cells (Schenk et al., 1999, Martina et al., 2010). Tie-2 (or TEK) is a tyrosine-kinase transmembrane receptor that is predominately expressed by endothelial cells. Its ligands are the angiopoietin family members ANGPT1, 2, and 4, which are secreted proteins with different functions in angiogenesis (Huang et al., 2010). VEGF-A and FGF-2 are both very potent inducers of angiogenesis. VEGF-A is part of the VEGF/PDGF growth factor family and strongly induces angiogenesis, vasculogenesis and endothelial cell growth (Ferrara et al., 2003) FGF-2 acts on many cell types and also greatly promotes angiogenesis (Szebenyi et al., 1998). However, these pro-angiogenic genes were downregulated upon endothelial culture conditions, i.e. in the presence of abundant angiogenic growth factors. Instead, genes with anti-angiogenic functions were upregulated: Serpin F1, angioarrestin and sprouty1. Serpin F1, or pigment epithelium-derived factor (PEDF), is one of the most effective natural angiogenesis inhibitors (Dawson et al., 1999). Angioarrestin, also known as angiopoietin-like 1, has been shown to inhibit angiogenic processes, including endothelial cell proliferation, migration, and tube formation in human umbilical vein endothelial cells (HUVEC) (O'Reilly et al., 1994). Sprouty1 acts as an antagonist of FGF signaling, and upregulation of sprouty1 in HUVEC inhibited FGF- and

VEGF-induced endothelial cell proliferation and network formation on Matrigel (Impagnatiello et al., 2001, Lee et al., 2010).

The gene expression results were confirmed on the protein level using an angiogenic protein antibody array. Again, FGF-2 and Tie-2 were downregulated upon endothelial induction, while the anti-angiogenic protein endostatin was clearly upregulated compared to non-induced controls. The expressions of VEGF-A and angioarrestin in this array were too low compared to the internal positive control to allow reliable quantification.

It seems as if hAMSC use this upregulation of anti-angiogenic and concomitant downregulation of pro-angiogenic genes and proteins as an autoregulatory mechanism to protect them against a differentiation into mature endothelial cells.

We assume that hAMSC resist a differentiation into mature endothelial cells which would be in accordance with the fact that amnionic membrane is one of the few avascular tissues that maintains its avascularity even though it is next to a highly vascularized tissue (the chorion) (Hao et al., 2000).

4.2.3 Angiogenic effects of amnionic membrane in clinical use

The gene and protein expression data might not only explain how hAMSC resist a differentiation into mature endothelial cells by upregulation of anti-angiogenic factors, but also gives some insight into the conflicting reports concerning the angiogenic properties of amnion in clinical use.

In the case of corneal reconstruction, it is essential that the implant remains avascular. Amnionic membrane transplantation has been shown to suppress corneal neovascularization and inflammation. Hence, the amnion was reported to have anti-angiogenic properties (Jiang et al., 2006) (Kim et al., 1995). However, for settings of wound healing and tissue engineering a sufficient vascularization of the affected tissue is crucial. Amnion application in leg ulcers resulted in improved formation of granulation tissue and vessel development. Thus, an angiogenesis inducing effect was ascribed to the amnionic membrane (Faulk et al., 1980). Several groups have looked into the factors responsible for these effects and again, controversial results were reported. Wolbank et al. and Marvin et al. identified mostly pro-angiogenic factors secreted by amnionic membrane and cells derived thereof (Marvin et al., 2002,

Wolbank et al., 2009). On the contrary, Hao et al. reported the expression of anti-angiogenic mRNA in human amnionic membrane (Hao et al., 2000).

Angiogenesis, or neovascularization, is a finely balanced process controlled by pro- and anti-angiogenic factors. Even though this is only speculative and needs to be further investigated using appropriate disease models, it might be possible that hAMSC can sense a pro-angiogenic environment and act as regulators between abundant and insufficient angiogenesis. This might explain the controversial angiogenic effects when applying amnion in different clinical settings and may account for a functional flexibility of hAMSC in response to environmental cues of the surrounding tissue.

4.2.4 Effect of hAMSC on endothelial cells

Even though hAMSC are capable of upregulating anti-angiogenic factors, they do not adopt actual anti-angiogenic properties towards endothelial cells. On the contrary, they supported endothelial network formation in the Matrigel assay and have a stabilizing effect on those networks. Also in a normal co-culture setting without Matrigel, induced hAMSC promoted the formation of vessel-like structures by endothelial cells.

These results are consistent with studies showing that MSC from bone marrow enhanced angiogenesis and supported blood vessel formation (Chen et al., 2003, Kinnaird et al., 2004, Gruber et al., 2005, Hung et al., 2007, Wu et al., 2007, Verseijden et al., 2010). Au et al. could demonstrate that MSC stabilized engineered blood vessels and kept them functional for 130 days *in vivo*. The authors showed that the MSC did not differentiate into endothelial cells but instead acted as pericyte-like cells (Au et al., 2008).

Pericytes are mural cells that closely encircle endothelial cells of arterioles, venules and capillaries, while larger vessels are usually invested by smooth muscle cells. They are involved in angiogenesis and vasculogenesis and play an essential role in the stabilization of vessels. Proposed modes of actions include matrix deposition and regulation of endothelial differentiation, blood flow and permeability (Gerhardt et al., 2003). So far, no specific marker to identify a pericyte has been agreed on. Most commonly they are characterized by the expression of NG2, PDGFR-ß and CD146 and the concomitant absence of hematopoietic and endothelial markers (Crisan et al., 2008).

In our study, the proteoglycan NG2 seemed to be one of the more reliable markers. It identified pericytes but also other mural cells in the vasculature of human term placenta and was expressed by hAMSC *in situ*. The expression of NG2 by hAMSC *in vitro* points to a possible pericyte-like function of these cells, similar to what has been shown for bone marrow-derived MSC (Au et al., 2008). In contrast to the expression of the previously described endothelial and angiogenic genes and proteins, NG2 expression did not change upon endothelial induction of hAMSC.

The endothelial cell-stabilizing effect of hAMSC is not restricted to direct cell-cell contact. Conditioned medium collected from induced hAMSC also stabilized network formation and enhanced viability of endothelial cells. The paracrine factors involved in these processes are currently under investigation.

5 Conclusion and outlook

Even though hAMSC do not differentiate into mature endothelial cells and are thus not ideal as a substitute for endothelial cells, they might be valuable in a variety of cell therapeutic or tissue engineering approaches where they can promote the survival of endothelial cells, the stabilization of pre-existing vessels and the revascularization of ischemic tissues.

From our results we conclude that an endothelial induction of hAMSC is advantageous for an application of these cells in therapeutic treatments, as it promotes a survival-enhancing effect on endothelial cells. Furthermore, careful analyses of primary cell isolations are mandatory to exclude misleading results due to contamination with endothelial cells.

In an upcoming study we will determine how a co-application of placental endothelial cells and hAMSC could be used in vascular therapies. In practice, we will investigate the endothelialization of vascular grafts in a bioreactor, where placental endothelial cells will be used for the seeding of the luminal surface. hAMSC will be applied as supporting stromal or mural cells to test if they enhance the viability of the endothelial cells and therefore the patency of the vascular graft.

Further, hAMSC and placental endothelial cells will be investigated both separately and combined for their potential to form vessels in a mouse model.

In the future, banking of both cell types may provide a convenient source for autologous therapy and for matching recipients with histocompatible donors.

6 References

Abramovitch R, Neeman M, Reich R, Stein I, Keshet E, Abraham J, Solomon A, Marikovsky M. 1998. Intercellular communication between vascular smooth muscle and endothelial cells mediated by heparin-binding epidermal growth factor-like growth factor and vascular endothelial growth factor. *FEBS Lett* 425:441-447.

Albig AR, Becenti DJ, Roy TG, Schiemann WP. 2008. Microfibril-associate glycoprotein-2 (MAGP-2) promotes angiogenic cell sprouting by blocking notch signaling in endothelial cells. *Microvasc Res* 76:7-14.

Alonso L, Fuchs E. 2003. Stem cells of the skin epithelium. *Proc Natl Acad Sci U S A* 100 Suppl 1:11830-11835.

Alviano F, Fossati V, Marchionni C, Arpinati M, Bonsi L, Franchina M, Lanzoni G, Cantoni S, Cavallini C, Bianchi F, Tazzari PL, Pasquinelli G, Foroni L, Ventura C, Grossi A, Bagnara GP. 2007. Term amniotic membrane is a high throughput source for multipotent mesenchymal stem cells with the ability to differentiate into endothelial cells in vitro. *BMC Dev Biol* 7:11.

Au P, Tam J, Fukumura D, Jain RK. 2008. Bone marrow-derived mesenchymal stem cells facilitate engineering of long-lasting functional vasculature. *Blood* 111:4551-4558.

Bailo M, Soncini M, Vertua E, Signoroni PB, Sanzone S, Lombardi G, Arienti D, Calamani F, Zatti D, Paul P, Albertini A, Zorzi F, Cavagnini A, Candotti F, Wengler GS, Parolini O. 2004. Engraftment potential of human amnion and chorion cells derived from term placenta. *Transplantation* 78:1439-48.

Barlow S, Brooke G, Chatterjee K, Price G, Pelekanos R, Rossetti T, Doody M, Venter D, Pain S, Gilshenan K, Atkinson K. 2008. Comparison of human placenta- and bone marrow-derived multipotent mesenchymal stem cells. *Stem Cells Dev* 17:1095-1107.

Ben-David U, Benvenisty N. 2011. The tumorigenicity of human embryonic and induced pluripotent stem cells. *Nat Rev Cancer* 11:268-277.

Benirschke K, Kaufmann P, Baergen R. 2006. Pathology of the human placenta. 5th Edition. Springer Science & Business Media.

Bilic G, Ochsenbein-Kolble N, Hall H, Huch R, Zimmermann R. 2004. In vitro lesion repair by human amnion epithelial and mesenchymal cells. *Am J Obstet Gynecol* 190:87-92.

Bilic G, Zeisberger SM, Mallik AS, Zimmermann R, Zisch AH. 2008. Comparative characterization of cultured human term amnion epithelial and mesenchymal stromal cells for application in cell therapy. *Cell Transplant* 17:955-968.

Caplan AI. 1991. Mesenchymal stem cells. *J Orthop Res* 9:641-650.

Cargnoni A, Di Marcello M, Campagnol M, Nassuato C, Albertini A, Parolini O. 2009a. Amniotic membrane patching promotes ischemic rat heart repair. *Cell Transplant* 18:1147-1159.

Cargnoni A, Gibelli L, Tosini A, Signoroni PB, Nassuato C, Arienti D, Lombardi G, Albertini A, Wengler GS, Parolini O. 2009b. Transplantation of allogeneic and xenogeneic placenta-derived cells reduces bleomycin-induced lung fibrosis. *Cell Transplant* 18:405-422.

Chang YJ, Hwang SM, Tseng CP, Cheng FC, Huang SH, Hsu LF, Hsu LW, Tsai MS. 2010. Isolation of mesenchymal stem cells with neurogenic potential from the mesoderm of the amniotic membrane. *Cells Tissues Organs* 192:93-105.

Chen J, Zhang ZG, Li Y, Wang L, Xu YX, Gautam SC, Lu M, Zhu Z, Chopp M. 2003. Intravenous administration of human bone marrow stromal cells induces angiogenesis in the ischemic boundary zone after stroke in rats. *Circ Res* 92:692-699.

Chia LA, Kuo CJ. 2010. The intestinal stem cell. *Prog Mol Biol Transl Sci* 96:157-173.

Copelan EA. 2006. Hematopoietic stem-cell transplantation. *N Engl J Med* 354:1813-1826.

Cremer M, Treiss I, Cremer T, Hager D, Franke WW. 1981. Characterization of cells of amniotic fluids by immunological identification of intermediate-sized filaments: Presence of cells of different tissue origin. *Hum Genet* 59:373-379.

Crisan M, Yap S, Casteilla L, Chen CW, Corselli M, Park TS, Andriolo G, Sun B, Zheng B, Zhang L, Norotte C, Teng PN, Traas J, Schugar R, Deasy BM, Badylak S, Buhring HJ, Giacobino JP, Lazzari L, Huard J, Peault B. 2008. A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* 3:301-13.

Davis JW. 1910. Skin transplantation with a review of 550 cases at the Johns Hopkins Hospital. *Johns Hopkins Med J*. 15:307-396.

Dawson DW, Volpert OV, Gillis P, Crawford SE, Xu H, Benedict W, Bouck NP. 1999. Pigment epithelium-derived factor: A potent inhibitor of angiogenesis. *Science* 285:245-248.

Decker T, Lohmann-Matthes M. 1988. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *J Immunol Methods* 115:61-69.

Demir R, Kayisli UA, Seval Y, Celik-Ozenci C, Korgun ET, Demir-Weusten AY, Huppertz B. 2004. Sequential expression of VEGF and its receptors in human placental villi during very early pregnancy: Differences between placental vasculogenesis and angiogenesis. *Placenta* 25:560-572.

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E. 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. the international society for cellular therapy position statement. *Cytotherapy* 8:315-317.

Dua HS, Gomes JA, King AJ, Maharajan VS. 2004. The amniotic membrane in ophthalmology. *Surv Ophthalmol* 49:51-77.

Edgar R, Domrachev M, Lash AE. 2002. Gene expression omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res 30:207-210.

Erices A, Conget P, Minguell JJ. 2000. Mesenchymal progenitor cells in human umbilical cord blood. *Br J Haematol* 109:235-242.

Fan W, Crawford R, Xiao Y. 2011. The ratio of VEGF/PEDF expression in bone marrow mesenchymal stem cells regulates neovascularization. *Differentiation* 81:181-191.

Faulk WP, Matthews R, Stevens PJ, Bennett JP, Burgos H, Hsi BL. 1980. Human amnion as an adjunct in wound healing. *Lancet* 1:1156-1158.

Ferrara N, Gerber HP, LeCouter J. 2003. The biology of VEGF and its receptors. *Nat Med* 9:669-676.

Fong CY, Gauthaman K, Bongso A. 2010. Teratomas from pluripotent stem cells: A clinical hurdle. *J Cell Biochem* 111:769-781.

Friedenstein AJ, Gorskaja JF, Kulagina NN. 1976. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp Hematol* 4:267-274.

Gage FH. 2000. Mammalian neural stem cells. *Science* 287:1433-1438.

Gerhardt H, Betsholtz C. 2003. Endothelial-pericyte interactions in angiogenesis. *Cell Tissue Res* 314:15-23.

Gronthos S, Franklin DM, Leddy HA, Robey PG, Storms RW, Gimble JM. 2001. Surface protein characterization of human adipose tissue-derived stromal cells. *J Cell Physiol* 189:54-63.

Gruber R, Kandler B, Holzmann P, Vogele-Kadletz M, Losert U, Fischer MB, Watzek G. 2005. Bone marrow stromal cells can provide a local environment that favors migration and formation of tubular structures of endothelial cells. *Tissue Eng* 11:896-903.

Hao Y, Ma DH, Hwang DG, Kim WS, Zhang F. 2000. Identification of antiangiogenic and antiinflammatory proteins in human amniotic membrane. *Cornea* 19:348-352.

Horwitz EM, Prockop DJ, Fitzpatrick LA, Koo WW, Gordon PL, Neel M, Sussman M, Orchard P, Marx JC, Pyeritz RE, Brenner MK. 1999. Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. *Nat Med* 5:309-313.

Horwitz EM, Gordon PL, Koo WKK, Marx JC, Neel MD, McNall RY, Muul L, Hofmann T. 2002. Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: Implications for cell therapy of bone. *Proc Natl Acad Sci U S A* 99:8932-8937.

Hsieh JY, Fu YS, Chang SJ, Tsuang YH, Wang HW. 2010. Functional module analysis reveals differential osteogenic and stemness potentials in human mesenchymal stem cells from bone marrow and wharton's jelly of umbilical cord. *Stem Cells Dev* 19:1895-1910.

Hu X, Yu SP, Fraser JL, Lu Z, Ogle ME, Wang JA, Wei L. 2008. Transplantation of hypoxia-preconditioned mesenchymal stem cells improves infarcted heart function via enhanced survival of implanted cells and angiogenesis. *J Thorac Cardiovasc Surg* 135:799-808.

Huang H, Bhat A, Woodnutt G, Lappe R. 2010. Targeting the ANGPT-TIE2 pathway in malignancy. *Nat Rev Cancer* 10:575-585.

Hung SC, Pochampally RR, Chen SC, Hsu SC, Prockop DJ. 2007. Angiogenic effects of human multipotent stromal cell conditioned medium activate the PI3K-akt pathway in hypoxic endothelial cells to inhibit apoptosis, increase survival, and stimulate angiogenesis. *Stem Cells* 25:2363-2370.

Impagnatiello MA, Weitzer S, Gannon G, Compagni A, Cotten M, Christofori G. 2001. Mammalian sprouty-1 and -2 are membrane-anchored phosphoprotein inhibitors of growth factor signaling in endothelial cells. *J Cell Biol* 152:1087-1098.

Jiang A, Li C, Gao Y, Zhang M, Hu J, Kuang W, Hao S, Yang W, Xu C, Gao G, Wang Z, Liu Z. 2006. In vivo and in vitro inhibitory effect of amniotic extraction on neovascularization. *Cornea* 25:S36-40.

Kaufmann P, Scheffen I. 1992. Placental Development. In Neonatal and Fetal Medicine. 1st edition. Saunders.

Kim JC, Tseng SC. 1995. The effects on inhibition of corneal neovascularization after human amniotic membrane transplantation in severely damaged rabbit corneas. *Korean J Ophthalmol* 9:32-46.

Kinnaird T, Stabile E, Burnett MS, Lee CW, Barr S, Fuchs S, Epstein SE. 2004. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. *Circ Res* 94:678-685.

Kirchmair R, Gander R, Egger M, Hanley A, Silver M, Ritsch A, Murayama T, Kaneider N, Sturm W, Kearny M, Fischer-Colbrie R, Kircher B, Gaenzer H, Wiedermann CJ, Ropper AH, Losordo DW, Patsch JR, Schratzberger P. 2004. The neuropeptide secretoneurin acts as a direct angiogenic cytokine in vitro and in vivo. *Circulatio*n 109:777-783.

Kita K, Gauglitz GG, Phan TT, Herndon DN, Jeschke MG. 2010. Isolation and characterization of mesenchymal stem cells from the sub-amniotic human umbilical cord lining membrane. *Stem Cells Dev* 19:491-502.

Klein-Soyer C, Azorsa DO, Cazenave J, Lanza F. 2000. CD9 participates in endothelial cell migration during in vitro wound repair. *Arterioscler Thromb Vasc Biol* 20:360-369.

Koc ON, Gerson SL, Cooper BW, Dyhouse SM, Haynesworth SE, Caplan AI, Lazarus HM. 2000. Rapid hematopoietic recovery after coinfusion of autologous-blood stem cells and culture-expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy. *J Clin Oncol* 18:307-316.

Lang I, Schweizer A, Hiden U, Ghaffari-Tabrizi N, Hagendorfer G, Bilban M, Pabst MA, Korgun ET, Dohr G, Desoye G. 2008. Human fetal placental endothelial cells have a mature arterial and a juvenile venous phenotype with adipogenic and osteogenic differentiation potential. *Differentiation* 76:1031-1043.

Le Blanc K, Rasmusson I, Sundberg B, Götherström C, Hassan M, Uzunel M, Ringdén O. 2004. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet* 363:1439-1441.

Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, Lanino E, Sundberg B, Bernardo ME, Remberger M, Dini G, Egeler RM, Bacigalupo A, Fibbe W, Ringden O, Developmental Committee of the European Group for Blood and Marrow Transplantation. 2008. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: A phase II study. *Lancet* 371:1579-1586.

Lee S, Bui Nguyen TM, Kovalenko D, Adhikari N, Grindle S, Polster SP, Friesel R, Ramakrishnan S, Hall JL. 2010. Sprouty1 inhibits angiogenesis in association with up-regulation of p21 and p27. *Mol Cell Biochem* 338:255-261.

Li H, Fredriksson L, Li X, Eriksson U. 2003. PDGF-D is a potent transforming and angiogenic growth factor. *Oncogene* 22:1501-1510.

Liu JW, Dunoyer-Geindre S, Serre-Beinier V, Mai G, Lambert J-, Fish RJ, Pernod G, Buehler L, Bounameaux H, Kruithof EKO. 2007. Characterization of endothelial-like cells derived from human mesenchymal stem cells. *J Thromb Haemost* 5:826-834.

Magatti M, De Munari S, Vertua E, Gibelli L, Wengler GS, Parolini O. 2008. Human amnion mesenchyme harbors cells with allogeneic T-cell suppression and stimulation capabilities. *Stem Cells* 26:182-92.

Magatti M, De Munari S, Vertua E, Nassauto C, Albertini A, Wengler GS, Parolini O. 2009. Amniotic mesenchymal tissue cells inhibit dendritic cell differentiation of peripheral blood and amnion resident monocytes. *Cell Transplant* 18:899-914.

Manini I, Gulino L, Gava B, Pierantozzi E, Curina C, Rossi D, Brafa A, D'Aniello C, Sorrentino V. 2011. Multi-potent progenitors in freshly isolated and cultured human mesenchymal stem cells: A comparison between adipose and dermal tissue. *Cell Tissue Res* 344:85-95.

Martina E, Degen M, Ruegg C, Merlo A, Lino MM, Chiquet-Ehrismann R, Brellier F. 2010. Tenascin-W is a specific marker of glioma-associated blood vessels and stimulates angiogenesis in vitro. *FASEB J* 24:778-787.

Marvin KW, Keelan JA, Eykholt RL, Sato TA, Mitchell MD. 2002. Expression of angiogenic and neurotrophic factors in the human amnion and choriodecidua. *Obstet Gynecol* 187:728-734.

Myatt L, Sun K. 2010. Role of fetal membranes in signaling of fetal maturation and parturition. *Int J Dev Biol* 54:545-553.

O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M, Lane WS, Cao Y, Sage EH, Folkman J. 1994. Angiostatin: A novel angiogenesis inhibitor that mediates the suppression of metastases by a lewis lung carcinoma. *Cell* 79:315-328.

Oswald J, Boxberger S, Jorgensen B, Feldmann S, Ehninger G, Bornhauser M, Werner C. 2004. Mesenchymal stem cells can be differentiated into endothelial cells in vitro. *Stem Cells* 22:377-384.

Parolini O, Alviano F, Bagnara GP, Bilic G, Buhring HJ, Evangelista M, Hennerbichler S, Liu B, Magatti M, Mao N, Miki T, Marongiu F, Nakajima H, Nikaido T, Portmann-Lanz CB, Sankar V, Soncini M, Stadler G, Surbek D, Takahashi TA, Redl H, Sakuragawa N, Wolbank S, Zeisberger S, Zisch A, Strom SC. 2008. Concise review: Isolation and characterization of cells from human term placenta: Outcome of the first international workshop on placenta derived stem cells. *Stem Cells* 26:300-311.

Parolini O, Alviano F, Bergwerf I, Boraschi D, De Bari C, De Waele P, Dominici M, Evangelista M, Falk W, Hennerbichler S, Hess DC, Lanzoni G, Liu B, Marongiu F, McGuckin C, Mohr S, Nolli ML, Ofir R, Ponsaerts P, Romagnoli L, Solomon A, Soncini M, Strom S, Surbek D, Venkatachalam S, Wolbank S, Zeisberger S, Zeitlin A, Zisch A, Borlongan CV. 2010. Toward cell therapy using placenta-derived cells: Disease mechanisms, cell biology, preclinical studies, and regulatory aspects at the round table. *Stem Cells Dev* 19:143-154.

Pasquinelli G, Tazzari P, Ricci F, Vaselli C, Buzzi M, Conte R, Orrico C, Foroni L, Stella A, Alviano F, Bagnara GP, Lucarelli E. 2007. Ultrastructural characteristics of human mesenchymal stromal (stem) cells derived from bone marrow and term placenta. *Ultrastruct Pathol* 31:23-31.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. 1999. Multilineage potential of adult human mesenchymal stem cells. *Science* 284:143-147.

Portmann-Lanz CB, Schoeberlein A, Huber A, Sager R, Malek A, Holzgreve W, Surbek DV. 2006. Placental mesenchymal stem cells as potential autologous graft for pre- and perinatal neuroregeneration. *Am J Obstet Gynecol* 194:664-673.

Pugh CW, Ratcliffe PJ. 2003. Regulation of angiogenesis by hypoxia: Role of the HIF system. *Nat Med* 9:677-684.

Regauer S, Franke WW, Virtanen I. 1985. Intermediate filament cytoskeleton of amnion epithelium and cultured amnion epithelial cells: Expression of epidermal cytokeratins in cells of a simple epithelium. *The Journal of Cell Biology* 100:997-1009.

Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A. 2000. Embryonic stem cell lines from human blastocysts: Somatic differentiation in vitro. *Nat Biotechnol* 18:399-404.

Rowe TF, King LA, MacDonald PC, Casey ML. 1997. Tissue inhibitor of metalloproteinase-1 and tissue inhibitor of metalloproteinase-2 expression in human amnion mesenchymal and epithelial cells. *Am J Obstet Gynecol* 176:915-921.

Sant'anna LB, Cargnoni A, Ressel L, Vanosi G, Parolini O. 2010. Amniotic membrane application reduces liver fibrosis in a bile duct ligation rat model. *Cell Transplant* 20:441-453.

Schenk S, Chiquet-Ehrismann R, Battegay EJ. 1999. The fibrinogen globe of tenascin-C promotes basic fibroblast growth factor-induced endothelial cell elongation. *Mol Biol Cell* 10:2933-2943.

Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC. 1995. Failure of blood-island formation and vasculogenesis in flk-1-deficient mice. *Nature* 376:62-66.

Soncini M, Vertua E, Gibelli L, Zorzi F, Denegri M, Albertini A, Wengler GS, Parolini O. 2007. Isolation and characterization of mesenchymal cells from human fetal membranes. *J Tissue Eng Regen Med* 1:296-305.

Stadler G, Hennerbichler S, Lindenmair A, Peterbauer A, Hofer K, van Griensven M, Gabriel C, Redl H, Wolbank S. 2008. Phenotypic shift of human amniotic epithelial cells in culture is associated with reduced osteogenic differentiation in vitro. *Cytotherapy* 10:743-752.

Stenderup K, Justesen J, Clausen C, Kassem M. 2003. Aging is associated with decreased maximal life span and accelerated senescence of bone marrow stromal cells. *Bone* 33:919-926.

Stolzing A, Scutt A. 2006. Age-related impairment of mesenchymal progenitor cell function. *Aging Cell* 5:213-224.

Szebenyi G, Fallon JF. 1999. Fibroblast Growth Factors as Multifunctional Signaling Factors. *Int Rev Cytol.* 185:45-106.

Takahashi K, Yamanaka S. 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126:663-676.

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131:861-872.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. 1998. Embryonic stem cell lines derived from human blastocysts. *Science* 282:1145-1147.

Verseijden F, Posthumus-van Sluijs SJ, Pavljasevic P, Hofer SO, van Osch GJ, Farrell E. 2010. Adult human bone marrow- and adipose tissue-derived stromal cells support the formation of prevascular-like structures from endothelial cells in vitro. *Tissue Eng Part A* 16:101-14.

Villaron EM, Almeida J, Lopez-Holgado N, Alcoceba M, Sanchez-Abarca LI, Sanchez-Guijo FM, Alberca M, Perez-Simon JA, San Miguel JF, Del Canizo MC. 2004. Mesenchymal stem cells are present in peripheral blood and can engraft after allogeneic hematopoietic stem cell transplantation. *Haematologica* 89:1421-1427.

Wijelath ES, Rahman S, Murray J, Patel Y, Savidge G, Sobel M. 2004. Fibronectin promotes VEGF-induced CD34 cell differentiation into endothelial cells. *J Vasc Surg* 39:655-660.

Wolbank S, Peterbauer A, Fahrner M, Hennerbichler S, van Griensven M, Stadler G, Redl H, Gabriel C. 2007. Dose-dependent immunomodulatory effect of human stem cells from amniotic membrane: A comparison with human mesenchymal stem cells from adipose tissue. *Tissue Eng* 13:1173-83.

Wolbank S, Hildner F, Redl H, van Griensven M, Gabriel C, Hennerbichler S. 2009. Impact of human amniotic membrane preparation on release of angiogenic factors. *J Tissue Eng Regen Med* 3:651-654.

Wu Y, Chen L, Scott PG, Tredget EE. 2007. Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis. *Stem Cells* 25:2648-2659.

Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA. 2007. Induced pluripotent stem cell lines derived from human somatic cells. *Science* 318:1917-1920.

Zhang SJ, Zhang H, Hou M, Zheng Z, Zhou J, Su W, Wei Y, Hu S. 2007. Is it possible to obtain "true endothelial progenitor cells" by in vitro culture of bone marrow mononuclear cells? Stem Cells Dev 16:683-690.

7 Abbreviations

Non-standard Abbreviations and Acronyms	
AEC	3-amino-9-ethylcarbacole
ANGPT	angiopoietin
BSA	bovine serum albumin
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CPD	cumulative population doubling
DAPI	4’,6-diamidino-2-phenylindole
DiI-Ac-LDL	1,1’-dioctadecyl-3,3,3’,3’ tetramethylindocarbo-cyanine perchlorate labeled acetylated low density lipoprotein
DMEM	Dulbecco’s modified eagle’s medium
EC	endothelial cells
EGF	epidermal growth factor
EGM	endothelial growth medium
FBS	fetal bovine serum
FC	fold change
FGF	fibroblast growth factor
hAMSC	human amnion-derived mesenchymal stromal cells
hAEC	human amnion-derived epithelial cells
HBSS	hank’s balanced salt solution
HLA	human leukocyte antigen
HUVEC	human umbilical vein endothelial cells
HRP	horseradish peroxidase
HSC	hematopoietic stem cells
Ig	immunoglobulin
IGF	insulin-like growth factor
IL	interleukin
ISCT	International Stem Cell Society
ITS	insulin-transferrin-sodium selenite
LDH	lactate dehydrogenase
MMP	matrix metalloproteinase

mRNA	messenger ribonucleic acid
MSC	mesenchymal stromal cells
NG2	chondroitin sulfate proteoglycan
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PDGFR	platelet derived growth factor receptor
PlEC	placental endothelial cells
PlGF	placental growth factor
RIN	ribonucleic acid integrity
RIPA	radioimmunoprecipitation assay buffer
RNA	ribonucleic acid
RPL30	ribosomal protein L30
RT-PCR	reverse transcriptase - polymerase chain reaction
Tie	tyrosine kinase with immunoglobulin-like and EGF-like domains
TIMP	tissue inhibitors of metalloproteinases
uPAR	urokinase-type plasminogen activator receptor
VE-cadherin	vascular endothelial-cadherin
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
vWF	von Willebrand Factor

8 Acknowledgements

First of all I want to thank Ingrid for being the best supervisor I could imagine. Thank you so much for your continuous help, motivation and encouragement, and for being a good friend. It was a great pleasure to learn from you and to work with you!
My sincere thanks also go to Berthold for his valuable help, friendly support and for sharing his immense knowledge.
I want to thank Gernot for his help and advices and the fruitful discussions at the Thesis committee meetings.
Many thanks to Prof. Dohr for his support and for giving me the opportunity to carry out the work at the Institute of Cell Biology, Histology and Embryology.
I also want to thank Ornella for her reliable help and for inviting me to her lab where I learned the method of hAMSC isolation.
Many thanks go to all my fellow lab mates at the Histology department: Amin, Christina, Gerit, Gregor, Julia F, Karin, Kiki, and Veronika for their help, for all the fun we had and for being friends. I want to especially thank Gregor and Christina for their assistance and for being great students.
I am particularly grateful to Monika Siwetz for her incessant helpfulness and for sharing her huge knowledge. Further I want to thank Angi for her help in the beginning of my PhD, Kerstin for the many immunostainings, Heidi for providing the culture media, Rudi for helping me with the preparation of the art files, Elisabeth for the electron microscopy analyses, and Sonja and Prof. Sedlmayr for the flow cytometry analyses.
Many thanks to Sandra and Frau Scheiber for their help with administrative matters and to Daniel for his help with computer problems.
Last but not least I want to thank my family and friends.

9 Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgment has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of "Good Scientific Practice".

Please note that parts of this thesis are already published:
König JM, Huppertz B, Desoye G, Parolini O, Fröhlich JD, Weiss G, Dohr G, Sedlmayr P, Lang I. *Amnion-derived mesenchymal stromal cells show angiogenic properties but resist differentiation into mature endothelial cells*. Stem Cells Dev, accepted July 15, 2011.

Graz, September 2011

Julia König