Dissertation

PARACRINE REGULATION OF FETO-PLACENTAL ANGIOGENESIS BY PLACENTAL MACROPHAGES AND TROPHOBLASTS

submitted by

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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 organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the "Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz".

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Jelena Lögl

Graz, September 2018

Disclosures

The contents of this thesis have been published in peer-reviewed journals and permission for re-print of the figures has been given within the Open-Access-Publication agreements of the respective publishing groups.

The thesis is based on the following two publications:

> Loegl J, Nussbaumer E, Hiden U, Majali-Martinez A, Ghaffari-Tabrizi-Wizy N, Cvitic S, Lang I, Desoye G, Huppertz B. Pigment epithelium-derived factor (PEDF): a novel trophoblast-derived factor limiting feto-placental angiogenesis in late pregnancy. Angiogenesis. 2016 Jul;19(3):373-88.
>
> Loegl J, Hiden U, Nussbaumer E, Schliefsteiner C, Cvitic S, Lang I, Wadsack C, Huppertz B, Desoye G. Hofbauer cells of M2a, M2b and M2c polarization may regulate feto-placental angiogenesis. Reproduction. 2016 Nov;152(5):447-55.

All co-authors gave their consent to re-use data from these publications within this thesis.

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Abbreviations

A

Adipose triglyceride lipase	ATGL
Angiopoietin 2	ANGPT2
Arrestin beta	ARRB1

C

CD59 complement fragment	CD59
Chick chorioallantoic membrane	CAM
Colony stimulating factor 1	CSF1
Collagen type IV alpha 1	COL4A1
Collagen type IV alpha 2	COL4A2
Collagen type IV alpha 3/Tumstatin	COL4A3
Collagen type XVIII alpha 1	COL18A1
Conditioned medium	CM
Cytokeratin 7	CK7
Chemokine ligand 10	CXCL10

E

Endothelial nitric oxide synthase	eNOS
Extracellular matrix	ECM
Extracellular-signal related kinases	ERK1/2

F

F1-ATPase beta subunit	ATP5B
Fas ligand	FasL
Feto-placental endothelial cell	fpEC
Fibroblast growth factor	FGF
Fibronectin	FN1
Fibulin 5	FBLN5
First trimester trophoblast cell	FTB
Fluorescence-labelled acetylated low-density-lipoprotein	Dil-Ac-LDL
Fms-related tyrosine kinase 1	Flt1
Fms-related tyrosine kinase 4	Flt4

Focal adhesion kinase	FAK
G	
Gro-beta	CXCL2
Growth-factor-receptor-bound protein	GRB2
H	
Heparin sulphate proteoglycan	HSP
Heparan sulphate proteoglycan of basement membrane	HSPGBM
I	
Insulin like growth factor 2	IGF2
Interferon alpha	IFNA1
Interferon beta	IFNB1
Interferon gamma	IFNG1
Interleukin 4	IL4
Interleukin 6	IL6
Interleukin 12 A	IL12A
Interleukin 18	IL18
Interleukin 1 receptor antagonist	ILR1N
K	
Kisspeptin	KISS1
Kinase insert domain receptor	KDR
L	
Lactate dehydrogenase	LDH
Laminin receptor 1	LR1
Leukocyte cell-derived chemotaxin/Chondromodulin	LECT1
Low density lipoprotein receptor-related protein 6	LRP6
M	
Maspin	SERPINB5
Metalloproteinase inhibitor 1	TIMP1
Metalloproteinase inhibitor 2	TIMP2
Metalloproteinase inhibitor 3	TIMP3
Metalloproteinase inhibitor 4	TIMP4

N	
Neurolipin 1	NRP1
Nitric oxide	NO
Non-catalytic region of tyrosine kinase adaptor protein	Nck
O	
Osteopontin	SPP1
P	
Pigment derived growth factor	PEDF
Phosphatidylinositol 3 kinase	PI3K
Phospholipase C γ	PLCγ
Placental growth factor	PlGF
Placental ribonuclease inhibitor	RNH
Placental tissue macrophage, Hofbauer cell	HBC
Plasminogen activator inhibitor/Serin peptidase inhibitor	SERPINE1
Plasminogen fragment 4	PLG
Platelet factor 4	PF4
Prolactin	PRL
Protein kinase B	PKB
Protein kinase C	PKC
Protein kinase D	PKD
S	
Secreted protein, acidic, cysteine-rich	SPARC
Serpin peptidase inhibitor, clade C/Antithrombin III	SERPINC1
SH2-domain-containing protein tyrosine phosphatase 2	SHP2
SHC (Src homology and collagen homology)-related adaptor protein	Sck
Soluble Fms-related tyrosine kinase 1	sFlt1
Syndecan 3	SDC3
T	
Third trimester trophoblast cell	TTB
Thrombospondin 1	THBS1
Thrombospondin 2	THBS2

Transforming growth factor beta	TGFB1
Trophoblast	TB
Troponin I	TNNI3
Tumor necrose factor α	TNFα
Tumor necrosis factor ligand superfamily, member 15/VEGI	TNFSF15
V	
Vascular endothelial growth factor	VEGF
Vascular endothelial growth factor receptor 1/	VEGFR1
Vascular endothelial growth factor receptor 2/	VEGFR2
Vascular endothelial growth factor receptor 3/	VEGFR3
Von Willebrand factor	vWF

Abstract

Within the placenta, the rapidly expanding feto-placental vasculature needs tight control by paracrine and endocrine mechanisms. Here, we focused on paracrine influence by trophoblasts (TB) and tissue macrophages (Hofbauer cells, HBC). First we aimed to characterize the specific polarisation and phenotype of HBC and investigated the role of HBC in feto-placental angiogenesis. Secondly, we intended to identify differences in regulation of feto-placental angiogenesis by early vs late trophoblast.

HBC: Placental macrophages were isolated from third trimester placentas and their phenotype was determined by presence of cell surface markers (FACS analysis) and secretion of cytokines (ELISA). HBC conditioned medium (CM) was analysed for pro-angiogenic factors, and the effect of HBC CM on angiogenesis, proliferation and chemoattraction of isolated primary feto-placental endothelial cells (fpEC) was determined *in vitro*. Our results revealed that isolated HBC possess an M2 polarisation, with M2a, M2b and M2c characteristics. HBC secreted the pro-angiogenic molecules VEGF and FGF2. Furthermore, HBC CM stimulated *in vitro* angiogenesis of fpEC. However, compared to control medium, chemoattraction of fpEC towards HBC CM was reduced. These findings demonstrate a paracrine regulation of feto-placental angiogenesis by HBC *in vitro*.

TB: The effect of conditioned media (CM) from early and late pregnancy trophoblast was tested on network formation, migration and proliferation of fpEC. Only CM of late pregnancy trophoblast reduced network formation and migration. Screening of trophoblast transcriptome for anti-angiogenic candidates identified pigment epithelium derived factor (PEDF) with higher expression and protein secretion in late pregnancy trophoblast. Addition of a PEDF neutralizing antibody abolished the anti-angiogenic effect of CM from late pregnancy trophoblast. Notably, human recombinant PEDF reduced network formation only in combination with VEGF (vascular endothelial growth factor). Analysis of phosphorylation of ERK1/2 (extracellular-signal related kinases) and FAK (focal adhesion kinase), two key players in VEGF-induced proliferation and migration, revealed that PEDF altered VEGF signalling, while PEDF alone did not affect phosphorylation of ERK1/2 and FAK.

This data suggest that the trophoblast derived anti-angiogenic molecule PEDF is involved in restricting growth and expansion of the placental endothelium predominantly in late pregnancy, whereas HBC are likely to support placental angiogenesis.

Zusammenfassung

Parakrine und endokrine Mechanismen regulieren das schnell wachsende feto-plazentare Blutgefäßsystem der Plazenta. Unser Fokus lag darin, die von Trophoblasten (TB) und Gefäßmakrophagen (Hofbauer Zellen, HBC) freigesetzten Moleküle welche die Angiogenese beeinflussen, zu untersuchen. Zuerst wollten wir den Phenotyp und die Polarisation der HBC charakterisieren, um dann deren Einfluss auf die feto-plazentare Angiogenese zu testen. Mit TB aus dem ersten und dem dritten Schwangerschaftstrimenon wollten wir Unterschiede in der Regulierung der feto-plazentaren Angiogenese ermitteln.

HBC: Plazentare Makrophagen wurden aus der Termplazenta isoliert und ihr Phenotyp wurde mit Hilfe von Zelloberflächenmarkern (FACS Analyse) und der Sekretion von Zytokinen (ELISA) bestimmt. HBC konditioniertes medium (CM) wurde auf pro-angiogene Faktoren untersucht, und der Effekt von dem CM wurde auf Angiogenese, Proliferation und Migrationsanreiz primär isolierter feto-plazentarer Endothelzellen (fpEC) untersucht. Unsere Resultate zeigten, dass isolierte HBC einen M2 Polarisierungstyp haben, wobei sie M2a, M2b und M2c Charakteristika aufweisen. HBC sekretieren die pro-angiogenen Moleküle VEGF und FGF2. Wir fanden, dass HBC CM die Angiogenese von fpEC stimuliert, wobei jedoch die Migration der fpEC zum HBC CM reduziert war. Diese Daten demonstrieren die parakrine Regulation der feto-plazentaren Angiogenese durch HBC *in vitro.*

TB: Der Effekt vom CM von TB aus dem ersten und dem dritten Trimenon wurde auf Netzwerkbildung, Migration und Proliferation von fpEC getestet. Dabei konnte nur das CM der TB aus dem dritten Trimenon die Netzwerkbildung und Migration reduzieren. Anschließend wurde das TB Transkriptom auf anti-angiogene Faktoren gescreent und erzielte ein Molekül, pigment epithelium derived factor (PEDF), welches im letzten Trimester eine hohe Expression und Protein Sekretion erwies. Durch Zugabe des PEDF Neutralisierungsantikörpers wurde der anti-angiogene Effekt des CM aufgehoben. Anzumerken ist, dass das rekombinante humane PEDF nur in Kombination mit VEGF (vascular endothelial growth factor) die Netzwerkbildung reduzierte. Hier fanden wir, dass die Phosphorylierung zwei essentielle Moleküle, ERK1/2 (extracellular-signal related kinases) und FAK (focal adhesion kinase), der VEGF-induzierten Proliferation und Migration erst durch PEDF und VEGF gemeinsam verändert wurden. Wobei PEDF alleine keinen Einfluss auf die Phosphorylierung von ERK1/2 und FAK hatte.

Diese Ergebnisse deuten darauf hin, dass hauptsächlich im dritten Trimenon das vom TB sekretierte anti-angiogene PEDF das Wachstum und die Ausbreitung des plazentaren Endotheliums einschränken, wohingegen HBC die plazentare Angiogenese unterstützen.

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1. Introduction - The Placenta

1.1. Placental function

The placenta as interface between the maternal and fetal compartment is responsible to separate the two blood circulation systems but also enables communication between them. Thus, the main function of this highly specialised organ is support of normal fetal growth and development. As the requests of the fetus change during pregnancy, the placenta needs to adapt to ensure an adequate supply of nutrients and appropriate elimination of waste products. Beside transport, other tasks include metabolism of substances and the release of metabolic products, protection of the fetus from infections and also endocrine homeostasis to maintain a healthy pregnancy (1).

1.2. Placental development and structure

Development of placenta and fetus is a continuous process starting with fertilization. By mitotic divisions, the zygote develops to a morula and enters the uterus. Further divisions and polarization form the blastocyst, which consists of an inner cell mass (embryoblast) and an outer layer of mononucleated trophoblasts (trophectoderm). Implantation of the blastocyst involves movement, adhesion and invasion. After appropriate attachment, the trophoblast layer of the blastocyst undergoes proliferation and fusion (2).

1.2.1. Villous development

Between 12 and 18 days post conception the villous development starts. Primary villi are formed, containing mononucleated trophoblast cells covered by a multinucleated syncytiotrophoblast. These primary villi evolve into secondary villi just a few days later by infiltration of embryonic mesenchyme into these structures. When the first fetal capillaries appear between day 18 and 20 post conception in the villous stroma, the villus enters the next stage and turns into a tertiary villus. This first generation of mesenchymal villi provides the first materno-fetal exchange. All these developmental steps are repeated throughout pregnancy as long as the villous tree expands (2).

Until week 5 post conception all placental villi show this mesenchymal type. Then, they start to transform into immature intermediate villi, which are characterised by an increased diameter and formation of stromal channels. These immature intermediate villi change again. This time, stromal fibrosis starts around central vessels and defines them into arteries and veins. Such villi are called stem villi (3).

Around week 7 the source for newly sprouting villi are no longer trabeculae but mesenchymal and immature intermediate villi. On the surface of these villi trophoblastic spots are generated, so-called hot spots, with a high number of trophoblast cells. These areas are characterised by enhanced trophoblast proliferation throughout gestation. The highly proliferative trophoblasts fuse with the syncytiotrophoblast and lead to syncytiotrophoblastic outgrowths. The next step is that highly proliferative mesenchyme invades these structures and transforms them into villous sprouts (Figure 1) (3).

Until week 23 continuous sprouting of mesenchymal villi ensures further mesenchymal and intermediate villi, which then turn into stem villi. This rapid villous tree expansion provides an appropriate materno-fetal exchange surface. From week 23 a meaningful alteration starts, mesenchymal villi transform into mature intermediate villi. This implies that these villi do not mature into stem villi but form terminal villi. These structures are mainly accountable for materno-fetal exchange, because they contain a higher capillarization along surfaces, a lower number of cytotrophoblasts and no stromal channels (3).

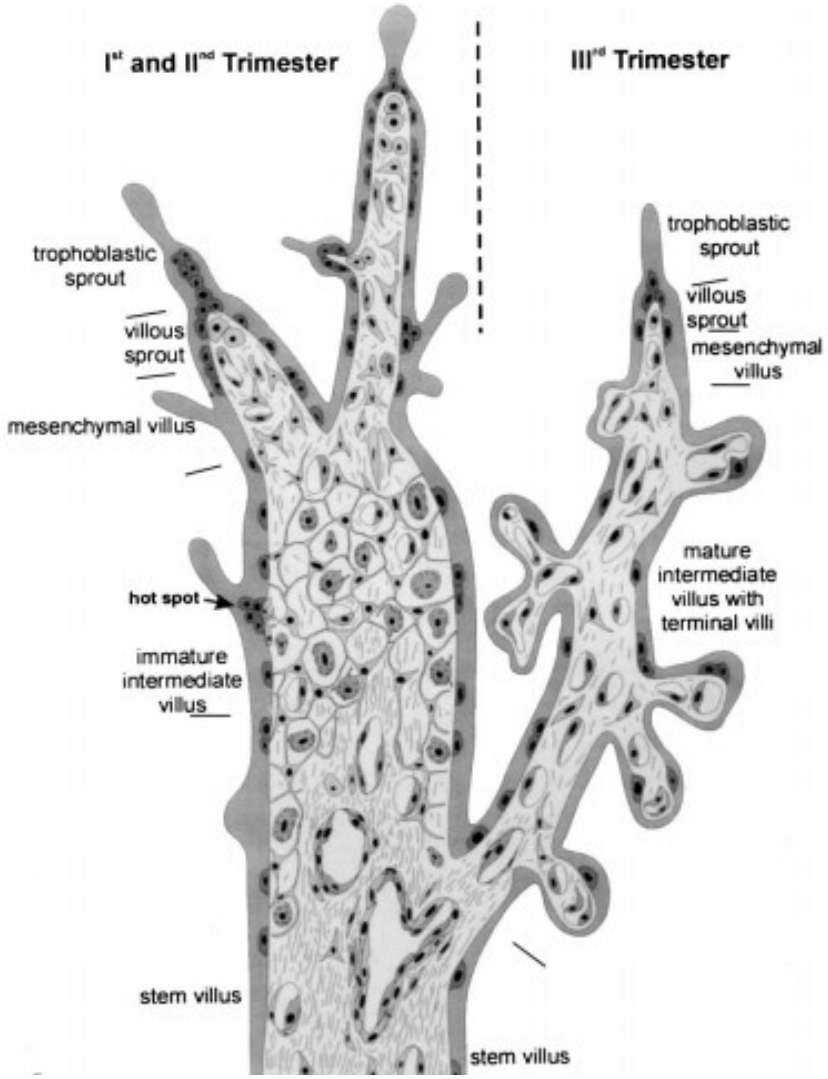

Figure 1. Various villous types during gestation. The left part of the picture represents villous types and structures in the first and second trimester. The right part shows villi from the third trimester. Adapted from (3) with permission (Human Reproduction Update).

1.2.2. Vascular development

In the placenta two distinct processes give rise to blood vessels: vasculogenesis and angiogenesis. Initially, placental vessel formation occurs by vasculogenesis in the early first trimester of pregnancy when endothelial progenitor cells form a primitive vascular network. Angiogenesis, the vessel formation from already existing vessels, is at first accomplished by sprouting of endothelial cells (sprouting angiogenesis) followed by elongation of tubes (non-branching angiogenesis) (4,5).

Placental vasculogenesis occurs solely in the first trimester, where first blood vessels appear from day 18 to day 35 post conception. This first vascularization also means that

secondary mesenchymal villi transform into tertiary mesenchymal villi. During vasculogenesis pluripotent mesenchymal cells pass through several differentiation steps. First, hemangiogenic stem cells develop, which further mature into angioblast cells, the progenitors for endothelial cells or into hemangioblast cells, the progenitors of hematopoietic cells (6). There is evidence that FGF 2 (fibroblast growth factor) plays a pivotal role in the first trimester when first villi with hemangiogenic progenitor cells are generated. VEGF (vascular endothelial growth factor) is required for all steps in vasculogenesis and angiogenesis and is needed from the first trimester until the end of pregnancy. PlGF (placental growth factor) seems to operate together with VEGF for angiogenic processes during second and third trimester (Figure 2). During the third trimester angiopoietins and many other growth factors are higher expressed to mediate branching and non-branching angiogenic processes (7).

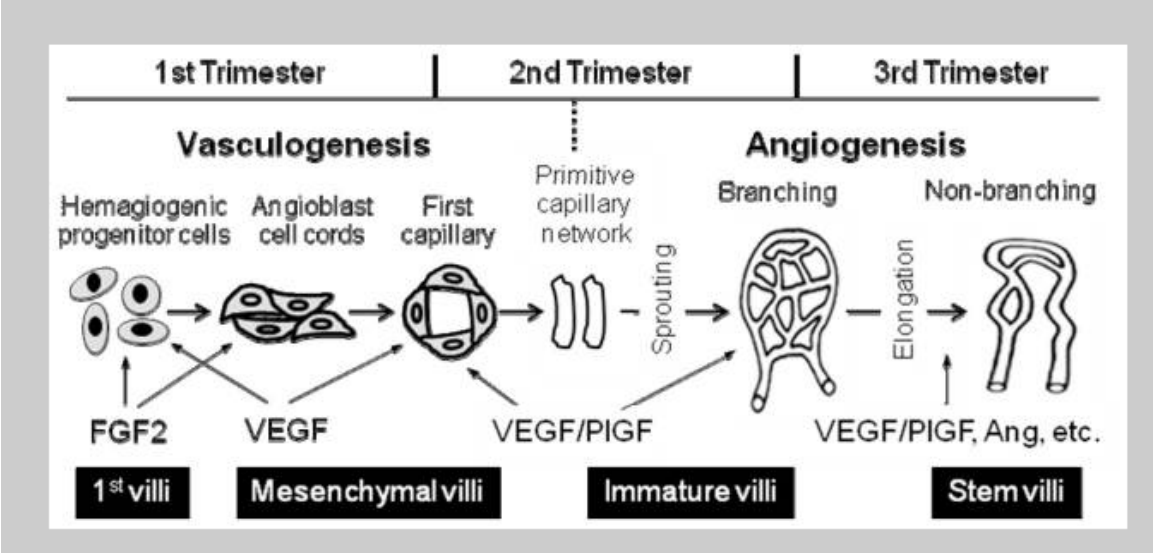

Figure 2. Regulation of placental vasculogenesis and angiogenesis during gestation. FGF2 supports vasculogenesis in the first trimester. VEGF is involved in all vasculogenic and angiogenic processes throughout gestation. PlGF together with VEGF mediates sprouting and elongating angiogenesis from the second trimester onwards. Taken from (7) with permission (Microcirculation).

Placental angiogenesis takes place from day 21 post conception until term. Thus, until week 10 to 12 villous capillaries develop from hemangioblast cells. From then on capillaries

coil, form sinusoids and protrude towards the trophoblastic layer. Around day 32 post conception villous endothelial tubes contact each other and fuse to build the first primitive capillary network (6).

In normal pregnancy, capillary growth occurs via two phases. First, branching angiogenesis forms high numbers of tightly looped capillaries and second, non-branching angiogenesis leads to formation of longer capillaries (5).

There is a suggestion that capillaries and villi are in close relation. Thereby, the trophoblastic layer is changeable and in parallel with, or in response to, the altering vascular structure. This is seen when first and third trimester villi are compared. Thus, immature intermediate villi from early gestational age have a large bulky structure covered by a thick layer of trophoblast with a complex capillary network inside. Whereas, villi from late gestational age predominantly possess filiform structures with a thin trophoblast layer and tightly looped capillaries (5).

The following steps of angiogenesis consist of three different phases, which overlap partly. Step 1: from day 32 post conception to week 25 post conception generation of capillary networks take place by prevalence of branching angiogenesis. Step 2: between week 15 and 32 post conception reconstitution of the peripheral capillaries occurs and central stem vessels form. Step 3: from week 25 post conception until term non-branching angiogenesis leads to terminal capillary loops (Figure 3) (5).

In general, vasculogenesis and angiogenesis need to be tightly regulated otherwise pathologic outcomes may happen. Both, abnormal vasculogenesis and angiogenesis are in correlation with impaired placental and fetal development and result in IUGR (intra-uterine growth restriction), PE (preeclampsia) or GDM (gestational diabetes mellitus).

Pregnancies complicated with IUGR suggest that an imbalance of angiogenesis regulating molecules like VEGF, PlGF and sFlt1 (soluble Fms-like tyrosine kinase 1) may be responsible for an inadequate vascular development (8). PE is known to be associated with decreased angiogenesis and dysfunctional endothelium. Studies displayed that from early gestation pro-angiogenic factors are expressed lower and anti-angiogenic ones higher, when compared to normal gestations. Further, MMPs (matrix metalloproteases) are decreased in PE and may be responsible for impaired trophoblast invasion in the decidua or decreased

endothelial network formation, proliferation and migration (9). Pregnancies complicated with GDM show endothelial dysfunction and increased angiogenesis (10).

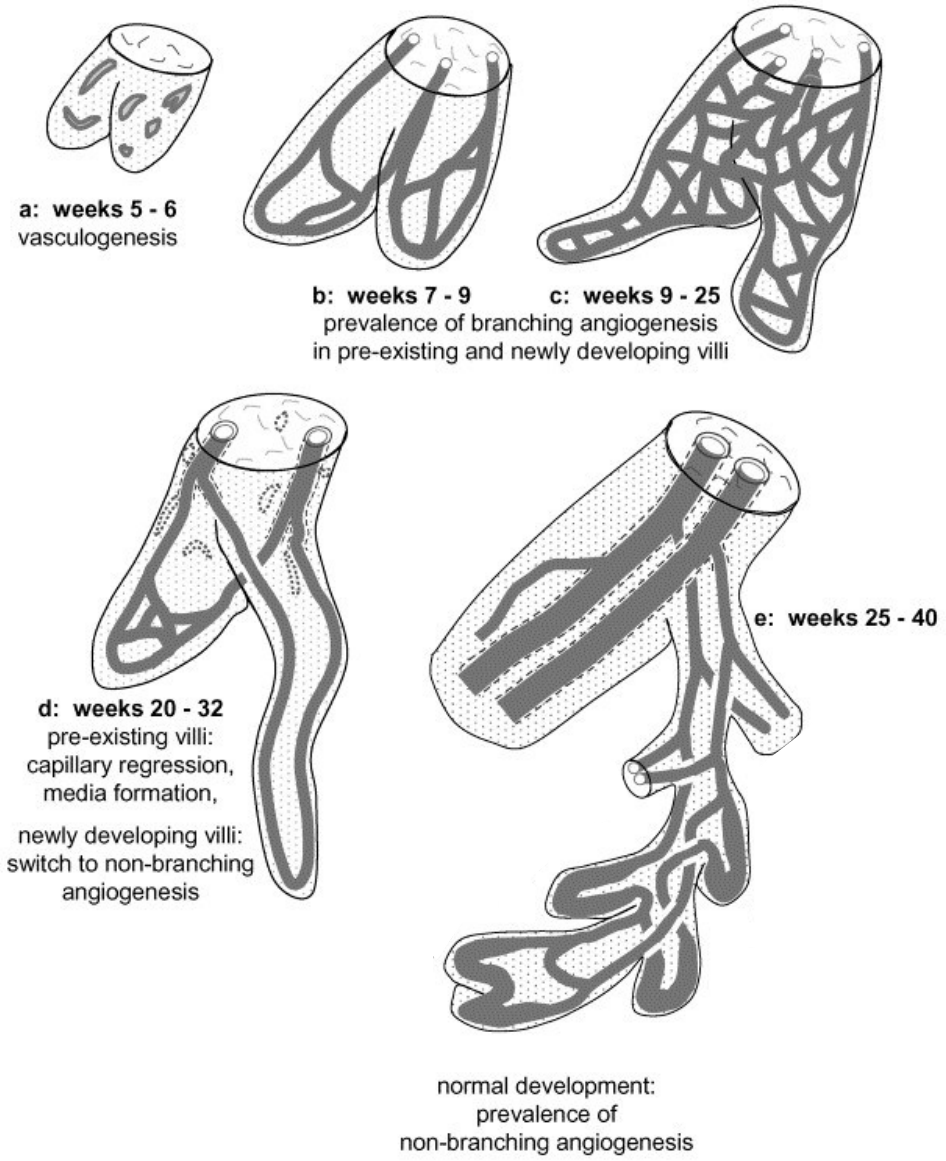

Figure 3. Placental vascular development via vasculogenesis and angiogenesis grouped into different gestational weeks. Adapted from (5) with permission (Placenta).

When angiogenesis takes place the endothelium responds to diverse signals derived from blood, subendothelium or interacting cells and also has influence on them. In general,

the angiogenic process is initiated by a triggering signal leading to increased permeability of the vessel and degradation of the extracellular matrix. Proliferation and chemotactic migration of endothelial cells results in lumen formation. Afterwards, the functional maturation is supported by recruitment of pericytes and smooth muscle cells. When the new vessels complete their assembly, endothelial cells become quiescent again (Figure 4).

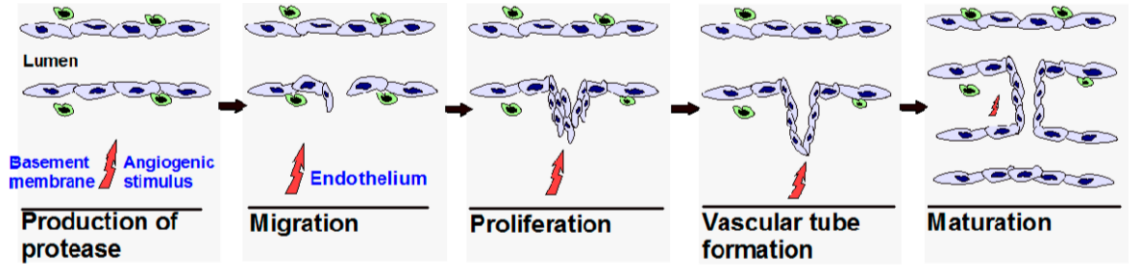

Figure 4. Angiogenic process. Angiogenesis is a step-wise process that starts with a triggering signal (i.e. VEGF) leading to vasodilation and increased permeability of the endothelium. Then degradation of the extracellular matrix enables endothelial cells to migrate and proliferate. After lumen formation pericytes/smooth muscle cells stabilize the vessel and endothelial cells become quiescent again. Adapted from (11) with permission (Biomedicines).

To maintain a normal and healthy vasculature a tight regulation is necessary. Therefore, angiogenesis is balanced by many factors: secreted soluble factors, oxygen, shear stress, components of the extracellular matrix and direct cell-cell interaction with distinct cell types, such as pericytes, fibroblasts and tissue resident macrophages (4,12,13).

1.3. Placental cells

1.3.1. Trophoblast cells

The epithelial layer of the placental villi is the trophoblast. In early pregnancy the villous cytotrophoblast is present as a complete cell layer below the multinucleated syncytial layer. Steady proliferation and differentiation of cytotrophoblast (CTB) cells maintain either the multinucleated syncytiotrophoblast (STB) layer, which is in contact with maternal blood, or the extravillous trophoblast (EVT), which is anchored to the maternal decidua (14). Although the number of cytotrophoblastic cells increases throughout pregnancy, in tissue sections it seems as if their number is reduced. This is caused by the rapid expansion of the mesenchymal villous core, thus separating the cytotrophoblast cells (2,15).

In early pregnancy and early trophoblast development oxygen tension plays a crucial role. This milieu in which low oxygen triggers placental vascularization as well as trophoblast progression changes when maternal blood flow and spiral arteries affect placenta and fetus (16,17). During the time of low oxygen tension in the frist trimester of pregnancy, the placenta expands significantly. Especially, EVT differentiation happens in the first trimester. Thereby, maternal endothelium is partially replaced by EVT to form transformed spiral arteries, which are low-resistance and high-capacity vessels. These ones are able to support the fast changing needs of the steady growing fetus (18).

The villous trophoblast epithelium is separated by a basement membrane from the mesenchymal core. The core comprises different cell types and connective tissue. In early stages the villous core is mostly filled with mesenchymal cells, from second trimester on reticulum cells are the majority within the terminal villi, whereas fibroblasts are within the stem villi (19). Later, various cell types such as endothelial cells, blood cells, tissue macrophages, myofibroblasts, smooth muscle cells/pericytes and fibroblasts fill the stromal space (Figure 5) (20-22).

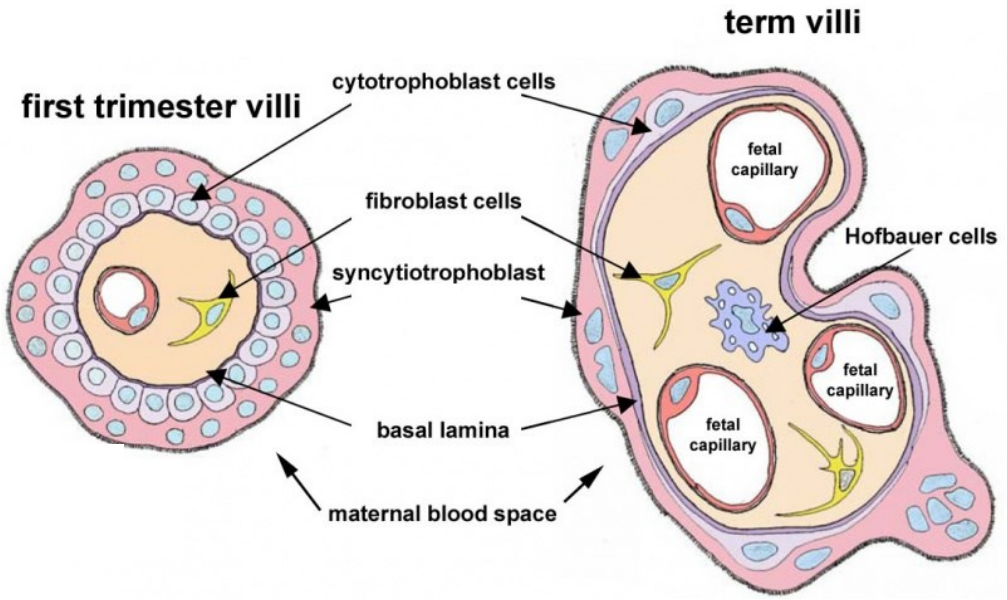

Figure 5. Cross section of chorionic villi from first and third trimester placenta. The outermost layer is the syncytiotrophoblast with the underlying cytotrophoblast cells. The villous core contains fetal capillaries and many other cell types such as fibroblasts and Hofbauer cells (placental macrophages). Adapted from (23) with permission (Biomedicines).

1.3.2. Hofbauer cells

Placental tissue macrophages termed Hofbauer cells (HBC) are abundant in the villous stroma. Although these immune cells have been identified more than 100 years ago, a uniform protocol for isolation and culture of HBC was still lacking. This fact did not discourage researchers to provide insight into potential functions of HBC. Early studies showed phagocytic activities (24) and suggested an anti-inflammatory role in the villus (25). Further studies revealed that placental macrophages may regulate uptake of triglycerides and transfer of fatty acids to the fetus (26) and the stimulating effect on proliferation (27) and differentiation (28) of trophoblasts. Successful isolation of HBCs by *Tang et al.* enabled to use them for *in vitro* studies (29). Cell morphology and shape from cultured HBC were similar to those described in placental villi (30). They are approximately 10–20 µm in size, and manifest a pleiomorphic phenotype with vacuoles. The shape varies from round to partially elongated forms, which arrange to colonies after several days in culture (29). Now it is widely accepted that HBC are of fetal origin. In the first trimester placental macrophages appear before circulation, therefore it is supposed that they arise from mesenchymal

progenitor cells (31). Their appearance during the early phase of vascularization suggests that they influence placental vasculogenesis (32). In the second and third trimester where circulation is completed monocytes may recruit from fetal blood cells and differentiate into HBC (33). As their cell number is very high at term, they might affect angiogenesis during gestation (34). In general, HBC were found to be in the villous stroma adjacent to the trophoblast and feto-placental capillaries (35,36). This proximity of HBC to trophoblasts and endothelial cells suggests on the one hand trophoblast activity (27,28) and on the other hand influence on regulation of feto-placental vascularization (32).

Macrophages play versatile roles, whereby their function is reflected by a heterogeneous profile of phenotypes, which depends on their polarity, i.e. state of activation. The manifestation of the macrophage activation status is determined by the surrounding milieu which they sense (37,38). In general, two distinct states of macrophage polarisation have been defined: M1 macrophages are activated classically, i.e. by interferon γ and LPS, and promote inflammatory processes. M2 macrophages are activated alternatively, i.e. by interleukins, and promote ECM construction, cell proliferation, and angiogenesis (39-41). M2 macrophages can be further discriminated depending on their phenotype and functional properties. M2a macrophages are activated by IL4/IL13 and possess tissue repair and immunoregulating features. Stimulation by immune complexes favours the activation of the M2b phenotype, which supports humoral immunity and allergic reactions. M2c macrophages are stimulated by IL10 or glucocorticoids and induce anti-inflammatory reactions by remodelling the extracellular matrix and suppression of immunity (42,43).

Placental tissue macrophages represent an M2 phenotype (44-46). Their role in the placenta is not yet clearly understood, but due to their location and paracrine abilities, they are thought to be involved in early placental development, placental immunology, as well as development and maturation of the placental mesenchyme throughout pregnancy (28,32,43,47,48). Moreover, the fact that HBC produce and secrete vascular endothelium growth factor (VEGF)(28,49) reinforces the assumption that similar to macrophages in other tissues, HBC may regulate angiogenesis in the placental vasculature (32,50). A study revealed that CSF1 (colony stimulating factor 1) and MIF (migration inhibiting factor) are involved in

trafficking placental macrophages through the placental stroma towards sites where they are needed (51).

1.3.3. Endothelial cells

The endothelium of villous vessels is derived from the mesoderm and participates in several processes including control of vascular tone and blood flow, trafficking of nutrients and gases, and developing and remodelling of the vasculature (5). Usually, veins and arteries display differences in morphology, which result in distinct functions and behaviour. Arteries show a larger diameter while veins have thinner walls. In the placenta veins transport oxygenated and nutrient loaded blood towards the embryo/fetus and arteries carry deoxygenated blood with waste products from the embryo/fetus. Isolated and cultured arterial and venous endothelial cells can be distinguished by their morphology and growth pattern. Arterial endothelial cells possess a polygonal cell shape with a smooth surface and grow in a characteristic endothelial cobble-stone pattern, while venous endothelial cells have a spindle-shaped appearance growing closely to each other, reaching a fibroblastoid form in confluence.

Further, they differ *in vitro* regarding proliferation and generation times, i. e. VEGF induces a higher proliferative response in arterial endothelial cells, while PlGF (placental growth factor) only has an effect on venous endothelial cells (52,53). Unpublished work from our group revealed that only arterial endothelial cells form network-like structures on Matrigel. Venous endothelial cells are also capable of building up network-like structures but need a higher cell density and stimulating factors such as IGF2 (insulin like growth factor 2) (not published). Plating cells on Matrigel prevalently leads to a more differentiated cell type, regarding morphology and gene expression (54). When endothelial cells are plated on Matrigel they attach to the substrate within an hour. In the next few hours they migrate to each other and develop cell-cell contacts. Within twelve hours they form networks by holding strong cell-cell and cell-matrix interactions. This kind of assay is mostly used, because it is highly reproducible, easy to perform and quantifiable, and a high throughput screening is possible (55).

A very important extracellular matrix is the basement membrane. Each tissue covering a surface possesses a specific basement membrane, which provides distinct functionality to this tissue. The matrix is composed of many proteins that vary in quantity and type. The placental basement membranes consist of the underlying layer of the trophoblast and also surround endothelial cells. The placental basement membranes play an important role in villous integrity and vascular maintenance. Collagen IV, laminins and heparan sulfat proteoglycans (HSP) are the main compounds of the placental basement membranes, additionally other components like growth factors, fibronectin, entactin/nidogen and matrix metalloproteinase proenzymes are involved (56,57). Table I gives an overview about basement membrane components and their function in general (57).

Table I. Basement membrane components and their function.

Component	**Function**
Collagens IV and VIII	Stability, adhesion, migration
Entactin/nidogen 1 and 2	Linker, adhesion
Heparan sulfate proteoglycan	Filtration, matrix stability, growth factor binding
Laminins 8 and 10	Adhesion, migration, proteases
Growth factors	Growth and migration
Fibronectin	Adhesion and migration
Matrix metalloproteinase proenzymes	Degradation

The basement membrane is a multifunctional site. Endothelial cells are tightly anchored to this matrix by cell surface receptors. Thus, the endothelial basement membrane enables the endothelial cell to resist blood flow pressure and to sustain vessel integrity. Furthermore, it serves as a barrier and filters waste and nutrients. Another important aspect about this multifunctional site is its capability to bind numerous growth factors, which may play an important role in angiogenesis (58).

1.5. Regulation of angiogenesis by soluble molecules

In general, the placenta is a very rich source of stimulating and inhibiting angiogenic molecules. Researchers already detected some significant factors such as VEGF, FGF2, PlGF, endocrine gland-derived-VEGF, TGF-β1 (transforming growth factor-β1), leptin and angiopoietins but this list is not complete. As counterparts anti-angiogenic factors like sFlt1 and soluble TGF-β1 receptor endoglin are present, and this list is also going to be extended (7).

1.5.1. VEGF and its receptors

The most studied pro-angiogenic molecule is VEGF, which is a crucial regulator of vascular development during embryogenesis as well as blood vessel formation in the adult (59). The human VEGF family consists of five members: VEGFA, VEGFB, VEGFC, VEGFD and PlGF. These factors are active as dimeric glycosylated proteins. They can form hetero- or homodimers and bind to VEGF receptors VEGFR1 (Flt1, Fms-related tyrosine kinase 1), VEGFR2 (KDR, Kinase insert domain receptor) and VEGFR3 (Flt4, Fms-related tyrosine kinase 4) in an overlapping pattern, as well as to co-receptors such as HSPG (heparin sulphate proteoglycans) and neuropilins. Flt1 recognises VEGFA, VEGFB and PlGF, while VEGFA also binds to KDR. Flt4 interacts with VEGFC and VEGFD (Figure 6).

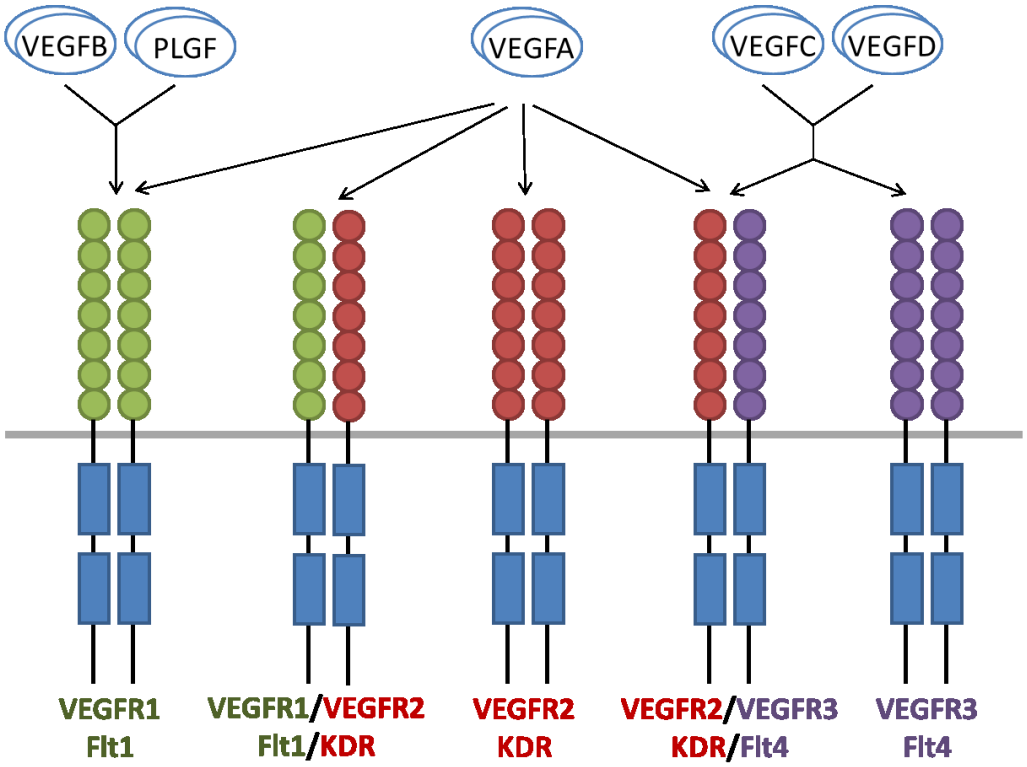

Figure 6. VEGF binding specificities and VEGFR signalling complexes. Five vascular endothelial growth factors, VEGFA, VEGFB, VEGFC, VEGFD, and PlGF, bind with different affinities to three VEGF receptor tyrosine kinases (VEGFR) and two NRP co-receptors initiating homo- and heterodimer formation.

Binding of VEGF to its cognate receptor induces receptor homo- or heterodimerization. The consequence is a receptor conformation change that leads to exposure of the ATP-binding site in the intracellular kinase domain. Then, phosphorylation of tyrosine and other amino acid residues occurs and forms binding sites to intracellular signalling molecules, which result in subsequent downstream signalling cascades. In general, all VEGF receptors have an extracellular portion consisting of 7 immunoglobulin-like domains, a single transmembrane spanning region and an intracellular part including a tyrosine-kinase domain (60,61).

As VEGFA was used for all experiments we focus on this molecule (it is entitled as VEGF in this study) and its main receptor KDR. VEGF exhibits endothelial cell activating properties, it has the capacity to stimulate endothelial cell proliferation and increases vascular permeability. Further, VEGF promotes survival and migration of endothelial cells (62,63).

VEGF is recognised by Flt1 and KDR. VEGF has a 10-fold higher affinity to Flt1 than to KDR (64). However, ligand binding to Flt1 only weakly induces its tyrosine kinase activity. It is suggested that a repressor sequence and the lack of positive regulatory tyrosine residues are responsible for the poor signalling (65,66). Beside its role as reservoir for VEGF, studies revealed that Flt1 is involved in migration and proliferation of endothelial cells. Flt1 activation and dimerization leads to phosphorylation of tyrosine sites which bind and activate molecules such as PLCγ (phospholipase C), GRB2 (growth-factor-receptor-bound protein), Nck (non-catalytic region of tyrosine kinase adaptor protein), SHP2 (SH2-domain-containing protein tyrosine phosphatase 2) and PI3K (phosphoinositide 3-kinase) (60,67), (68,69) leading to migratory and proliferative activities of endothelial cells (Figure 7) (70,71).

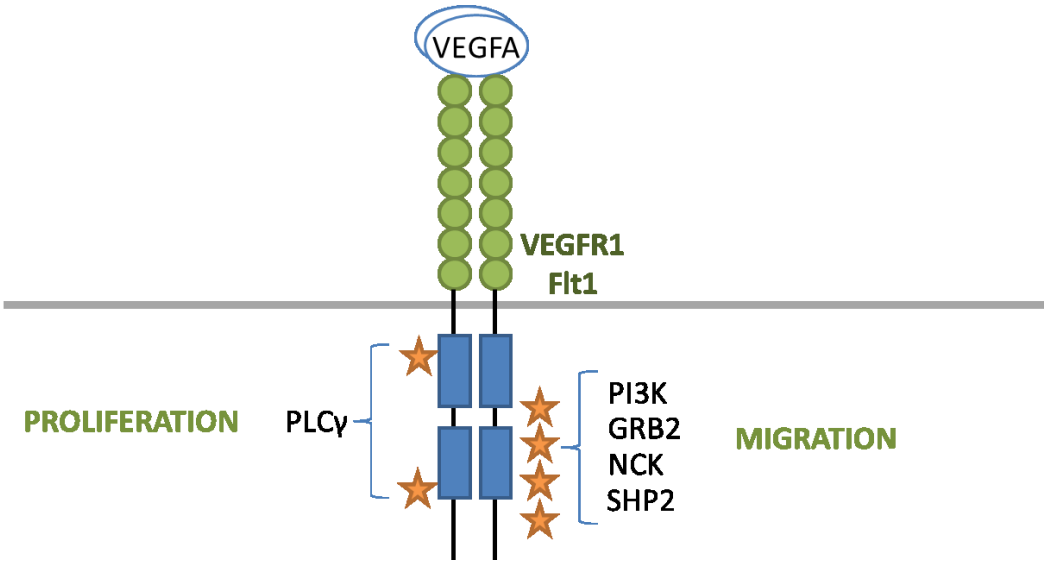

Figure 7. VEGF-mediated signal transduction of VEGFR1. VEGFA-binding to VEGFR1, autophosphorylates tyrosine residues (stars) which recruit signalling molecules and activate downstream mediators resulting in biological responses such as proliferation and migration.

KDR is responsible for the VEGF-mediated major steps of angiogenesis including endothelial survival, proliferation, migration and formation of the vascular tube. VEGF-induced proliferation is progressed by the activation of the ERK1/2 (extracellular signal-regulated kinase) pathway. Here, PLCγ-mediated activation of PKC (protein kinase C) or recruitment of adapter molecules Sck [SHC (Src homology and collagen homology)-related adaptor protein] or GRB2 result in the downstream activation of ERK1/2 (72-75). PKC

activation can further activate PKD (protein kinase D) and regulate migration of endothelial cells (76). VEGF-mediated migration is also regulated by activation of the Src-IQGAP1-complex, the SHB-FAK complex and the Nck-Fyn-CDC42-complex (77-79). Endothelial cell survival and permeability are controlled by PKB (protein kinase B) which is activated by PI3K (phosphatidylinositol 3 kinase), inhibition of caspase 9, 3 and 7 and stimulation of NO (nitric oxide) production by eNOS (endothelial nitric oxide synthase) (Figure 8) (80-81).

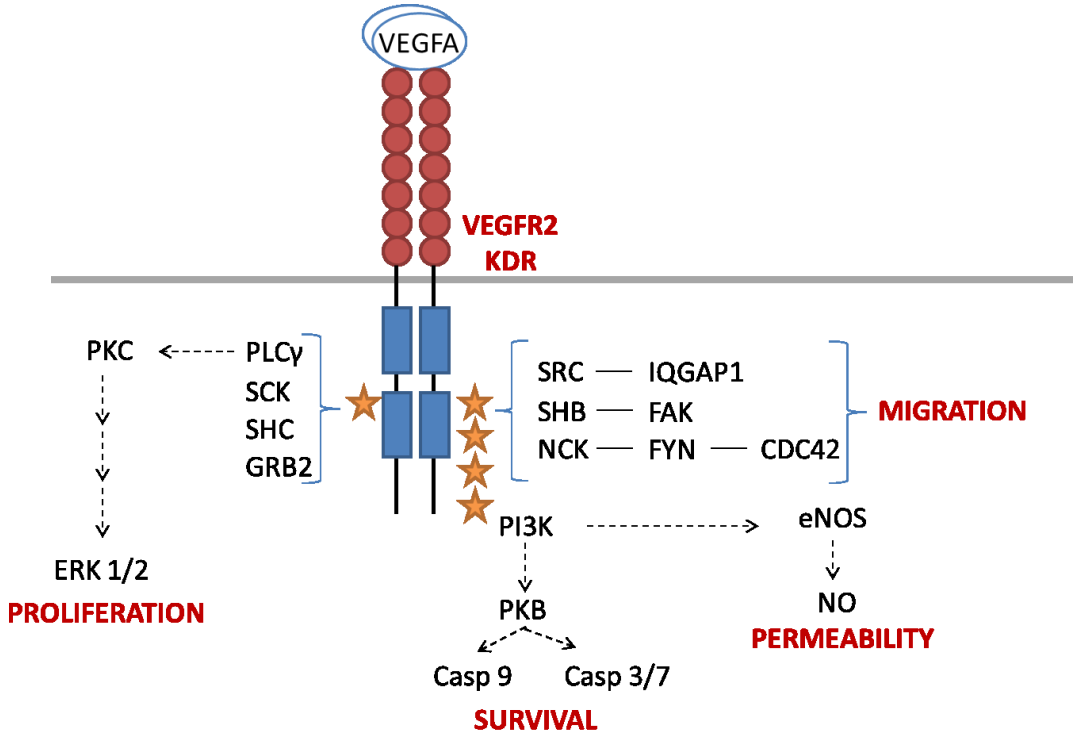

Figure 8. VEGF-mediated signal transduction of VEGFR2. VEGFA-binding to VEGFR2, autophosphorylates tyrosine residues (stars) which recruit signalling molecules and activate downstream mediators resulting in biological responses such as proliferation, migration, survival and permeability. Doted arrows indicate that further molecules are involved.

Flt1 and Flt4 were detected mainly in the syncytiotrophoblast, whereas KDR was identified in endothelial cells of the placenta (82). In the first weeks of gestation placental VEGF is produced intensely by cytotrophoblast cells, whereas the hemangiogenic cell cords show the strongest expression for KDR. During pregnancy, the expression pattern alters and the villous tissue macrophages and mesenchymal cells become strongly immunopositive for VEGF. This switch may support angiogenic remodelling of early vessels, stimulating the formation of a capillary network within the mesenchymal villous core. Studies showed that VEGF and KDR in mice are essential to initiate vasculogenesis (83,84,50).

1.5.2. sFlt1

An alternatively spliced form of Flt1 that encodes a soluble truncated form of the receptor lacking transmembrane and intracellular signalling domains is soluble Flt1 (sFlt1) (85). Soluble Flt1 antagonizes pro-angiogenic proteins such as VEGF and PlGF, which are essential for normal vascular endothelial homeostasis. VEGF activity is inhibited by sFlt1 by sequestering VEGF from signalling receptors and by forming non-signalling heterodimers with Flt1 or KDR (86). With regard to physiological conditions *in vivo*, sFlt1 is known to be essential for avascularity in the cornea (87). Sources of sFlt1 in humans are vascular endothelial cells, vascular smooth muscle cells and placental villous trophoblast (Figure 9) (88).

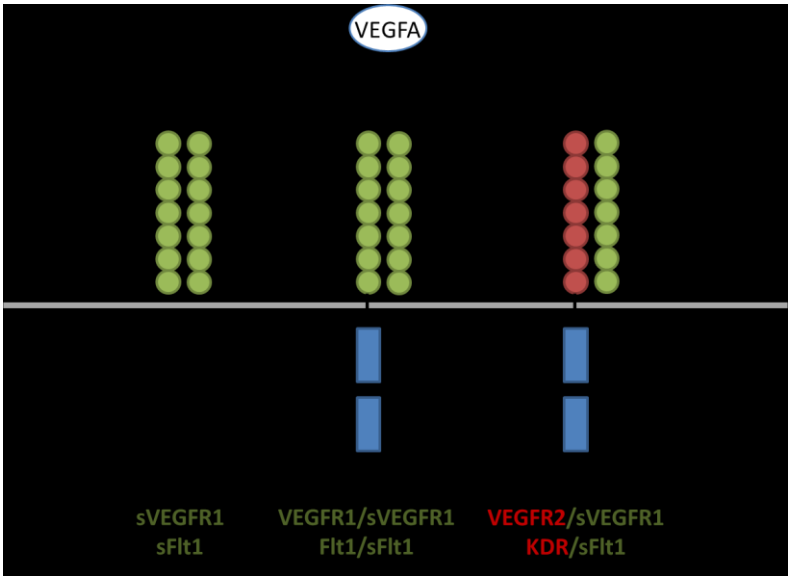

Figure 9. sFlt-1 prevents VEGF signalling. sFlt1 binds free VEGF and prevents its binding to full-length receptors. sFlt-1 also forms heterodimers with membrane-bound VEGFRs but no intracellular signal is initiated.

Elevated levels of sFlt1 in serum and plasma were detected in patients with the pathogenesis of preeclampsia (91). Furthermore, an intimate relationship between the serum levels of sFlt1 and the degree of preeclampsia was demonstrated. A report showed that alterations in sFlt1 and PlGF expression were more pronounced before the onset of PE in women who had preeclampsia before term than in women who had an onset of preeclampsia at term (92). This strongly suggests that abnormal suppression of VEGF by sFlt1 causes hypertension and proteinuria in the patients. Studies revealed that artificial

expression of sFlt1 with a vector system in pregnant rats induces symptoms such as hypertension and proteinuria, suggesting that high levels of sFlt1 may be involved in causing preeclamptic symptoms (91,93).

1.5.3. PEDF and its receptors

PEDF is a further strong anti-angiogenic molecule which is a key factor in this work. It is a non-inhibitory member of the serine protease inhibitor (SERPIN) gene family (94-96). PEDF has anti-angiogenic (97), anti-tumorigenic (98) and anti-inflammatory properties (99). Several binding partners were identified, through which PEDF exerts its anti-angiogenic effects: adipose triglyceride lipase (ATGL) (100), laminin receptor 1 (LR1) (101), F1-ATP synthase (102) and low density lipoprotein receptor-related protein 6 (LPR6) (103). Furthermore, PEDF was shown to bind to VEGFR2 and to compete with VEGF binding (104,105).

There is no direct evidence showing that PEDF's anti-angiogenic activities are mediated through ATGL in endothelial cells, but it has been demonstrated that the nuclear factor kB (NFkB) is regulated in those cells expressing the apoptotic Fas ligand (FasL) (106,107). LR1 in endothelial cells was shown to mediate anti-proliferative, anti-migratory and pro-apoptotic effects by PEDF binding (108). Binding of PEDF to F1-ATPase results in a reduced F1-ATP synthesis activity and subsequently lower ATP levels, which hinders the energy supply for angiogenic events in endothelial cells (102). Stimulation of LRP6 by PEDF induces the Wnt/β-catenin pathway that plays important pathogenic roles in retinal inflammation, neovascularization and vascular leakage in angiogenic eye models (Figure 10) (103,104).

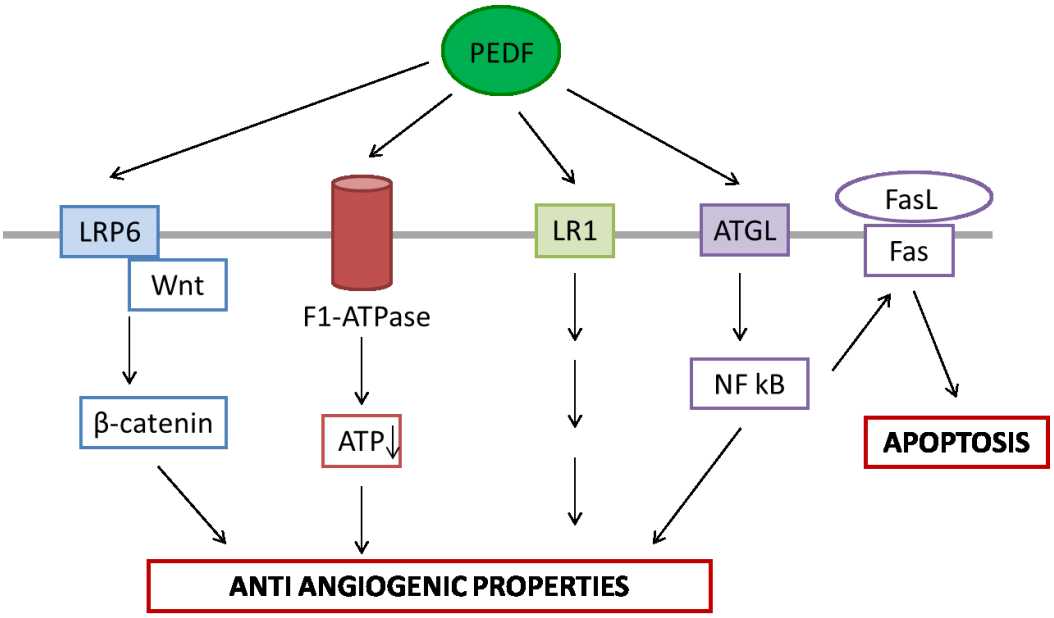

Figure 10. PEDF signaling via different receptors. PEDF binding to LRP6, F1-ATPase, LR1 and ATGL leads to anti-angiogenic effects by blocking survival, proliferation and migration of endothelial cells. ATGL-induced PEDF signalling leads to apoptosis by Fas/FasL activation.

It has been demonstrated that PEDF acts as an inhibitor of VEGF-induced angiogenesis by translocation of the intracellular VEGFR1 domain and by regulating the phosphorylation state of VEGFR1 (71). Another study suggested that PEDF inhibits VEGF-induced vascular permeability and angiogenesis through the SRC kinase pathway (109). PEDF demonstrated a dose dependent stimulation of survival or apoptosis in choroidal neovascularization in mice, pointing out an opposing effect of this molecule (110). Additionally, it was reported that survival and apoptosis occur simultaneously, with the crucial factor being the balance between PEDF and VEGF (111).

2. Hypothesis and Objectives

Successful pregnancy requires appropriate development and adaption of the placental vascular system, as inadequate placental vascular development and angiogenesis result in pregnancy failure or pregnancy pathologies, i.e. fetal growth restriction or preeclampsia (112). The angiogenic process is tightly controlled by several aspects like angiogenesis modulating factors, oxygen, shear stress and components of the extracellular matrix.

In the placenta many different cell types are involved in production and secretion of angiogenesis-related molecules. The trophoblast is a source of soluble pro- and anti-angiogenic factors, suggesting a key role in regulating placental vascular growth. A prominent example of a trophoblast-derived pro-angiogenic factor is VEGF, which has been suggested to promote growth of feto-placental vessels (50). A natural endogenous VEGF inhibitor produced by the trophoblast is sFlt1. Soluble Flt1 is a truncated splice variant of Flt1 (VEGF receptor 1), lacking the membrane-spanning and intracellular kinase domain (86,113).

Another prominent cell type is the placental macrophage, Hofbauer cell (HBC). The role of HBC in the placenta is not yet understood, but due to their location and paracrine abilities, they are thought to play a role in early placental development, vasculogenesis, and development and maturation of the placental mesenchyme throughout pregnancy (114-117,27,28,47). The fact that HBC produce pro-angiogenic factors including vascular endothelium growth factor (VEGF), fibroblast growth factor (FGF) and vasculotropin (118,28,49,50) reinforces the assumption that similar to macrophages in other tissues, HBC may regulate placental angiogenesis in the placental vasculature (32,50).

Against this background we hypothesized that

1) the trophoblast secretes anti-angiogenic factors, which increase in late pregnancy to limit angiogenesis.

2) a paracrine effect of primary human trophoblasts from early and late pregnancy has an effect on the angiogenic potential of isolated feto-placental endothelial cells from term placenta.

3) isolated primary HBC possess a determined activation status, namely M2.

4) HBC derived factors influence the angiogenic potential of feto-placental endothelial cells.

3. Material and Methods

3.1. Tissue collection

Placentas from third trimester were obtained after uncomplicated vaginal delivery or caesarean section, between gestational weeks 36 and 40. First trimester placentas were obtained from elective pregnancy terminations, between gestational weeks nine and eleven. The study was approved by the local ethics committee and all patients gave written informed consent (approval number 25-008 ex 12/13, 12-095 ex 01/02 and 27-265 ex 14/15).

3.2. Cell isolation, culture and characterization

3.2.1. Primary third trimester placental endothelial cells (fpEC)

Primary placental endothelial cells were isolated from third trimester placentas (n=14 for TB CM experiments and n=6 for HBC CM experiments) following a standard protocol (53). In brief, chorionic blood vessels were dissected and endothelial cells isolated by perfusion with a collagenase/dispase (Roche, Germany) solution. Cells were resuspended in endothelial basal medium (EBM, Lonza, USA) supplemented with the EGM-MV BulletKit (Lonza) containing gentamicin/amphotericin, hydrocortisone, human epidermal growth factor (EGF), bovine brain extract, and fetal bovine serum (FBS) (Thermo Scientific, USA), and plated on culture plates precoated with 1% gelatine (Sigma-Aldrich, USA). All cell preparations were subjected to immunocytochemical characterization for identity, purity, and functionality. Isolated placental endothelial cells were grown at 37°C and 20% oxygen, and used up to passage 10.

3.2.2. Primary third trimester placental macrophages (Hofbauer cells, HBC)

For isolation of HBC the protocol developed by *Tang et al.* (2011) was partly modified. Villous tissue (60-100g) was dissected from fetal membranes and large vessels, finely minced and stored overnight at 4°C in phosphate buffered saline buffer (PBS). The next day, the tissue was subjected to three sequential enzymatic digestion steps containing 0.25% trypsin, 0.2% DNase I, 25mM HEPES, 2mM $CaCl_2$, and 0.8mM $MgSO_4$ in Hanks'

balanced salt solution (HBSS, Gibco, Lofer, Austria) at 37°C. The first step was carried out for 15min in 150ml digestion solution, and the subsequent two steps for 30min each in 150ml and 200ml of digestion solution, respectively. After each step, tissue was washed with PBS. The last washing ensued through gauze and a 100µm sieve. Then, tissue was further digested with collagenase A (1mg/ml)/DNase I (0.2mg/ml) in RPMI-1640 containing 25mM HEPES, 5% FBS, and 1% antibiotics–antimycotics for 1h at 37°C. The cell suspension was filtered through gauze and a 70µm sieve and centrifuged at 4°C and 300xg for 10min. Cells were resuspended in RPMI medium and loaded onto a discontinuous Percoll gradient ranging from 70% to 10% and centrifuged without break (27). The band containing cells was taken off and after extensive washing, cells were immunopurified by negative selection using sequential treatment with anti-EGFR (clone528, Cat#MS-609-P0, Thermo scientific, United Kingdom) and anti-CD10 (clone MEM-78, Cat#ab659, Abcam, UK) conjugated to magnetic beads. Remaining cells in the supernatant were counted and plated at 37°C and 20% oxygen in macrophage medium (ScienCell Research Laboratories, San Diego, USA). One gram of tissue gave between 1.2 and $2.9*10^6$ of immunopurified cells. After 1h non-attached cells were removed and the remaining highly adhesive macrophages were cultured further. All cell preparations were subjected to immunocytochemical characterization for identity, purity, and functionality.

3.2.3. Primary third trimester trophoblast cells (TTB)

Primary villous trophoblasts were isolated from third trimester placentas (n=6) as described (27,119). Briefly, minced villous tissue was digested with a trypsin/dispase/DNase solution (Gibco, UK; Roche, Germany; Sigma, USA). After Percoll-gradient (Sigma) centrifugation a negative selection with MCA-81-conjugated magnetic beads (Sigma) was performed to obtain pure cultures of villous trophoblast. Isolated villous trophoblasts were cultured in DMEM (Gibco) supplemented with 10% FBS, 20mM HEPES pH7.4 (Sigma), and penicillin/streptomycin (Sigma). After isolation cells were tested for viability and differentiation by measuring β-human chorionic gonadotropin secretion (Dade Behring, USA) (120). Purity was determined by immunocytochemical staining for the trophoblast marker cytokeratin 7 (CK7, Dako, Denmark) (see table II for details on antibodies) (27). The cells were plated on plastic dishes and cultured at 37°C and 20% oxygen.

3.2.4. Primary first trimester trophoblast cells (FTB)

First trimester villous trophoblasts were isolated (n=4) by enzymatic digestion with trypsin/dispase. Percoll centrifugation and negative magnetic bead immunopurification with the anti-leukocyte marker CD45 (Invitrogen, Norway) and anti-fibroblast marker CD90 (Dianova, Germany) were performed as described earlier (121)(121). Purity was checked by immunocytochemical staining for cytokeratin 7 (CK7, Dako). After isolation first trimester trophoblasts were resuspended in keratinocyte medium (Gibco) supplemented with the keratinocyte SFM kit (Gibco) containing epidermal growth factor (EGF1-35), bovine pituitary extract (BPE) and FBS. Cells were seeded on plastic dishes and cultured at 37°C and 20% oxygen.

3.3. DiI-Ac-LDL uptake

Fluorescence-labelled acetylated low-density-lipoprotein (DiI-Ac-LDL) (Biomedical Technologies, Stoughton MA) was used to indicate presence of the M2 marker and LDL binding protein CD36 and thus to determine purity and viability of isolated HBC (122). DiI-Ac-LDL was added in a final concentration of 10µg/ml to the culture medium. After incubation for 4h at 37°C cells were washed with PBS and DiI-Ac-LDL uptake was observed and quantified by a Zeiss Cell Observer microscope with an AxioCam HRm camera and an A-Plan 5x/0.12 Ph0 objective using AxioVision software (Carl Zeiss Imaging Solutions GmbH). Feto-placental endothelial cells were also capable of ingesting fluorescent-conjugated ac-LDL, but in a much lower extent, while placental trophoblasts did not incorporate ac-LDL (Figure 11).

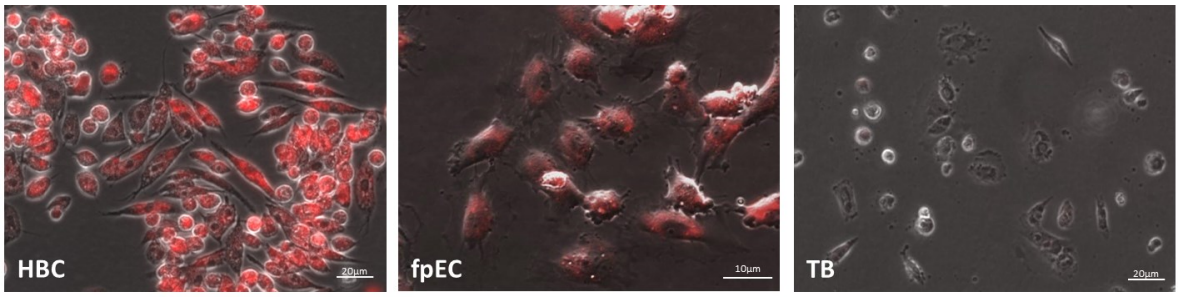

Figure 11. Ac-LDL uptake by HBCs and fpEC. HBC, fpEC and TB were incubated with 10µg/ml dil-ac-LDL for 4h at 37°C. Ac-LDL ingestion was highest by HBC and occurred at lower levels also in fpEC. TB did not incorporate ac-LDL. A representative image of three different experiments is shown. Taken from (123) with permission (Reproduction).

3.4. Growth of HBC on different extracellular matrices

To identify optimal culture conditions for HBC, cell adhesion on different extracellular matrices (ECM) was tested. Tissue culture polystyrene, gelatine and two major ECM components of the placenta, i.e. fibronectin and collagen I (Cao et al. 2003) were used. Culture plates were coated for 1h at 37°C either with 1% gelatine, with 3µg/cm^2 rat collagen I (Culturex, R&D systems, Minneapolis, USA), or for 30min at 37°C with 1µg/cm^2 of bovine fibronectin (Culturex, R&D systems). Prior to plating of HBC (1.5x10^{6}cells/ml), the coated wells were washed with PBS. After 1h, cells were washed with PBS to remove non-adhered cells. HBC were monitored by a Zeiss Cell Observer microscope with an AxioCam HRm camera and an A-Plan 5x/0.12 Ph0 objective (Carl Zeiss Imaging Solutions GmbH) for five days. Since best attachment of HBCs was observed by using tissue culture polystyrene and fibronectin, cells were grown under this condition for all further experiments (Figure 12).

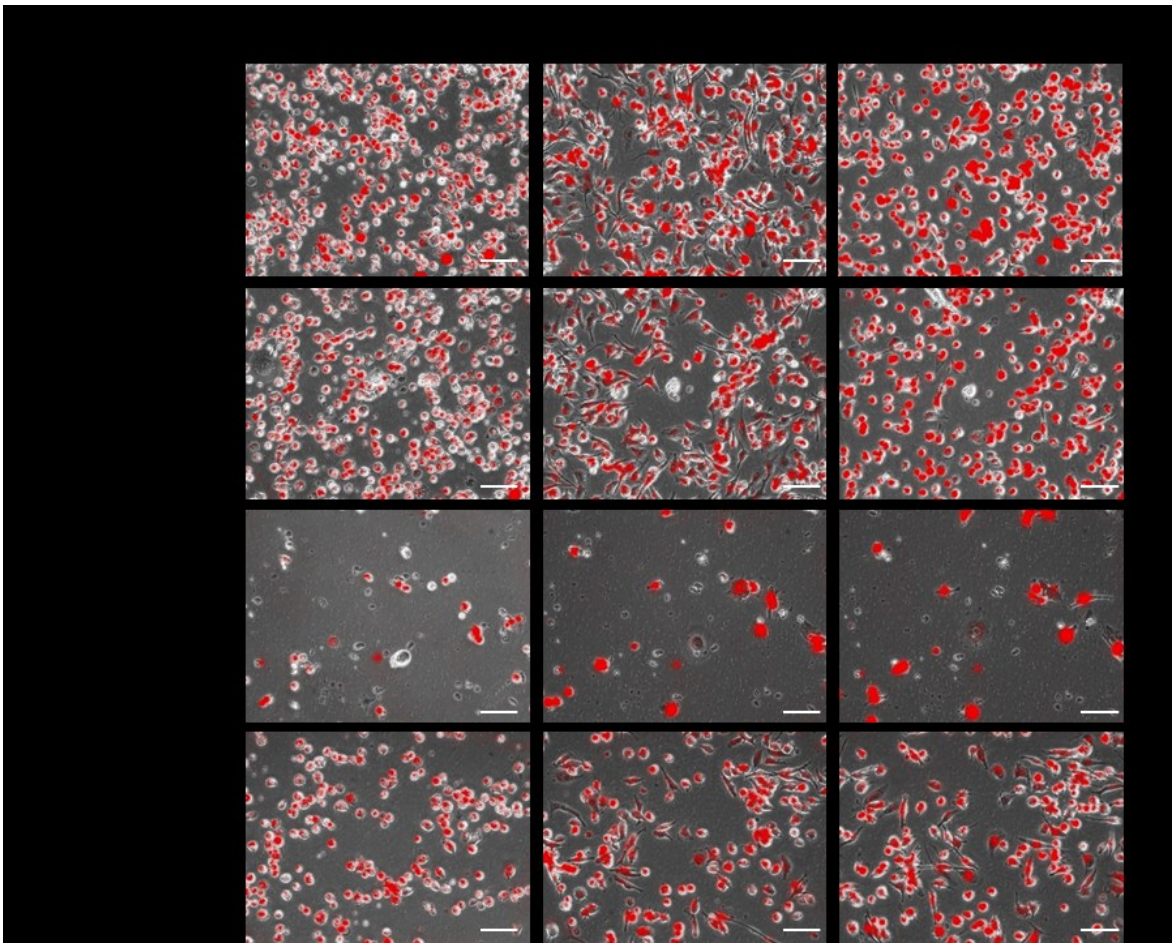

Figure 12. HBC adherence to different extracellular matrices. Freshly isolated HBC were seeded on tissue culture polystyrene, fibronectin, collagen I and gelatine coated culture dishes. After one hour cells were washed and revealed different adherence, morphology and movement. Best attachment was achieved on tissue culture polystyrene and fibronectin. Representative images of three different experiments are shown. Scale bar 40µm. Taken from (123) with permission (Reproduction).

3.5. Conditioned medium

Freshly isolated first and third trimester trophoblasts were seeded at a density of $3x10^6$ cells per 2ml in their appropriate medium to recover. Isolated HBC ($1.5x10^6$/ml) were seeded in macrophage medium with supplements (ScienCell Research Laboratories, San Diego, USA) to recover. After 24h the medium was changed to DMEM/EBM (DE, 1:1) with 7.5% FBS. After another 48h incubation, the conditioned medium (CM) was aspirated and centrifuged for 5min with 300xg to remove dead cells and cell debris. CM was aliquoted and stored at -80°C. CM was pooled to enable comparable testing with various assays using the same CM pool. At least 2 pools of first and third trimester trophoblast from two to four

different isolations were used. As a control (non-CM), DMEM/EBM with 7.5% FBS was incubated at the same conditions, but without cells.

3.6. Flow Cytometry

HBC specific cell surface markers were determined by flow cytometry. 4 days after isolation, cells were detached by gently scraping them (Costar, New York, USA) in HBSS. Cell suspension was centrifuged at 300xg at 4°C for 5min. Then, Fc-receptors were blocked by incubation with 3%FBS/HBSS for 10min at 4°C. PE-conjugated mouse anti-human CD45 (Cat#555483, BD), V450-conjugated mouse anti-human CD80 (Cat#560442, BD), APC-conjugated mouse anti-human CD163 (Cat#333609, BioLegend), V450-conjugated mouse anti-human CD11b (Cat#560481, BD), PerCP-Cy5.5 conjugated mouse anti-human CD209 (#558263, BD), V450-conjugated mouse anti-human CD86 (Cat#560367, BD), and isotype-matched controls (Cat#345816, Cat#561504, Cat#345818, BD) were added and incubated for 30min at 4°C. For identification of potential contaminating fibroblasts, PE anti-human CD90 (Cat#328110, BioLegend) was used. Afterwards, cells were washed twice with PBS and resuspended in 150µl PBS for analysis.

After setting the forward scatter threshold to exclude cell debris, ten thousand events were collected using FACS LSRII and Diva software (BD Biosciences, Franklin Lakes, NJ, USA). The results were analyzed using FlowJo software (Tree Star, Ashland, OR, USA). The percentage of positive cells was based on comparison with the isotype-matched control antibody, for which gating was set at 1%.

3.7. *In vitro* network formation assay

To observe network formation, $1x10^4$ feto-placental endothelial cells were resuspended in conditioned/treatment medium and plated on growth factor reduced Matrigel (BD Bioscience, USA). Cellular network structures were visualized after 12h incubation by a Zeiss Cell Observer microscope with an AxioCam HRm camera and an A-Plan 5x/0.12 Ph0 objective using the software AxioVision (Carl Zeiss Imaging Solutions GmbH). For quantification the total tube length, the branching points and the number of meshes were analysed by the ImageJ software (NIH) using the AngioJ-Matrigel assay plugin, kindly provided by Diego Guidolin (Department of Human Anatomy and Physiology, Section of Anatomy, University of

Padova, Italy) (124). Total network length, number of branching points and meshes were counted. As representative parameter total tube length can be used because branching points and number of meshes show the same trend (not shown).

3.8. Migration/Chemoattractant assay

Chemo attraction of feto-placental endothelial cells towards the conditioned medium was observed using a 96-well chemotaxis microplate system (Neuro Probe Inc., UK). After serum starvation of feto-placental endothelial cells for 3h in EBM, $1x10^4$ cells per well were placed in the upper part of the chemotaxis system, which was separated from the lower well by a fibronectin-coated polycarbonate filter with 8µm pores. Cells were allowed to migrate toward chemo attractants in the lower well (CM) for 4h at 37°C. As positive control, DE medium supplemented with FBS and growth factors (EGM-MV BulletKit, Lonza) was used. The upper surface of the filter was wiped clean of non-migrating cells. Cells were fixed with 4% formaldehyde and stained with DAPI (Invitrogen, USA). Subsequently, the microplate was observed by a Zeiss Axioplan fluorescence microscope and a 10x objective using the AxioVision software (Carl Zeiss Imaging Solutions GmbH). From each filter well 35 pictures were taken. Out of these, 7 pictures were randomly selected and analysed using DotCount v1.2 (online provided by Martin Reuter, MIT).

3.9. Proliferation assay

Proliferation of feto-placental endothelial cells was assessed after treatment with conditioned/treatment medium using the BrdU ELISA kit (Cyclex, Japan) according to the manufacturer's recommendations. $6x10^3$ cells per well were seeded in a 96-well plate. After 24h the medium was replaced by CM and cells were incubated for another 24h. Subsequently, BrdU was added to a final concentration of 10µM and incubated for 2h. Cells were fixed, denatured and incubated with the fluorescence-conjugated monoclonal antibody against BrdU. Absorbance was measured immediately at 450nm/540nm using the FluoSTAR Optima 413 spectrofluorometer (BMG Lab technologies, Germany).

3.10. LDH assay

Cytotoxicity of conditioned/treatment medium on feto-placental endothelial cells was tested by measurement of released lactate dehydrogenase (LDH, Takara, Japan) according to the manufacturer's instructions. $6x10^3$ cells per well were seeded in a 96-well plate and incubated with the CM for 24h. Absorbance was measured immediately at 490nm/650nm using the spectroMax 250 molecular devices microplate reader (MWG-Biotech, Germany).

3.11. Chicken chorioallantoic membrane (CAM) assay

To determine the effect of CM on angiogenesis, the *ex ovo* chorioallantoic membrane (CAM) assay was performed. Briefly, fertilized white leghorn chicken (Gallus domesticus L.) eggs (Schropper GmbH, Gloggnitz, Austria) were incubated for three days at 37.6°C and 70 to 75% relative humidity (J. Hemel Brutgeräte, Am Buschbach, Germany). Eggs were then opened into plastic weigh boats covered with square Petri dishes and returned to the incubator. On day ten, six on-plants per egg were placed on the CAM vasculature. The on-plants consisted of a silicone ring containing either FTB CM, TTB CM or non-conditioned control medium, each on 4 different eggs. On day 3, vascularisation of the on-plants was scored by a blinded observer using a 5 partite scale between -2 and +2.

The anti-angiogenic potential of PEDF in combination with VEGF was evaluated. Silicone rings contained either collagen (1mg/ml) mixed with PEDF (10ng/ml) or VEGF (25ng/ml), or both, in DMEM:EBM supplemented with 7.5% FCS. As control, collagen mixed with medium alone was used. For both settings, vessel sprouting was monitored under a microscope (Olympus stereomicroscope SZX16, Tokyo, Japan) immediately after application of the silicone ring on day 0 and every 24h for four days.

3.12. RNA isolation, array hybridization and data analysis

RNA was isolated with Trizol (MRC, Cincinnati, OH, USA) followed by quality assessment using a bioanalyser (Agilent, Palo Alto, CA, USA). Experimental procedures and data analysis followed recommended standards (125). Total RNA from ten preparations per cell type (FTB and TTB), isolated from different placentas, was pooled. Using 5µg of pooled

RNA, cDNA was synthesised (SuperScript Double-Stranded cDNA Synthesis Kit; Invitrogen, Carlsbad, CA, USA), transcribed *in vitro* (RNA Transcript Labeling Kit; Enzo diagnostics, Farmingdale, NY, USA) and then fragmented. To test the quality of the cRNA, it was hybridised against Test-3 arrays (Affymetrix, Santa Clara, CA, USA). As samples passed the quality criteria (bioC, bioD and cre were present, the 3':5' ratio of the polyA controls was <3), the cRNAs were hybridised against Affymetrix HU133A chips. RNA preparation and hybridisation followed the Affymetrix user manual.

Data analysis of raw data was normalised globally and processed with Microarray Suite, version 5.0 (Affymetrix) and Data Mining Tool (Affymetrix) software (126). Genes that met the following three criteria were classed as being differentially expressed: (1) fold change ≥1.5 or ≤−1.5; (2) change in p value ≥0.992 or ≤0.008; and (3) at least one signal intensity (control or treatment) >100. Annotations were obtained from NetAffx (available at http://www.affymetrix.com, last accessed in December 2013).

3.13. Quantitative reverse transcription PCR (RT-qPCR)

Total RNA was isolated from FTB and TTB using the RNeasy mini Kit (Qiagen, Hilden, Germany). The quality and integrity of the RNA was determined by the ratio of spectrophotometric absorbance 260nm/280nm measured with the Scandrop 250 (Analytik Jena AG, Germany). The cDNA was synthesized from 250 ng total RNA according to the manufacturer's instructions (SuperScript II Reverse Transcriptase protocol from Invitrogen, USA). 10ng/µl of cDNA were used on a total reaction volume of 10µl in the ABI Prism 5,700 Sequence Detection System. RT-qPCR for PEDF was performed using the TaqMan assay Hs01106937_m1 (Applied Biosystems, CA, USA). Mean expression of the housekeeping gene ribosomal protein L30 (RPL30 Hs00265497_m1; Applied Biosystems) was used to normalize gene expression with the $2^{-\Delta\Delta ct}$ method.

3.14. Quantification of PEDF and sFlt1 in trophoblast conditioned medium

The PEDF and sFlt1 concentrations in CM were measured using immunoassays (Biovendor-R&D Products, Minneapolis, USA) according to the manufacturer's instructions.

3.15. Quantification of VEGF, FGF, TNFα and PlGF in HBC conditioned medium

VEGF, FGF2, PlGF and TNFα concentrations in CM were measured using immunoassays (Peprotech, Vienna, Austria) according to the manufacturer's instructions.

3.16. ELISA and multiplex immunoassay

IL1RN, IL6, TNFα, and PlGF concentrations in CM were measured by using immunoassays (Peprotech, Vienna, Austria) according to the manufacturer's instructions. Secretion of FGF2 and VEGF from HBC was assessed by multiplex immunoassay on beads (Aimplex, YSL Bioprocess Development Co.). HBC (n=5) were cultured up to 4 days, CM taken every 24h. The multiplex experiment was carried out according to the manufacturer's instructions. Bead signals were quantified using a FACS Calibur instrument (Becton Dickinson), and FlowCytomixPro software (eBioscience) was used for calculation of standard curves and sample concentrations.

3.17. Dot blot assay

To determine VEGF concentrations in trophoblast conditioned medium, total protein concentration of CM was determined by a BCA protein assay (Thermo Scientific), according to the manufacturer's instructions. 100µg of each sample and increasing concentrations of VEGF (Sigma) were spotted on a nitrocellulose membrane and air dried. For blocking of non-specific sites the membrane was soaked in 5% non-fat milk for 30min. Then, the membranes were incubated with the primary antibody (anti-VEGF, Proteintech, 1:1000) for 1h, washed three times (10min) and the secondary antibody (anti-rabbit, Biorad, California, USA, 1:1000) was applied for 30min. After three washing steps, the membrane was incubated with SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific) for 5min and observed by a Biorad lumino image analyser. The signals were quantified by Alpha DigiDoc software and standards used to determine the concentrations of the samples.

3.18. Neutralizing anti-PEDF antibody

Trophoblast-released PEDF in CM was blocked by using a neutralizing anti-PEDF antibody (BioProducts, Middletown, USA). A non-specific antibody (Biorad) served as isotype control. Antibodies were used at a concentration of 5µg/ml.

3.19. Treatment with pro- and anti-angiogenic factors

For all treatments and dilutions DMEM/EBM with 7.5% FBS was used. Pigment epithelium derived factor (PEDF, Prospec, Israel) was used at final concentrations of 0.05; 0.25; 0.5; 2.5; 5 and 10ng/ml. The concentrations of 5 and 10ng/ml were also combined with 25ng/ml VEGF (Sigma). Soluble Flt1 (sFlt1, Genway Biotech, USA) was used at final concentrations of 1; 5; 10; 20; 100; 200 and 1000ng/ml. The concentrations of 100 and 200ng/ml were also combined with 25ng/ml VEGF.

3.20. Immunocytochemistry

Hofbauer cells (250.000 cells per 1.7 cm^2 chamber) were grown on chamber slides and fixed with ice-cold acetone (Merck, Darmstadt, Germany) for 5min. The presence of HLA-DR, CD206 and CD14 was detected using Ultra Vision HRP Polymer (Thermo Scientific, Runcorn, UK). Slides were washed in PBS (phosphate buffered saline) pH7.5 for 5min. Non-specific binding sites were blocked with UV block (Lab vision) for 10min. Subsequently, the primary antibodies rabbit anti-CD206 (#NBP1-90020PEP, Novus Biologicals, Littleton, CO), rabbit anti-human HLA-DR (#ab92511, Abcam), mouse anti-human CD14 (#MS-1080, Thermo Scientific) diluted in Dako antibody diluent were applied for 30min. After three washing steps in PBS primary antibody enhancer was applied and incubated for 10min. Following further washing steps in PBS, the slides were incubated with HRP Polymer for 15min and washed again. Then, chromogenic reaction was started by addition of peroxidase-compatible chromogen (Thermo Scientific). After washing in aqua dest, Haemalaun (Sigma) solution was used for nuclear counterstaining. Slides incubated with rabbit or mouse unspecific immunoglobulin fractions (rabbit and mouse IgG1 negative control, Dako) using the same concentration as the primary antibody served as negative controls.

3.21. Immunohistochemistry: Fluorescence staining

For co-localization of soluble factors and their receptors to distinct cell types, 5µm sections were cut and placed on Superfrost Plus slides (Menzel, Braunschweig, Germany). Paraffin embedded sections were deparaffinised in xylene and rehydrated through a series of graded alcohol. Heat-induced antigen retrieval was performed in epitope retrieval solution at pH9 (Leica Biosystems Newcastle Ltd., Newcastle, UK) for VEGF/cytokeratin 7 (CK7), PEDF/CK7 and VEGF receptor 2 (KDR) /von Willebrand factor (vWF). For laminin receptor 1 (LR1)/vWF antigen retrieval was performed in citrate puffer pH6. Slides were boiled in a pressure cooker for 7min at 120°C and allowed to cool down for 20min before being rinsed in wash buffer [phosphate-buffered saline, 0.05% Tween-20 (PBS/T), pH 7.4]. All further steps were performed at room temperature. Slides were incubated with an Ultra V-Block (Thermo Scientific) for 7min. Then, slides were rinsed in PBS/T three times before applying both primary antibodies for 45min. Primary antibodies (Table II) were diluted in antibody diluent with Background Reducing Components (Dako). Negative controls were incubated with nonspecific IgG fractions of the appropriate isotype from mouse (IgG/IgM) (Dako) or with a negative control for rabbit IgG (Thermo Scientific). All incubation steps were performed in a dark, humidified chamber. After three washing steps in PBS/T slides were incubated for 30min with fluorescent labelled secondary antibodies (Alexa Fluor®555 goat anti-mouse IgG and Alexa Fluor®488 goat anti-rabbit IgG; both diluted 1:2000, Invitrogen, Lofer, Austria). Afterwards, slides were stained with 4,6-diamidino-2-phenylindoledihydrochloride (DAPI; diluted 1:2000 in PBS; Invitrogen) for 10min. Slides were rinsed in deionized water, air dried and mounted with ProLong Gold antifade reagent (Invitrogen). Sections were assessed with a Leica DM 6000B microscope and photographed using an Olympus DP 72 Camera (Leica Microsystems, Wetzlar, Germany).

Table II. Primary antibodies used for immunofluorescent staining.

Antigen (clone/cat. No.)	Company	Concentration	Host/Isotype
Cytokeratin 7 (OV-TL 12/30)	Thermo Scientific (Rockford, USA)	0.1µg/ml	Mouse/IgG monoclonal
KDR (sc-6251)	Santa Cruz Biotechnology (Santa Cruz, USA)	0.2µg/ml	Mouse/IgG monoclonal
Laminin receptor 1 (MLuC5)	Thermo Scientific (Rockford, USA)	4µg/ml	Mouse/IgM monoclonal
PEDF (AB-PEDF1)	BioProducts MD (Middletown, USA)	1µg/ml	Rabbit/IgG polyclonal
VEGF (19003-1-AP)	Proteintech (Manchester, UK)	0.7µg/ml	Rabbit/IgG polyclonal
Von Willebrand Factor (A0082)	Dako (Glostrup, Denmark)	3µg/ml	Rabbit/IgG polyclonal

3.22. Immunofluorescence of Hofbauer cells, trophoblasts and feto-placental endothelial cells

For co-localization of markers for HBC, TB and feto-placental endothelial cells, sections (5µm) were cut and placed on Superfrost Plus slides (Menzel, Braunschweig, Germany). Sections were deparaffinised in xylene and rehydrated through a series of graded alcohol. Heat-induced antigen retrieval was performed in epitope retrieval solution at pH9 (Leica Biosystems Newcastle Ltd., Newcastle, UK). Then, slides were boiled in a pressure cooker for 7min at 120°C and allowed to cool down for 20min before being rinsed in wash buffer [Tris-buffered saline, 0.05% Tween-20 (TBS/T), pH 7.4]. All further steps were performed at room temperature in a dark, humified chamber. Slides were incubated with UltraVision Hydrogen Peroxidase Block (Thermo Scientific, Runcorn, UK) for 5min and rinsed in TBS/T three times before applying blocking solution (TBS/T with 10% goat serum (Thermo

Fisher, Rockford, USA) and 6% BSA (Sigma-Aldrich, St. Louis, USA) for 30min. After 3 washing steps, primary antibodies were applied (rabbit anti-CD34; 1:500, Abcam, UK), mouse anti-CD163 (1:25, Thermo Scientific) and anti-CK7-FITC (1:50, USBiological, Swampscott, USA) diluted in antibody diluent with Background Reducing Components (Dako, Glostrup, Denmark). After three washing steps in TBS/T slides were incubated for 30min with fluorescent labelled secondary antibodies (Dylight 633 goat anti-mouse IgG; 1:100, Thermo Fisher) and CY3 goat anti-rabbit IgG (1:25, Abcam). Afterwards, slides were stained with 4,6-diamidino-2-phenylindoledihydrochloride (DAPI; diluted 1:2000 in PBS; Invitrogen, Carlsbad, USA) for 10min, rinsed in deionized water, air dried and mounted with ProLong Gold antifade reagent (Invitrogen). Sections were assessed with a Leica DM 6000B microscope and photographed using an Olympus DP 72 Camera (Leica Microsystems, Wetzlar, Germany).

3.23. Immunoblot analysis for VEGF and PEDF signalling

Feto-placental endothelial cells (150,000/well) were seeded in supplemented EBM medium and gelatin coated 6-well plates. After 48h, cells were serum starved for 4h. Then, PEDF (10ng/ml), VEGF (25ng/ml) and the combination of both was added for 10min. For experiments where VEGF activity was blocked, the VEGFR2 inhibitor Ki8751 (Calbiochem, Merck Millipore, Darmstadt, Germany) was added to a final concentration of 10ng/ml 30min prior to the VEGF treatment. Protein was isolated using RIPA puffer with complete protease inhibitor (Sigma, 1 tablet/10ml) and used for immunoblot analysis for P-ERK1/2 (Tyr 576) (Millipore; 1:1000) and P-FAK (Tyr 397) (Cell Signalling, Merck Millipore; 1:1000). Antibodies against unphosphorylated ERK1/2 (Abcam; 1:1000) and FAK (Abcam; 1:1000) were used as loading controls.

3.24. Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis used Sigma Plot 12.0 software and a p value of less than 0.05 was considered as significant. Statistical differences were assessed by Student's t-test (Shapiro-Wilk-test for normal distribution; Mann-Whitney U-test for non-parametric values) and ANOVA. The indicated number of independent experiments is provided in the figure legends.

4. Results

The following parts ‘results’ and ‘discussion’ are taken from two publications by *Loegl et al.* (123,127).

4.1. HBC often localize close to placental vessels

To identify and localize HBC in human third trimester placentas, placental cross sections were stained with the cell type specific markers cytokeratin 7 (CK7) for trophoblast (TB), CD34 for placental endothelial cells and CD163 for HBC. The immunofluorescent staining shows the placental villus framed by the TB layer (CK7, green) with the feto-placental vessels inside the stroma (CD34, white) (Figure 13). HBC (CD163, red) are distributed in the villous stroma, sometimes in close proximity to the basal membrane of the trophoblast layer. Moreover, HBC are often in close proximity to placental vessels, suggesting an interaction with endothelial cells (Figure 13). Inserts of figure 13 show isolated and cultured primary HBC (HBC) and placental endothelial cells (fpEC) (123).

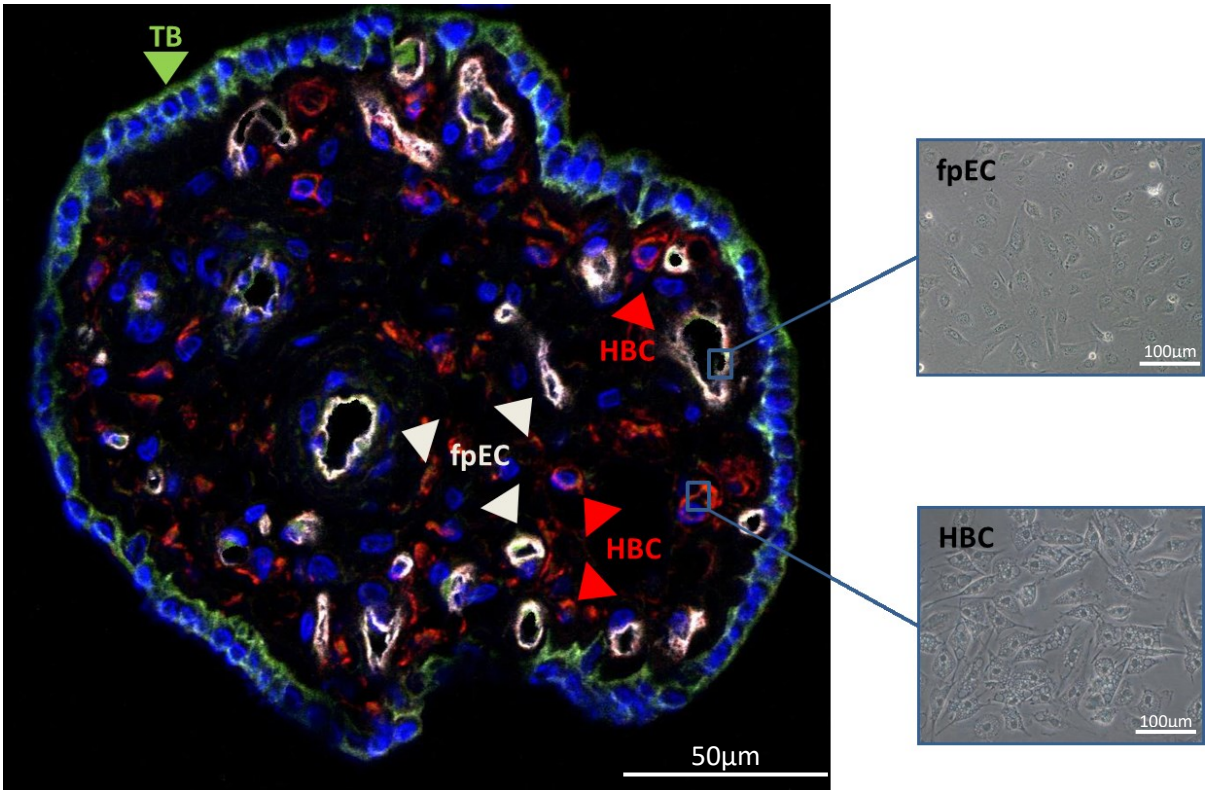

Figure 13. Immunofluorescent staining of a third trimester placental villus. The cross section of the villus is bordered by the trophoblast (TB) layer (CK7, green, green arrowhead). Inside the villus, feto-placental endothelial cells (fpEC) line the placental vessels (CD34, white, white arrowhead). Hofbauer cells (HBC) are evenly distributed, but often close to placental vessels (CD163, red, red arrowhead). A representative image of images taken from three individual placentas is shown. Original magnification: 200x, scale bar = 50µm. The upper insert shows cultured primary fpEC that have a polygonal shape, grow in loose arrangements and exhibit the classical cobblestone-like appearance. The lower insert shows cultured HBC that exhibit a pleiomorphic phenotype with many vacuoles. Original magnification of the inserts: 200x, scale bars = 100µm. Taken from (123) with permission (Reproduction).

4.2. Primary HBC represent a heterogeneous cell population with M2 characteristics

Several studies demonstrated that HBC provide M2 characteristics (29,43-46). We wanted to further identify the specific subtype of HBC polarisation in normal pregnancy. Therefore, we analysed the expression of surface markers for macrophage phenotypes (CD11b (128), CD80, CD86, CD163, and CD209 (37,129,130)) using flow cytometry, and measured the secretion of interleukin 1 receptor antagonist (IL1RN), tumor necrosis factor α (TNFA) and interleukin 6 (IL6) by HBC by ELISA. The expression of the common leukocyte antigen CD45 (131), which has been shown on placental macrophages *in situ* (132), was used as a general marker for flow cytometry (figure 14 A).

Almost no staining (2.0±1.3%) was observed when CD80, a marker for M1 polarisation, was used. Absence of the M1 marker was paralleled by positive staining of cells for CD163 (95±5%) and CD11b (84±11%), both M2 markers, by the majority of cells (Figure 14 B-D). Moreover, the ability to uptake Dil-Ac-LDL by visually all HBC (Figure 11) indicates presence of the M2 marker and LDL binding protein CD36 (207). Further analysis of makers for M2 subtypes revealed 12±9% of HBC positive for the M2a marker CD209 (Figure 14 E) (123).

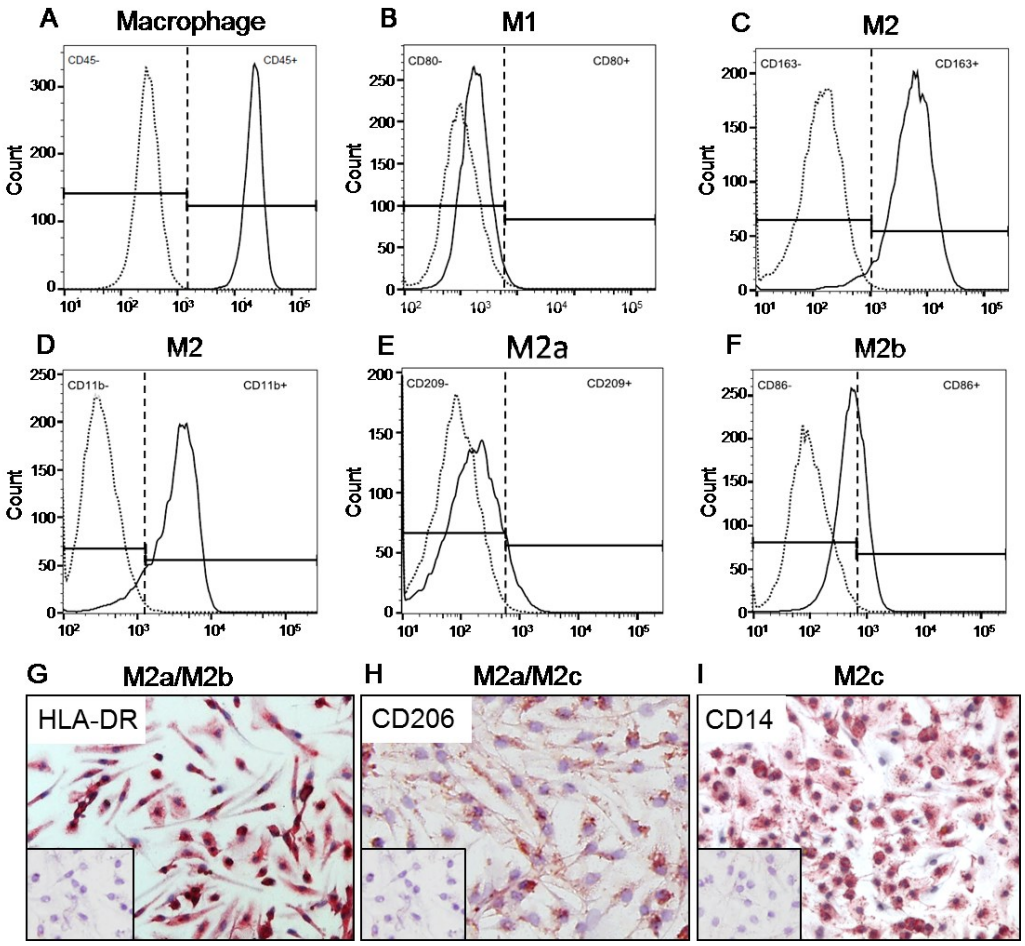

Figure 14. Characterization of HBC by flow cytometry and immunocytochemistry. Isolated HBC were cultured for four days before staining for flow cytometry analysis. HBC were stained for the general macrophage marker CD45 (A), and for markers of macrophage subtypes, i.e. CD80 (B), CD163 (C), CD11b (D), CD209 (E) and CD86 (F). The histograms are representatives of independent experiments. CD45: n=4; CD80: n=7; CD163: n=9; CD11b: n=4; CD209: n=6; CD86: n=4. Dotted lines represent unstained cells, continuous lines represent positively stained cells. Immunocytochemistry for HLA-DR, CD206 and CD14 was performed after 5 days of culture. Photographs are representative of independent experiments of n=4 different isolations. Inserts show negative controls incubated with unspecific IgG of the same isotype as the specific antibody. Taken from (123) with permission (Reproduction).

The presence of CD209 was paralleled by secretion of IL1RN (Figure 15 A), which also indicates M2a polarisation (133). 31±30% of HBC were positive for the M2b marker CD86 (Figure 14 F) which was paralleled by secretion of TNFA and IL6 (Figure 15 B, C), two M2b macrophage specific cytokines (37,129,130).

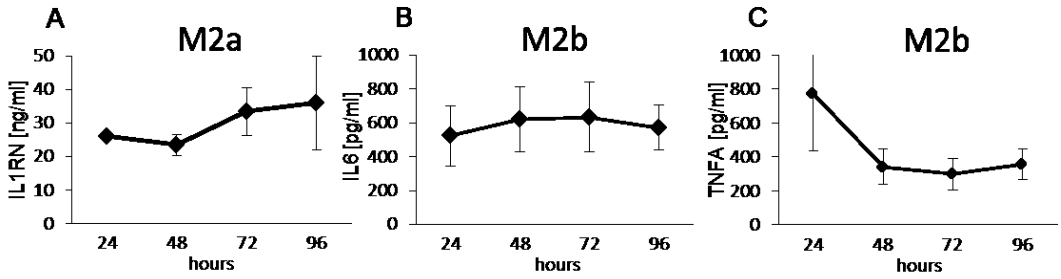

Figure 15. Secretion of IL1RN, TNFA and IL6 by HBC. Cytokines were measured by ELISA up to 96 hours after isolation. In the non-conditioned control medium, IL1RN, TNFA and IL6 concentrations were below the detection limit of the ELISAs. Conditioned media of n=5 independent HBC isolations were analysed. Taken from (123) with permission (Reproduction).

Immunocytochemistry revealed positive staining for the M2a/M2b marker HLA-DR (major histocompatibility complex, class II, DR) (39,130), the M2a/M2c marker CD206 (Mannose Receptor C, Type 1) (130) and the M2c marker CD14 (130,134) (Figure 14 G-I) (123). Surface markers and cytokines used to characterize HBC phenotypes are summarized in table III.

Table III. Macrophage markers and cytokines used in this study to characterize the HBC phenotype.

	M1	M2	M2a	M2b	M2c
surface molecules:	CD80 [1]	**CD11b [1]** **CD36 [3]**	**CD163 [1]** **CD206 [2]** **HLA-DR [2]** **CD209 [1]**	**CD163 [1]** **HLA-DR [2]** **CD86 [1]**	**CD163 [1]** **CD206 [2]** **CD14 [2]**
cytokines:			**IL1RN [4]**	**TNFA [4]** **IL6 [4]**	

Superscript numbers indicate the method of analysis: [1] FACS, [2] immunocytochemistry, [3] dil-ac-LDL uptake, [4] ELISA. Expressed surface proteins and secreted cytokines are printed in bold.

4.3. Secretion of pro-angiogenic factors by HBC

Since M2 macrophages have the capacity to regulate vessel development in other tissues, we determined whether HBC (n=5) secrete pro-angiogenic factors. Levels of VEGF, FGF2 and PlGF as key factors to stimulate angiogenesis were determined in HBC CM by multiplex immunoassay and ELISA. VEGF and FGF2 were identified in HBC CM (Figure 16), whereas PlGF could not be detected by the assay and was below 30pg/ml (data not shown).

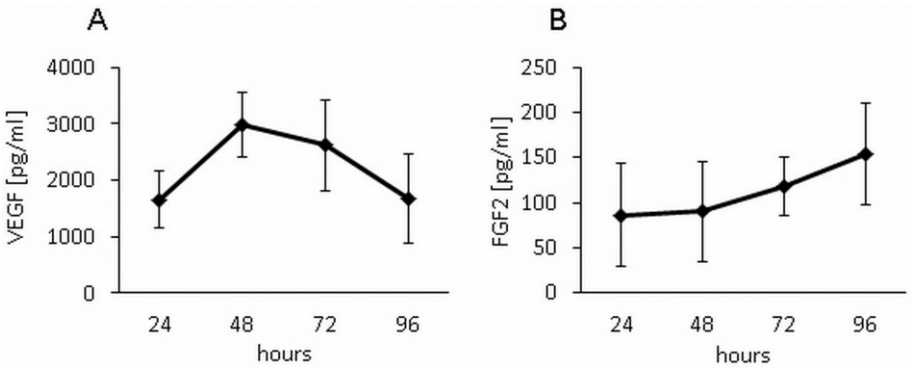

Figure 16. Secretion of VEGF and FGF2 by HBC. VEGF (A) and FGF2 (B) were measured by multiplex immunoassay up to 96 hours after isolation. In the non-conditioned control medium, VEGF and FGF2 were below the detection limit of the assays. Conditioned media of n=5 independent HBC isolations were analysed. Taken from (123) with permission (Reproduction).

4.4. Paracrine effects of HBC on placental endothelial cell angiogenesis

The presence of pro-angiogenic factors in HBC CM suggests a stimulatory effect of HBC derived paracrine factors on placental angiogenesis. We analysed the impact of HBC CM on network formation of primary term placental endothelial cells. CM of HBC induced network formation of placental endothelial cells, increased length of the network by 26 ± 12% (p=0.018) and the number of branching points by 15 ± 9% (p=0.048) as compared to control medium (Figure 17).

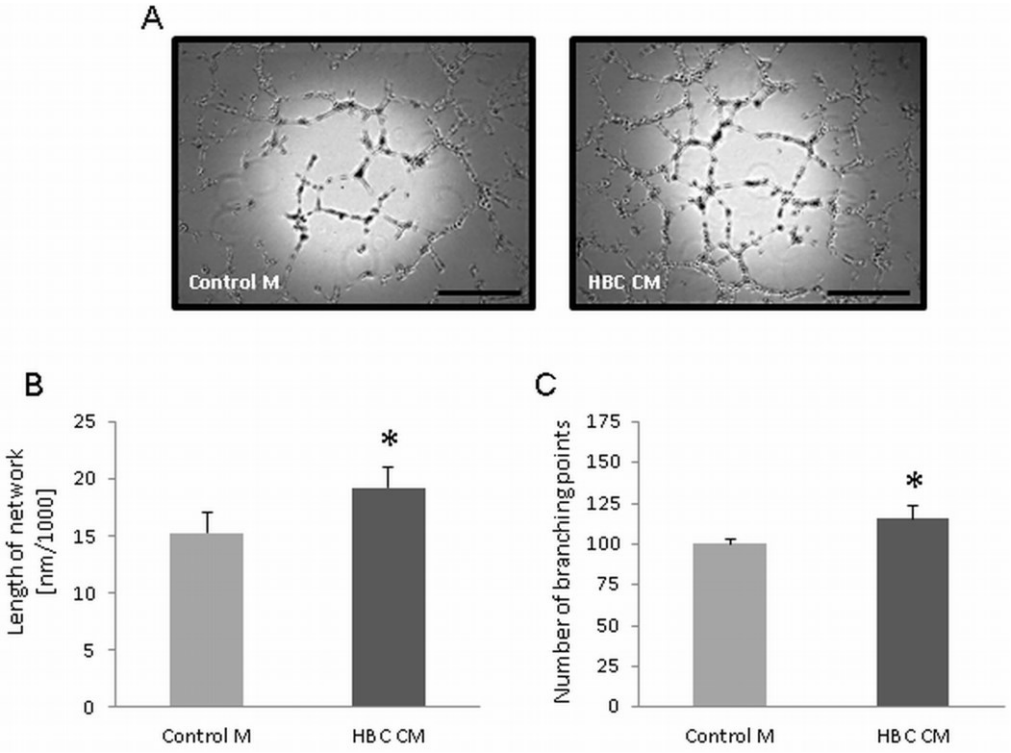

Figure 17. HBC conditioned medium stimulates network formation of fpEC. CM from HBC (n=5, pooled in two different stocks) was used for network formation assays with fpEC. (A) Representative phase contrast light microscopic images show network formation of fpEC after 12h on growth factor reduced Matrigel. (B, C) Conditioned medium of HBC increased length of network (B) and number of branching points (C) of fpEC. Original magnification: 50x; scale bar = 500µm. Data are given as mean ± SEM. Statistical analysis used the mean of the triplicates of the number of individual biological replicates of fpEC (n=6). Control M (control medium): medium without cells. *$p<0.05$. Taken from (123) with permission (Reproduction).

The angiogenic event comprehends different processes, and, besides network formation, includes chemoattraction, proliferation and survival of placental endothelial cells. We investigated the paracrine effect of HBC on these placental endothelial cell functions. Proliferation of placental endothelial cells was slightly increased by HBC CM, but this did not reach significance (Figure 18 A). Interestingly, chemoattraction of placental endothelial cells to the CM was reduced by 28 ± 12% ($p=0.037$) when compared to non-conditioned control medium (Figure 18 C). HBC CM did not affect LDH release from placental endothelial cells (Figure 18 B) indicating that the decrease in chemoattraction did not result from reduced cell survival and viability (123).

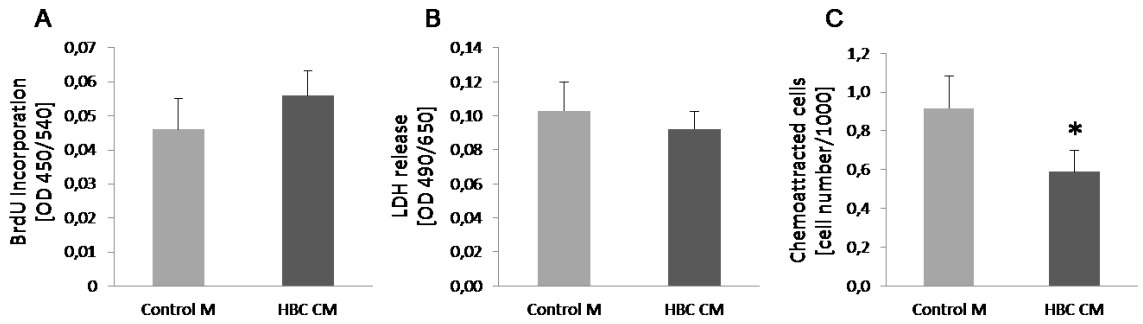

Figure 18. HBC conditioned medium affects fpEC functions. CM from HBC (n=5 pooled in two different stocks) was used for functional assays with fpEC. (A, B) CM of HBC stimulated proliferation of fpEC by trend and had no effect on viability. (C) Chemo-attracted migration of fpEC towards the HBC CM was reduced. Data are given as mean ± SEM. Statistical analysis used the mean of the triplicates of the number of individual biological replicates of fpEC (n=6). Control M (control medium): medium without cells. * $p<0.05$. Taken from (123) with permission (Reproduction).

4.5. Paracrine regulation of placental endothelial cell angiogenesis by trophoblast from early vs late pregnancy

To investigate the paracrine effect of trophoblast from early vs late pregnancy on angiogenesis, the influence of CM on network formation, migration, proliferation and survival of placental endothelial cells was analysed (Figure 19). CM of trophoblast from late pregnancy (term trophoblasts; TTB) reduced network formation of placental endothelial cells by 30 ± 14% ($p=0.024$) (Figure 19 a, b), while CM of trophoblast from early pregnancy (first trimester trophoblasts; FTB) had no effect. CM of peripheral blood monocyte cells (PBMC) used as positive control (135) stimulated network formation by 21 ± 10% ($p=0.049$). Similar results were obtained with CM that had been ultracentrifuged prior to use (not shown), indicating that soluble factors secreted by trophoblast induce the anti-angiogenic effects (127).

To determine paracrine regulation of trophoblast CM from early vs late pregnancy on migration, transwell migration assays were performed. TTB CM reduced migration by 44 ± 7% ($p=0.026$), whilst FTB CM reduced migration only by trend (Figure 19 c). Medium

supplemented with FBS and growth factors was used as positive control and enhanced feto-placental endothelial cell migration by 32 ± 22% (p=0.007).

The effect of trophoblast CM from early and late pregnancy was further determined on proliferation, which is a further step of angiogenesis. CM of both, FTB and TTB tended to increase BrdU incorporation as compared to control medium, but without reaching significance (Figure 19 d). Medium supplemented with FBS and growth factors was used as positive control and stimulated proliferation 8-fold (p=0.039).

Neither FTB nor TTB CM increased LDH release from placental endothelial cells (Figure 19 e), indicating that the negative effect of CM on network formation and migration did not result from reduced cell survival and viability.

To evaluate the effect of the CM in a physiological 3D model of embryonic angiogenesis we employed chicken chorioallantoic membrane (CAM) assays. In parallel to the data obtained from placental endothelial cells, TTB CM reduced vasculogenesis in the CAM assay (Figure 20 a, b) (127).

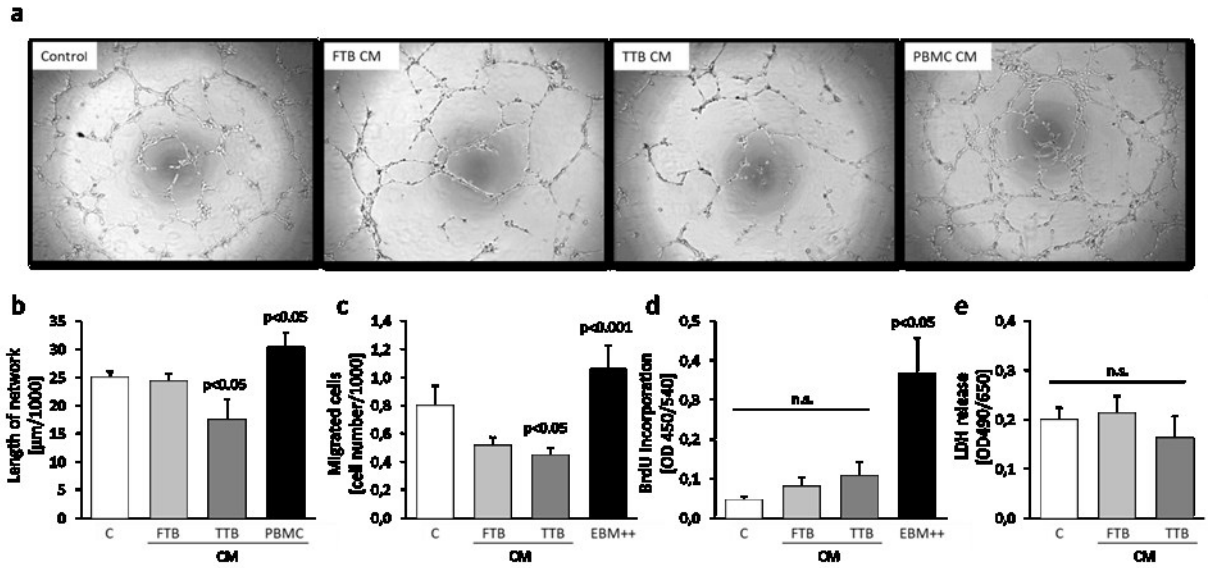

Figure 19. Effect of first (FTB) and third (TTB) trimester trophoblast conditioned medium (CM) on network formation, migration, proliferation and survival of placental endothelial cells. (a) Representative phase contrast light microscopic images show network formation of placental endothelial cells after 12 hours on growth factor reduced Matrigel. CM of peripheral blood monocyte cells (PBMC) was used as positive control. (b) Quantitative analysis of networks depicts the inhibitory effect of TTB CM on placental endothelial cells (n=6). (c) CM of FTB and TTB reduced migration of placental endothelial cells compared to control (n=6). Endothelial basal medium (EBM) supplemented with FBS and growth factors (EBM++) was used as positive control. (d) Proliferation was unchanged between early and late trophoblast CM (n=4). EBM supplemented with FCS and growth factors (EBM++) was used as positive control. (e) FTB and TTB CM did not increase LDH release of placental endothelial cells (n=4). Data are given as mean ± SEM. Statistical analysis used the mean of the triplicates of the number of individual biological replicates. Control medium (C) = DMEM/EBM+7.5% FCS for 48h at 37°C without cells. Taken from (127) with permission (Angiogenesis).

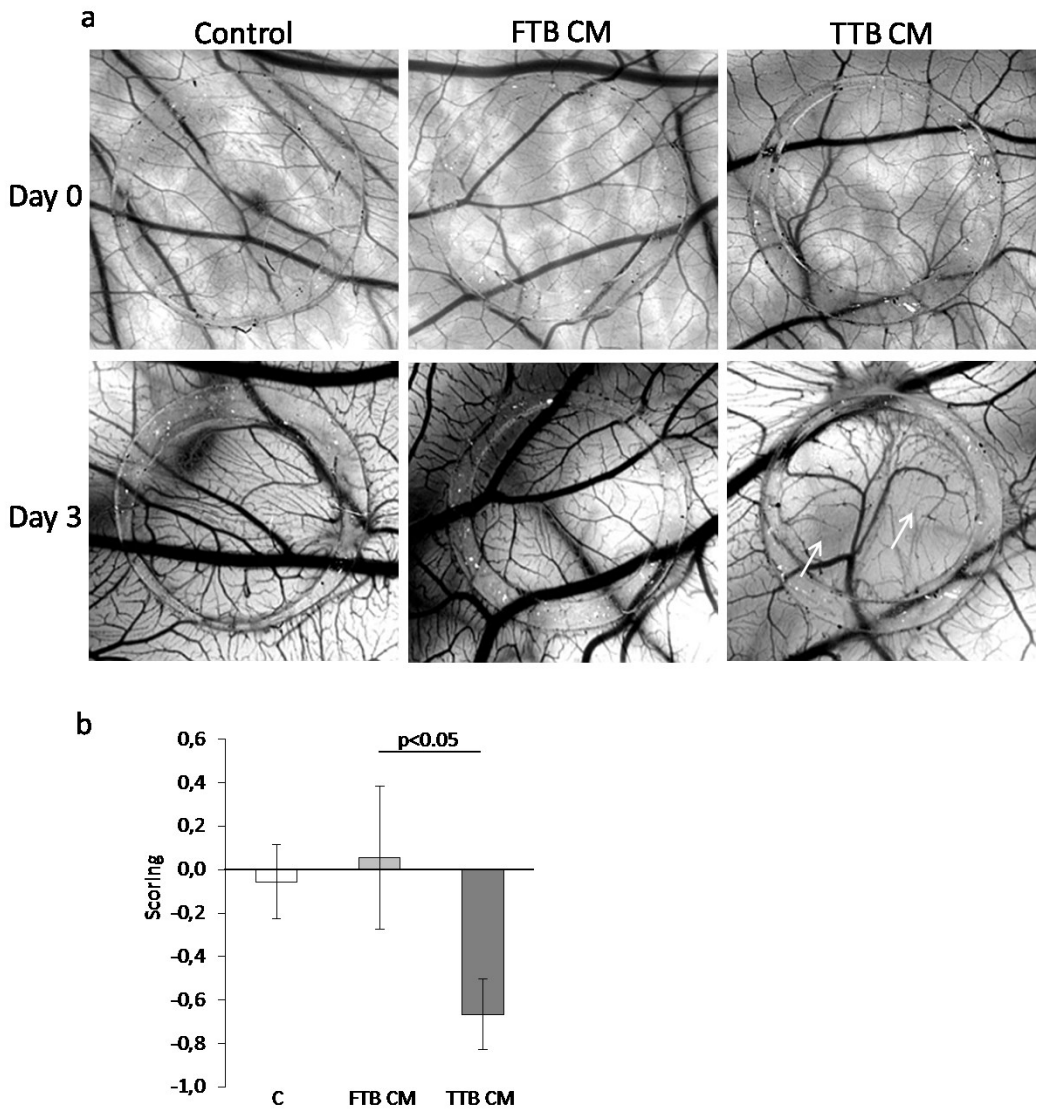

Figure 20. Anti-angiogenic effect of first (FTB) and third (TTB) trimester trophoblast conditioned medium (CM) on the vessel formation of the chicken chorioallantoic membrane (CAM). CAMs were treated with on-plants (silicone rings) containing FTB CM, TTB CM or control medium. TTB CM reduced the tertiary and quaternary vessels (white arrows). Per condition, four different eggs were treated, each performed with six on-plants. (a) Representative pictures of each condition immediately after application of the silicone ring on day 0 and at day 3 of the treatments are shown. (b) Data were scored on day 3 of the treatment. Taken from (127) with permission (Angiogenesis).

4.6. Anti-angiogenic molecules expressed by trophoblasts from early vs late pregnancy

The reduction of network formation and migration in presence of CM from late pregnancy indicates the presence of anti-angiogenic factors secreted by trophoblast. To identify potential targets causing the anti-angiogenic effect of late pregnancy CM, the expression of genes encoding angiogenesis limiting factors were compared in FTB vs TTB. Thus, their transcriptomes were screened for 42 candidate angiogenesis related genes obtained from literature (Table IV) (136-159). Of these, 21 were expressed either in early or late pregnancy trophoblast, or both (as threshold for expression a signal > 200 was used). In general, the expression of anti-angiogenic factors was higher in early compared to late pregnancy trophoblast. Eight genes differed in their expression by >2 fold between early and late pregnancy trophoblast (Table V) with only PEDF (pigment epithelium-derived factor) showing higher (3.8-fold) expression in late pregnancy trophoblast. Real-time qPCR confirmed the microarray results and revealed a fold change of 6.6±2.9 for PEDF (Figure 21 a). This suggests PEDF as a potential contributing factor to the anti-angiogenic paracrine effect of late pregnancy trophoblast and the effect of PEDF on feto-placental endothelial cells was further studied (127).

Table IV. Genes encoding anti-angiogenic molecules expressed in first and third trimester trophoblast (signal > 200) as determined by microarray analysis. a = absent.

Gene symbol	Gene name	FTB	TTB
ANGPT2	Angiopoietin2	735	a
ARRB1	Arrestin beta1	336	446
CD59	CD59 complement fragment	713	724
COL4A1	Collagen type IV alpha1	9985	2830
COL4A2	Collagen type IV alpha2	7011	2570
COL4A3	Collagen type IV alpha3/Tumstatin	a	a
COL18A1	Collagen type XVIII alpha1	a	a
CXCL2	Gro-beta	307	377
CXCL10	Chemokine ligand10	227	a
FN1	Fibronectin	1165	681
FBLN5	Fibulin5	a	a
HSPGBM	Heparan sulphate proteoglycan of basement membrane	744	a
IFNA1	Interferon alpha	a	a
IFNB1	Interferon beta	a	a
IFNG1	Interferon gamma	a	a
IL4	Interleukine 4	a	a
IL12A	Interleukin12 A	a	a
IL12B	Interleukin12 B	a	a
IL18	Interleukin18	a	a
KISS1	Kisspeptin	1121	a
LECT1	Leukocyte cell-derived chemotaxin (Chondromodulin)	a	a
NRP1	Neurolipin1	a	a
PF4	Platelet factor4	a	a
PLG	Plasminogen fragment	a	a
PRL	Prolactin	a	233
RNH	Placental ribonuclease inhibitor	1932	2377
SDC3	Syndecan3	212	a
SERPINB5	Maspin	a	a
SERPINC1	Serpin peptidase inhibitor, clade C (Antithrombin III)	a	a
SERPINE1	Plasminogen activator inhibitor/Serin peptidase inhibitor	11887	9413
SERPINF1	Pigment epithelium derived factor (PEDF)	776	2914
SPARC	Secreted protein, acidic, cysteine-rich	2458	555
SPP1	Osteopontin	228	323
TGFB1	Transforming growth factor beta	a	a
THBS1	Thrombospondin1	a	a
THBS2	Thrombospondin2	a	a
TIMP1	Metalloproteinase inhibitor1	1226	292
TIMP2	Metalloproteinase inhibitor2	2849	2711
TIMP3	Metalloproteinase inhibitor3	11176	7901
TIMP4	Metalloproteinase inhibitor4	a	a
TNNI3	Troponin I	a	a
TNFSF15	Tumor necrosis factor ligand superfamily, member 15/VEGI	a	a

Table V. Fold change of genes encoding anti-angiogenic proteins differentially expressed between first and third trimester trophoblast (signal > 200, fold change >2 or < -2) as determined by microarray analysis.

Gene symbol	Gene name	fold change
SERPINF1/PEDF	Pigment epithelium derived factor	-3.8
THBS1	Thrombospondin1	2.6
TIMP1	Tissue inhibitor of metalloproteinases1	4.2
SPARC	Secreted protein, acidic, cystein-rich	10.2
SDC3	Syndecan3	11.8
TGFB1	Transforming growth factor beta1	16.8
ANGPT2	Angiopoietin2	17.5
KISS1	Kisspeptin	101.9

a = absent. Positive fold change indicates higher expression in first trimester trophoblast, whilst a negative fold change indicates higher expression in third trimester trophoblast.

4.7. Identification of trophoblast-derived PEDF as negative regulator of network formation and proliferation in feto-placental endothelial cells

In line with mRNA expression, TTB secreted more PEDF (2.6ng/ml/1x10^6 cells) than the same number of trophoblasts from early pregnancy (0.9ng/ml/1x10^6 cells; p=0.025) (Figure 21 b). This difference in PEDF remained similar when PEDF levels were normalized to total protein content of the CM (2.9 fold higher levels in TTB; p<0.001).

To determine whether PEDF accounts for the anti-angiogenic effect of CM from late pregnancy trophoblast, *in vitro* network formation assays with late pregnancy trophoblast CM were repeated in the presence of PEDF neutralizing antibody (Figure 21 c). TTB CM with unspecific IgG reduced *in vitro* network formation by 26±10% (p=0.025). This reduction diminished in the presence of PEDF neutralizing antibodies (127).

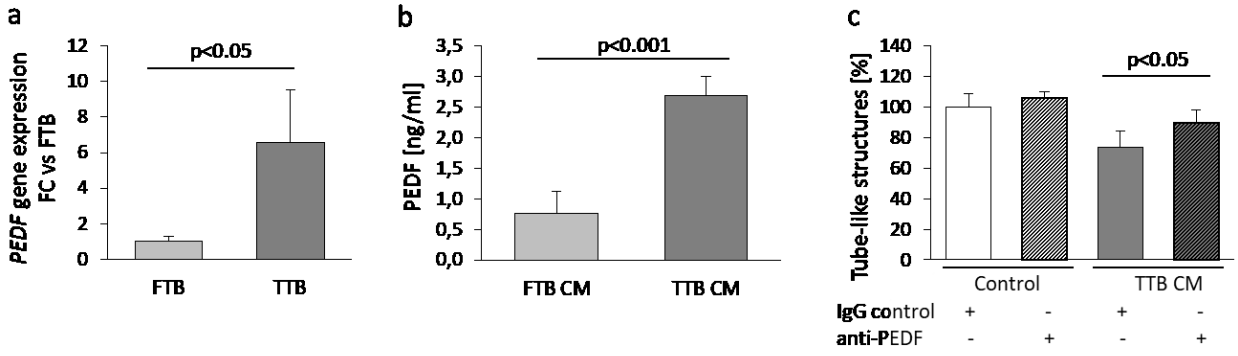

Figure 21. Expression and secretion of PEDF from first (FTB) and third (TTB) trimester trophoblasts. (a) Quantification of *PEDF* mRNA in FTB and TTB by real-time qPCR using the expression of the ribosomal protein RPL30 as internal control. (b) Quantification of PEDF in FTB vs TTB CM measured with ELISA. (c) PEDF neutralizing antibody significantly reduced the inhibitory effect of TTB CM on network formation of placental endothelial cells. Data are given as mean ± SEM. Statistical analysis used the mean of the triplicates of the number of individual biological replicates. n (real-time qPCR) =5, n (ELISA) = 8, n (network formation) = 3. Control medium (C) = DMEM/EBM+7.5% FCS for 48h at 37°C without cells. Taken from (127) with permission (Angiogenesis).

4.8. Effect of PEDF on VEGF-activated network formation and proliferation of placental endothelial cells

Stimulation of placental endothelial cells with different concentrations (0.25-25ng/ml) of human recombinant PEDF did not affect network formation (Figure 22 a). However, when 25ng/ml VEGF was used to activate feto-placental endothelial cells for angiogenesis, PEDF reduced the VEGF effect in a dose-dependent manner to a maximum of 31 ± 7% (Figure 22 b) when compared to VEGF alone. PEDF concentrations between 0.5 and 10ng/ml reduced network formation to levels even below control levels, i.e. endothelial cells without any treatment. In the setting of these *in vitro* experiments with super-physiological VEGF levels and absence of other trophoblast secreted angiogenesis regulating factors, also PEDF levels similar to that in FTB CM reduced 2D network formation. Since 5 and 10ng/ml PEDF produced the strongest and most significant effects, these concentrations were used for further experiments.

Proliferation and LDH secretion were not affected by PEDF treatment alone (Figure 22 c, e). However, when cells were co-stimulated with VEGF, PEDF (10ng/ml) reduced the stimulatory effect of VEGF on proliferation (Figure 22 d) by 27 ± 8% ($p<0.05$). Moreover, PEDF increased LDH release by 42 ± 11% ($p<0.001$) (Figure 22 f) when compared to VEGF treatment alone (127).

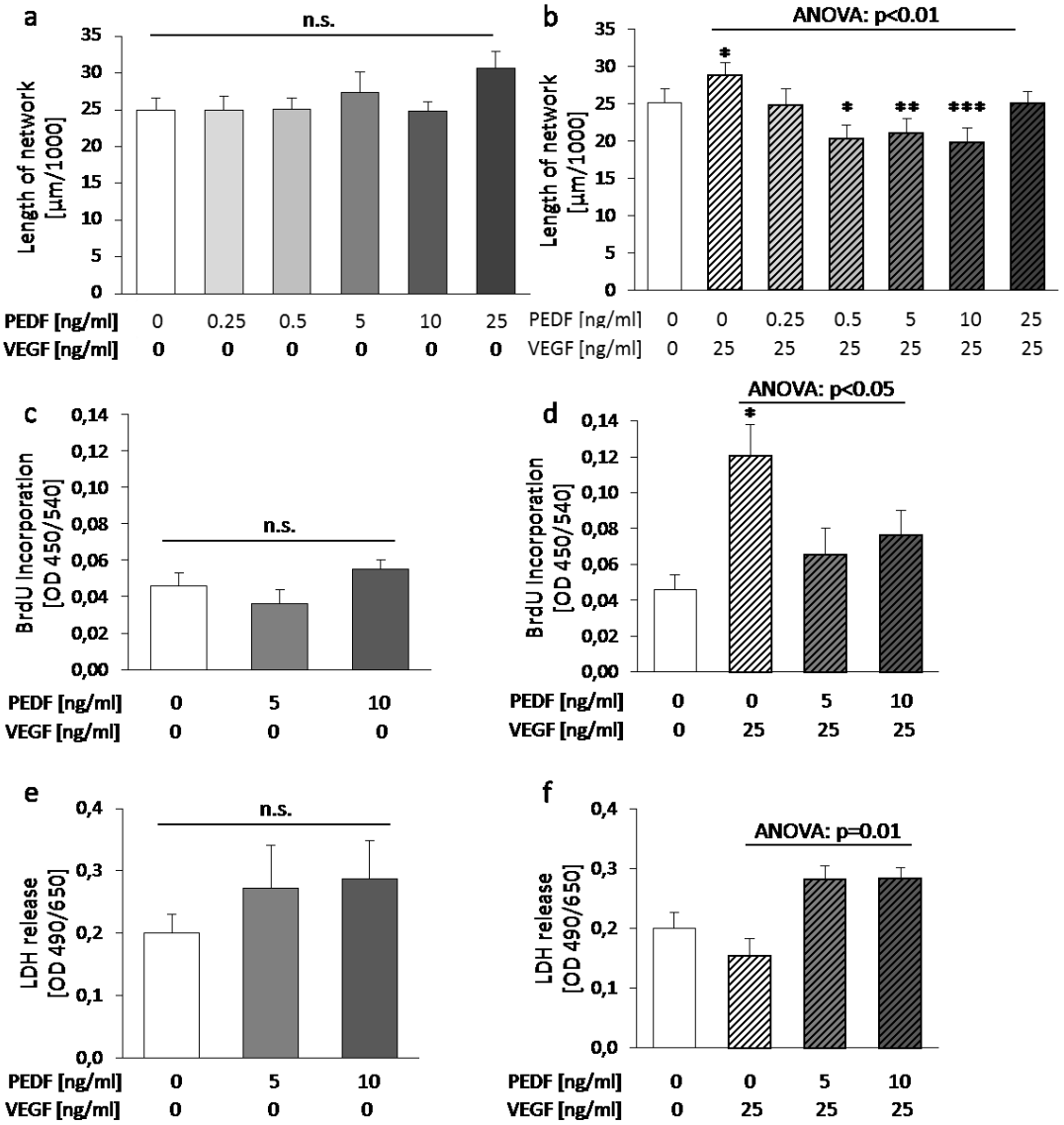

Figure 22. Effects of human recombinant PEDF on network formation, proliferation and survival of placental endothelial cells depends on VEGF. (a, c, e) Addition of PEDF alone did not affect network formation, proliferation and LDH release of placental endothelial cells. (b) In cells stimulated with VEGF (25ng/ml), PEDF reduced network formation in a concentration-dependent manner, (d) reduced proliferation and (f) increased cell death. Data are given as mean ± SEM. Statistical analysis used the mean of the triplicates of the number of individual biological replicates. n (network formation) = 8, n (BrdU incorporation) = 9, n (LDH release) = 6. n.s. = not significant; *** $p<0.001$, ** $p<0.01$, * $p<0.05$ compared to untreated control without VEGF. Taken from (127) with permission (Angiogenesis).

In order to compare and relate the anti-angiogenic effects of PEDF with sFlt1, experiments were also performed with sFlt1 (Figure 23). As a molecule that sequesters VEGF, sFlt1 blocked the VEGF effects to control levels, but did not have further inhibiting effects.

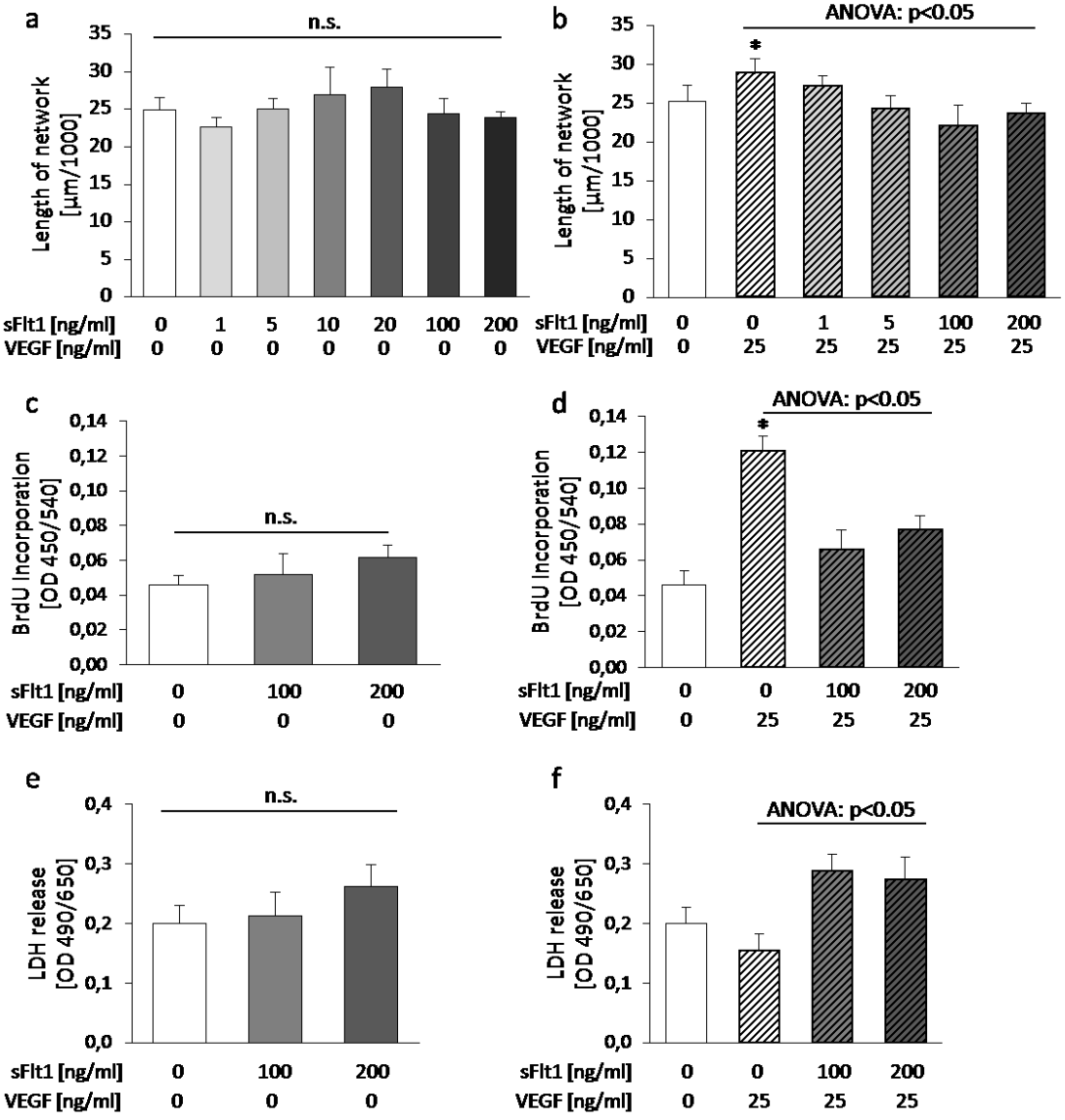

Figure 23. Effects of human recombinant sFlt1 on network formation, proliferation and survival of placental endothelial cells depends on VEGF. (a, c, e) Addition of sFlt1 alone did not affect network formation, proliferation and LDH release of placental endothelial cells. (b) In cells stimulated with VEGF (25ng/ml), sFlt1 reduced network formation in a concentration-dependent manner, (d) reduced proliferation and (f) increased cell death. Data are given as mean ± SEM. Statistical analysis used the mean of the triplicates of the number of individual biological replicates. n (network formation) = 8, n (BrdU incorporation) = 9, n (LDH release) = 6. n.s. = not significant; *** p<0.001, ** p<0.01, * p<0.05 compared to untreated control without VEGF. Taken from (127) with permission (Angiogenesis).

The combined effect of PEDF and VEGF was also tested in CAM assays. Similar to the 2D network formation, the combined application of PEDF and VEGF reduced formation of tertiary and quaternary vessels after 4 days of treatment (Figure 24), whilst PEDF and VEGF alone had no effect (127).

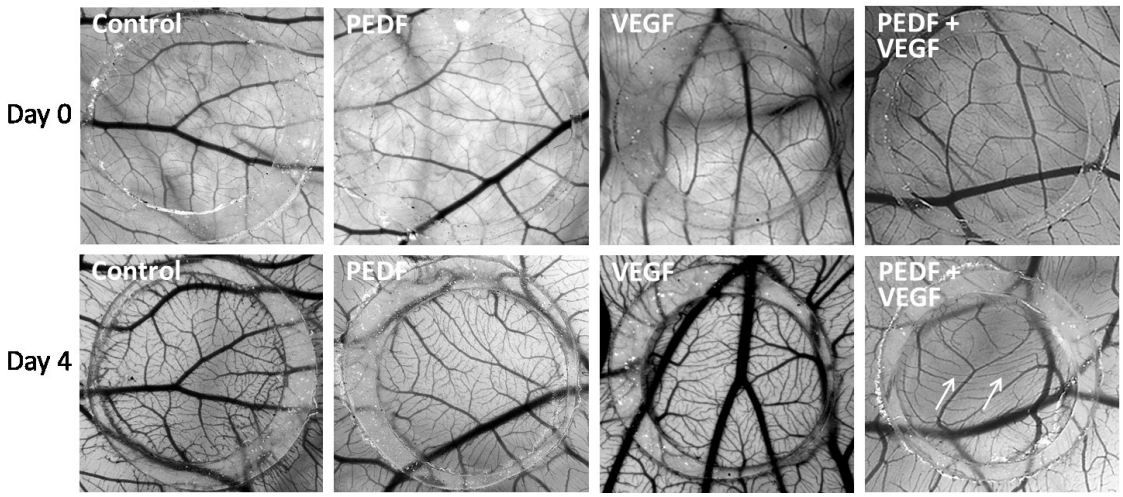

Figure 24. Anti-angiogenic activity of the combination of PEDF and VEGF on vessel formation of the chorioallantoic membrane (CAM). The same CAMs were treated with on-plants (silicone rings) containing 10ng/ml PEDF with 25ng/ml VEGF and control on-plants. Representative pictures of each condition immediately after application of the silicone ring on day 0 and at day 4 of the treatments are shown. After 4 days the vessel structure shows tertiary and quaternary vessels (white arrows). Addition of PEDF or VEGF alone did not affect vessel formation. Treatment with the combination of PEDF and VEGF CAM showed decreased angiogenesis by formation of fewer tertiary and quaternary vessels (white arrows). Taken from (127) with permission (Angiogenesis).

Since the PEDF effect was dependent on the presence of VEGF, we measured VEGF levels and the levels of the VEGF-capture molecule sFlt1 in FTB vs TTB CM. FTB secreted more sFlt1 (4.6ng/ml/1x10^6 cells) and VEGF (6.2ng/ml/1x10^6 cells) than TTB (sFlt1: 2.7ng/ml/1x10^6 cells, p=0.028; VEGF: 3.3ng/ml/1x10^6 cells, p=0.024) (Figure 25 a, b). The difference in VEGF and sFlt1 concentration remained when normalized to the total protein content of the CM (both factors had 1.6 fold higher levels in FTB CM; p=0.02).

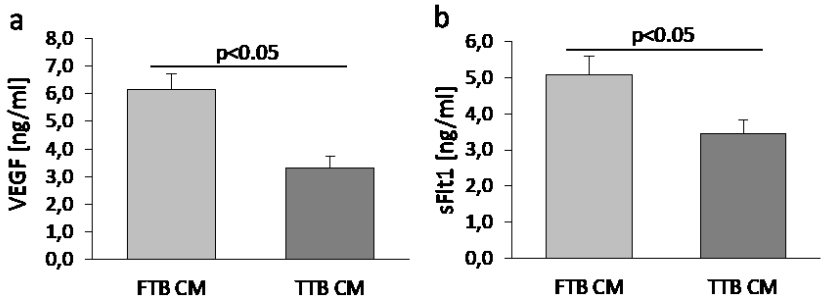

Figure 25. Protein secretion of VEGF and sFlt1 from first (FTB) and third (TTB) trimester trophoblast. (a) Quantification of VEGF in CM of FTB vs TTB measured by dot blot analysis. (b) Quantification of sFlt1 in CM of FTB vs TTB measured by ELISA. Data are given as mean ± SEM. Statistical analysis used the mean of the triplicates of the individual biological replicates. n (dot blot) = 4, n (ELISA) = 8. Taken from (127) with permission (Angiogenesis).

4.9. Localization of PEDF, VEGF and VEGFR2 in placenta in late pregnancy

Although PEDF mRNA expression in placenta was already demonstrated (94), the specific cell types expressing PEDF have not yet been identified. In line with the finding that isolated trophoblasts secrete PEDF, staining for PEDF revealed a prominent signal in the trophoblast, which was co-stained with the trophoblast marker cytokeratin 7 (Figure 26 b). Feto-placental endothelial cells co-stained with the endothelial cell marker CD31, while some other stromal cells showed a weak signal (Figure 26 a).

Several putative receptors for PEDF are known: adipose triglyceride lipase (ATGL) (102,160,162), laminin receptor-1 (LR1) (101), beta subunit of F1-ATPase (ATP5B) (161), and low density lipoprotein receptor-related protein 6 (LRP6) (103). Gene expression analysis for the expression levels of these receptors in primary feto-placental endothelial cells revealed that all of these receptors are expressed (Table VI). PEDF can reduce VEGF induced angiogenesis also by competing with VEGF binding to the VEGF receptor 2 (VEGFR2; KDR) (163). The observed PEDF effects were dependent on the presence of VEGF. Therefore, we also stained for VEGF and the VEGFR2. In line with published literature (90,164) both VEGF and VEGFR2 were produced in the trophoblast and in feto-placental endothelial cells (Figure 26 c-f).

Table VI. Expression of genes encoding PEDF binding partners in feto-placental endothelial cells as determined by microarray analysis.

Gene symbol	Gene name	Signal
ATGL/PNPLA2	Adipose triglyceride lipase	2460
ATP5B	F_1-ATP synthase beta subunit	37640
KDR/VEGFR2	Kinase insert domain receptor	11261
LR1/RPSA	Laminin receptor1	62601
LRP6	Low density lipoprotein receptor-related protein 6	454

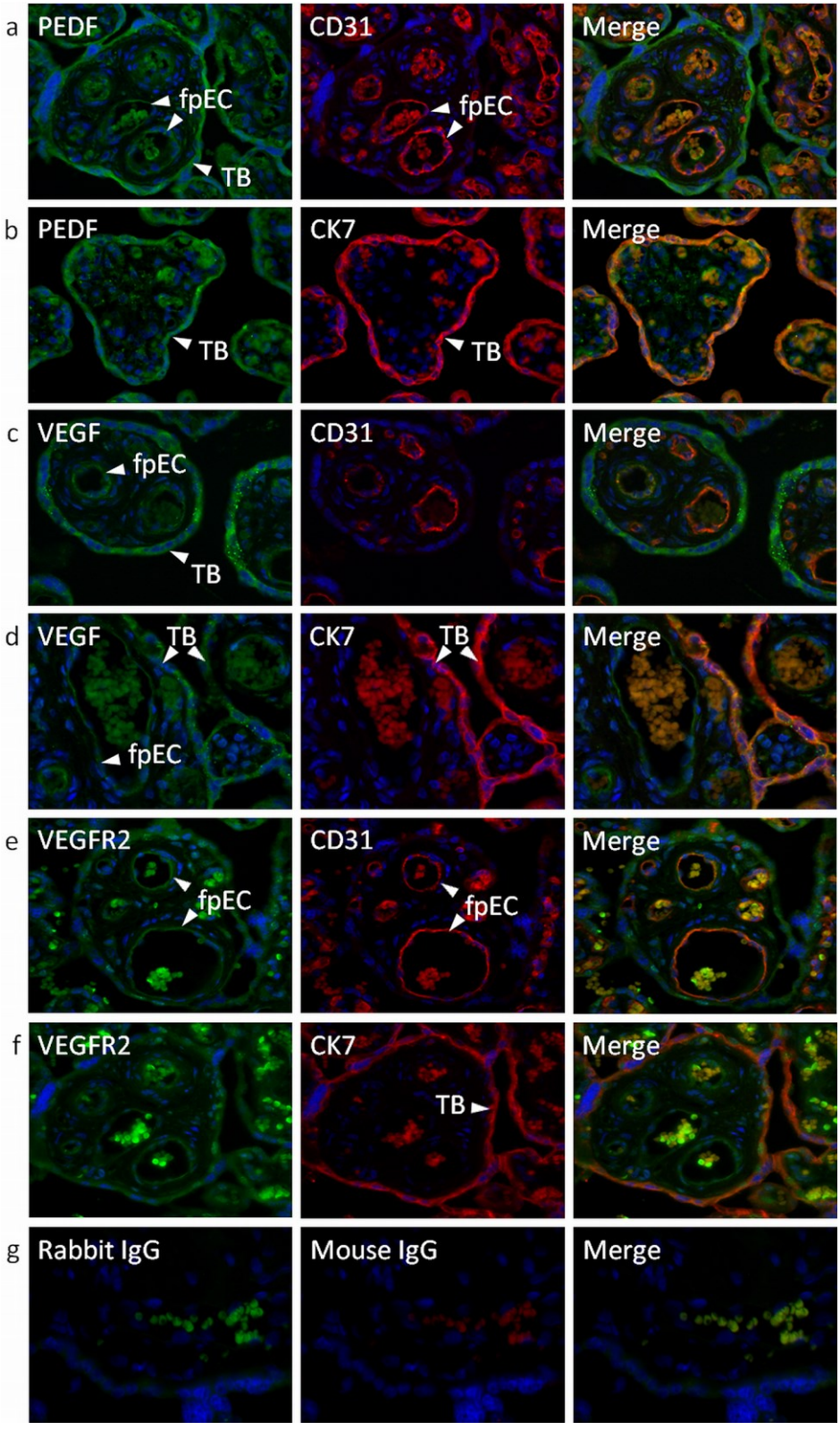

Figure 26. Localization of PEDF, VEGF and VEGFR2 in third trimester placenta. (a, b) PEDF and its receptor are predominantly expressed by villous trophoblast. (c-f) VEGF is expressed in trophoblasts and endothelial cells, similar to its pro-angiogenic receptor VEGFR2. (g) Isotype controls. Green staining: PEDF, VEGF, VEGFR2; red staining: CK7, CD31; blue staining: Dapi. TB = Trophoblast layer, fpEC = feto-placental endothelial cells. Original magnification: 200x. Taken from (127) with permission (Angiogenesis).

4.10. PEDF modifies VEGF signalling in placental endothelial cells

Since PEDF exerts its anti-angiogenic effects on placental endothelial cells only in combination with VEGF we were interested in VEGF signalling in presence and absence of PEDF. We measured phosphorylation of two signalling molecules in response to either VEGF, PEDF, or both. Two key molecules involved in proliferation and endothelial cell migration were chosen, extracellular signal related kinase 1/2 (ERK1/2) (165) and focal adhesion kinase 1 (FAK) (166). Whilst PEDF inhibited the VEGF induced phosphorylation of ERK1/2, FAK phosphorylation was not significantly stimulated by VEGF. However, in presence of VEGF, PEDF increased FAK phosphorylation by 82±14% (Figure 27) (127).

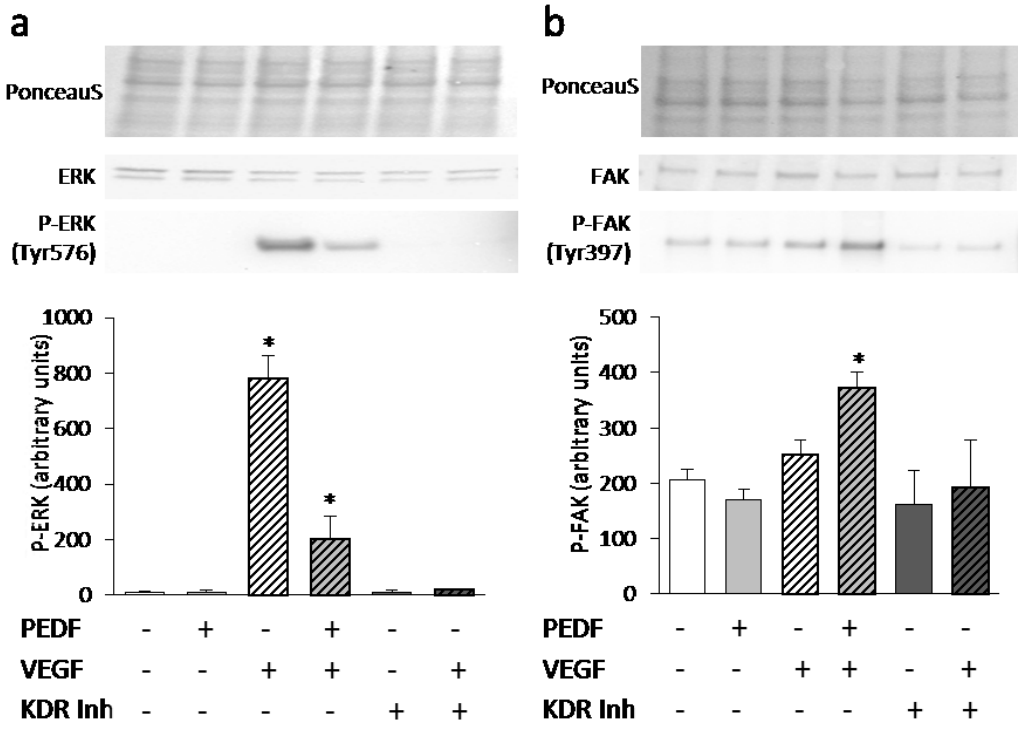

Figure 27. Signalling induced by the combination of PEDF and VEGF in feto-placental endothelial cells. Addition of PEDF (10ng/ml) and VEGF (25ng/ml) reduced VEGF dependent phosphorylation of ERK1/2 at Tyr576, and caused phosphorylation of FAK at Tyr397 that was not activated by PEDF or VEGF alone. Signals were normalized to total blotted protein (PonceauS staining). Blots stained with antibodies against unphosphorylated forms of ERK1/2 and FAK were used as loading controls. Representative immunoblots are shown on top. Results are given as mean ± SEM of three different cell isolations, each in duplicates. Taken from (127) with permission (Angiogenesis).

5. Discussion

5.1. Effect of HBC secreted molecules on placental angiogenesis

This study investigated the effect of isolated placental tissue macrophages on angiogenic processes of placental endothelial cells. Key findings were that HBC displayed a strong M2a, M2b and M2c polarization. Moreover, paracrine factors secreted by HBC modulated angiogenesis related processes in placental endothelial cells, i.e. the stimulation of network formation and the reduction of chemoattracted migration.

In line with previous studies, absence of CD80 on HBC indicates an M2 polarisation of macrophages (43-46). Moreover, we determined protein expression of markers for M2 subpopulations. Based on these markers ascribed to different human macrophage phenotypes in recent literature (37,129,130,167-169) we revealed that HBC represent a macrophage phenotype that cannot be readily classified into one M2 macrophage subset phenotype, as M2a, M2b and M2c specific proteins and markers were present (123). This indicates presence of all three M2 macrophage subtypes in the placenta. Furthermore, fetal macrophages may represent a unique polarisation pattern (170) and hence, do not fit into the above polarisation classification.

The observed heterogeneity of HBC, however, is probably not only a result of the influencing micro-environment. Moreover, the origin of HBC may prime their polarization. There is evidence that HBC appear within the placenta even before the establishment of the feto-placental circulation in the first trimester. This implies that subsets of HBC derive from placental mesenchymal cells, whereas later in pregnancy, it is supposed that HBC arise from recruited fetal monocytes (171,172). Considering that HBC derive from different progenitor cells it may be possible that differentiation is a programmed process and only secondarily determined by the environment (37).

Presence of M2a, M2b and M2c macrophages in the placenta implies that both, regenerative and tissue remodelling functions such as angiogenesis, but also pro- and anti-immunological functions are present in the normal HBC population. As HBC display a potentially pro-angiogenic phenotype, we analysed the secretion of angiogenesis-related

factors of HBC in the CM and identified VEGF and FGF2 secreted by HBC, whilst PlGF was not detected (127).

PlGF was thought to have a marginal role *in vitro*, it was assumed that its stimulation is too weak or it was considered as a VEGF antagonist (173,174). *In vitro* and *in vivo* research discovered PlGF as a stimulator for endothelial proliferation and further angiogenesis (53,175). Although PlGF seems to be involved in placental angiogenesis probably its source in term placenta is not the HBC. TNFA, a further factor secreted by HBC was shown to possess not only pro-inflammatory but also pro-angiogenic features (176).

Furthermore, we tested the paracrine effect of HBC CM on primary placental endothelial cells. In fact, HBC CM increased network formation, and stimulated proliferation by trend, that may be related to the secretion of the pro-angiogenic factors. A role of FGF2 in the stimulation of angiogenesis in Matrigel plugs by M2 macrophages in mice was already suggested (41). Interestingly, chemotactic migration of placental endothelial cells was impaired by HBC CM, but without affecting cytotoxicity. There are several possibilities how HBC derived molecules may affect placental endothelial cell migration. On the one hand, HBC may secrete factors that limit migration such as migration inhibiting factor (MIF), or reduce MMP secretion, whereas on the other hand, HBC derived molecules may affect expression of feto-placental endothelial cell integrins or receptors influencing chemotactic migration of feto-placental endothelial cells (123).

These results would be more meaningful when all experiments would be performed on different three dimensional (3D) assays, as the *in vivo* state of all cells is arranged in a three dimensional order, going along with cell-cell and cell-matrix interactions. Therefore, cell culture experiments should also be achieved in a 3D approach. Commonly, 3D cultured cells have a greater stability and lifespan, which means that they can be cultured longer compared to two dimension (2D) cultured cell monolayers. A 3D environment enables cells to grow without disturbance, because trypsinization occurs infrequently (177)(178). Further, a study revealed that a 2D culture is not that effective for examination of anti-angiogenic and vasculogenic agents, but permits identification of anti-proliferative factors (179). An appropriate *ex vivo* 3D model for our feto-placental endothelial cells would probably be a spheroid model. In the spheroid format cells could be used solely, with an extracellular matrix or with other cells. In a further step, co-culture of feto-placental endothelial cells with

tissue resident macrophages would give new insight into cell-cell interactions of endothelial cells and macrophages.

Whether the paracrine effect of HBC on placental endothelial cells is specific for the placenta or a common feature of M2 macrophages on endothelial cells remains speculative. Since both, macrophages as well as endothelial cells are well known for their organ specific heterogeneity (180,181) the observed paracrine effect may well be specific for the placenta. However, the ability to reduce migration of endothelial cells towards the macrophage and thus, the regulation of angiogenesis without altering the direction of vascular growth, may occur also in other situations and organs than in the placenta. Further, macrophages are described to be motile, in contrast to endothelial cells, which possess a restricted motility because of their integration in a vessel assembly. Thus, it is not really surprising, that we found a reduced migration towards the HBC CM, because HBC are the ones which move towards the site they are needed. Probably, macrophages are able to modulate endothelial cells when they are in direct contact and not only by released factors.

5.1.1. HBC and placental pathologies

Given a role of HBC in placental angiogenesis, conditions and pathologies altering macrophage abundance and polarisation may affect this process. In fact, a shift in HBC phenotype was observed in placentas exposed to maternal type 1 diabetes (45), although this study analysed only a very limited number of samples. However, a recent study from our laboratory revealed that placental macrophages keep their anti-inflammatory, tissue remodelling M2 phenotype despite the hyperglycemic environment of the mother. This reliable polarization of the HBC may be important for the fetus, to be protected from a low-grade inflammatory milieu (182). Furthermore, maternal obesity is associated with an increase of placental macrophages (183). For both conditions, analysis of the placental vascular tree revealed altered morphology with increased vascular growth (184,185).

Also preeclampsia was shown to be associated with reduced numbers of HBC (46), but results are conflicting about whether or not preeclampsia is associated with reduced, or unaltered placental vascularity (186,187). Whether fetal growth restriction, a pathology

hallmarked by reduced feto-placental angiogenesis (187), is also associated with an altered number of HBC, remains to be studied.

5.1.2. Strengths and weaknesses with HBC

The strength of the study is the use of isolated human primary cells. HBC CM was applied on placental endothelial cells from late pregnancy. However, this represents also the limitation of the study, since some aspects of placental angiogenesis may be more important for placental development in earlier stages of pregnancy. Because methods for the isolation of HBC and feto-placental endothelial cells from early pregnancy with high yield and purity have not been established so far, we had to restrict our study to the third trimester (123). If an appropriate and established protocol for isolation of HBC from early gestational age is developed, isolation and culture may work. But as these cells do not proliferate, it can be assumed that the cell number is very small compared to the effort.

Another aspect, why isolation from term placenta is more effective is that from one placenta four different cell types can be isolated and cultured, namely endothelial cells, trophoblast cells, Hofbauer cells and fibroblasts. As the tissue from a first trimester placenta is very small, a decision for the isolation of one cell type would be needed.

Although experiments with primary cells are important it is also necessary to mention that the work with these cells is quite difficult. First of all, the protocol for HBC isolation was already established by *Tang et al.* (29)*,* nonetheless intense communication with co-workers from this research group was essential to establish this protocol in our laboratory. A great challenge with HBC is that there is no appropriate freezing method discovered until now. Strong exchange of several freezing methods was performed by these two laboratories, however with no proper solution.

5.1.3. Summary of HBC effects on placental angiogenesis

In summary, primary isolated HBC represent an M2 activated phenotype with diverse, i.e. M2a, M2b and M2c subtypes, and secrete pro-angiogenic and mitogenic

molecules and survival factors for endothelial cells. These characteristics are in fact referred to the tissue remodelling macrophages and thus, HBC are likely to support placental angiogenesis. This was verified by our in vitro experiments showing that HBC CM stimulates 2D network formation of primary placental endothelial cells (123).

5.2. Effect of FTB and TTB on placental angiogenesis

This study identified PEDF secretion as a novel paracrine mechanism of human trophoblasts to limit placental angiogenesis and vascular expansion in late pregnancy.

We report the following key findings: (1) CM from late pregnancy trophoblast reduced *in vitro* network formation and migration of primary placental endothelial cells, and PEDF contributes to this effect. (2) The anti-angiogenic effect of PEDF on placental endothelial cells depends on the concomitant activation of endothelial cells with VEGF where it modulates VEGF signalling.

Our study demonstrates the trophoblast as major source of PEDF in the placenta (127). The dependence of PEDF effects on VEGF in our experiments highlights the prominent role of VEGFR2 in anti-angiogenic PEDF effects and thus, was further investigated. Indeed, the finding that the PEDF effect was depended on VEGF, was observed also in other studies (105,167,188-190). VEGF is part of the trophoblast secretome and acts pro-angiogenic when applied alone. PEDF not only blocked the stimulatory VEGF effect, but some PEDF doses reduced network formation even below control levels, i.e. endothelial cells without VEGF-stimulation. This indicates that PEDF can also act through mechanisms other than only interfering with VEGF action. This was supported by the finding that PEDF modulates VEGF signaling in different ways, i.e. by reducing VEGFR2 mediated ERK1/2 phosphorylation, and by increasing FAK phosphorylation that was not affected by VEGF alone (127). The fact that PEDF competes with VEGF for binding to VEGFR2 and thus, attenuates VEGFR2 signaling was already observed by Zhang *et al*. and Yang *et al*. (105,167). We here, however, observed that the combination of PEDF with VEGF generates new signaling events that are not produced by PEDF or VEGF alone. Thus, while sFlt1 as a classical capture molecule can block VEGF effects, PEDF alters VEGF signaling and can produce additional effects (127).

Reduced ERK1/2 phosphorylation by VEGF in the presence of PEDF in fact parallels our findings that the combination of VEGF and PEDF reduced network formation and proliferation of placental endothelial cells, and attenuated vascular growth in the chorioallantoic membrane. However, increased FAK phosphorylation at tyrosine 397 in presence of VEGF and PEDF is difficult to interpret, since FAK phosphorylation and thus, FAK activity promotes cellular movement and proliferation (191,192). Thus, in a situation of reduced angiogenesis and proliferation caused by the combination of VEGF and PEDF, increased FAK phosphorylation seems counter intuitive. However, we here determined the effect of PEDF and VEGF on immediate signaling events, investigated only one of several FAK phosphorylation sites and did not analyze downstream effects. Hence, we observed that the combination of PEDF and VEGF produces distinct signaling events compared to both factors alone, but we cannot yet conclude that this combination ultimately stimulates FAK mediated cellular processes (127).

More PEDF was expressed in trophoblasts of late pregnancy, whereas sFlt1 secretion was stronger from trophoblasts of early pregnancy. Accordingly, sFlt1 appears to be the predominant regulator of placental angiogenesis in early pregnancy, whilst PEDF mediated anti-angiogenic effects prevail in late pregnancy (127).

To assess the effect of CM and PEDF angiogenesis, we employed a Matrigel network formation assay. In this highly reproducible assay, cells develop cord-like structures, which however, do not necessarily reflect the complex formation of vascular networks *in vivo*. At the same time, this kind of assay was and still is used to identify distinct pro- or anti-angiogenic molecules. A first screen was performed to examine stimulation or inhibition of network formation of endothelial cells. To date, all identified angiogenesis regulating factors started with this assay and showed similar activity *in vivo* afterwards (193).

To determine the effect of CM and PEDF on vascular development *in vivo* we employed the CAM assay, which allows monitoring of vessel growth in response to pro- and anti-angiogenic factors in real time in an *in vivo* setting (194).

In literature there are several receptors described to be binding partners of PEDF, such as (ATGL) (100), LR1 (101), F1-ATP synthase (102) and LPR6 (103). Here, deeper investigation on binding, activation and pathway discovery of LR1 and PEDF would be of

great interest. In general, LR1 mediates and signals the anti-angiogenic effect of PEDF (101). LR1 binds laminin with high affinity and affects an array of cellular processes such as cell adhesion, invasion, viability, proliferation and migration (195,196). Downregulation of LR1 induces apoptosis and potentially hampers proliferation of tumor cell lines (197). Neutralizing LR1 signaling with anti-LR1 antibodies completely blocks angiogenesis (198). Hence, in this context it is tempting to speculate that VEGF stimulates endothelial cell activation and migration and causes LR1 to detach from extracellular laminin. This would make LR1 available for binding of PEDF to mediate PEDF's anti-angiogenic effect.

5.2.1. PEDF and pregnancy pathologies

The effect of PEDF to limit placental angiogenesis may have implications for pregnancy pathologies that are related to altered feto-placental angiogenesis. Indeed, PEDF expression was decreased in total placental tissue after unexpected stillbirth, a condition associated with vasculopathy and increased angiogenesis (199). Since we identified trophoblast as the major site of placental PEDF production, this finding provides indirect evidence that trophoblast-derived PEDF is secreted towards the placental endothelium to regulate angiogenesis (127). Here, measurement of PEDF in maternal blood as well as placental blood may give a precise insight.

5.2.2. Oxygen tension

A further condition that needs to be considered is oxygen tension, especially when working with first trimester placental cells. Several studies already showed that oxygen levels in the first trimester are very low. This low oxygen milieu has different functions and affects several processes of placental growing and progression, such as vascular and villous development. However, in this study we worked with standard oxygen tension (20% O_2), because it would be quite difficult to compare results, when first and third trimester trophoblast cell were cultured under different conditions. A new onset in which first trimester trophoblast cells are cultured under standard (20% O_2) and low oxygen (5% O_2) conditions would probably give a deeper insight into PEDF expression in the first period of

gestation. Here, a perfect condition would be to use the same isolated cells from one individual in the two different oxygen conditions. This would give very comprehensible facts. The next challenging step would be the addition of first trimester trophoblast conditioned medium to feto-placental endothelial cells to observe network formation. Because the feto-placental endothelial cells used in this study are derived from third trimester and are therefore cultured under standard oxygen tension (20% O_2).

5.2.3. Advantages and disadvantages of primary cells

Another disadvantage specifically when using primary isolated trophoblast cells in culture is that once separated from their underlying basement membrane *in vivo* they lose their proliferative capability.

At the same time, primary isolated human placental cells possess many advantages. Each cell isolation is characterised and controlled, which means that misidentification and contamination are excluded even before cells or conditioned medium are used. Misidentified and contaminated cell lines are now discussed by all scientists. Today researchers have become increasingly cautious when interpreting data generated from cell lines only (200,201). A very important and reliable advantage of primary isolated cells *in vitro* is their perpetuation of specific markers and functions, which were previously seen *in vivo* (202,203). Very essential for comparative studies is the use of cells from different patients/donors. This is easily achievable when using isolated cells from several individuals. To investigate physiological processes *in vitro* primary human cells are more reliable. The primary cell-based assays are a key parameter to improve prediction for *in vivo* approaches (204).

5.2.4. Summary of first and third trimester TB effects on placental angiogenesis

In summary, trophoblasts, particularly in late pregnancy, secrete PEDF to restrict growth and expansion of the placental endothelium. The trophoblast forms the placental epithelium, which is in contact with the maternal circulation, in contrast to the placental endothelium, which has no direct contact with maternal blood. The important implication of

our findings is that the maternal environment may affect the placental endothelium through modulating the trophoblast-endothelial paracrine network (127).

5.3. Overview and future prospective

This study gives an overview of how the investigation with primary cells can achieve a variety of findings. We found that placental angiogenesis in late pregnancy can be regulated in a paracrine manner by non-endothelial cells like trophoblasts and Hofbauer cells. Third trimester trophoblasts revealed a reduction on the angiogenic potential of placental endothelial cells presumably by the anti-angiogenic molecule PEDF, whereas Hofbauer cells possess stimulatory properties. Probably, HBC-secreted VEGF, FGF2 and TNFA are involved in angiogenic processes such as migration, proliferation, cell survival and network formation of endothelial cells. All these findings and several further molecules from literature (7,205,206, 208-212) are shown in figure 28.

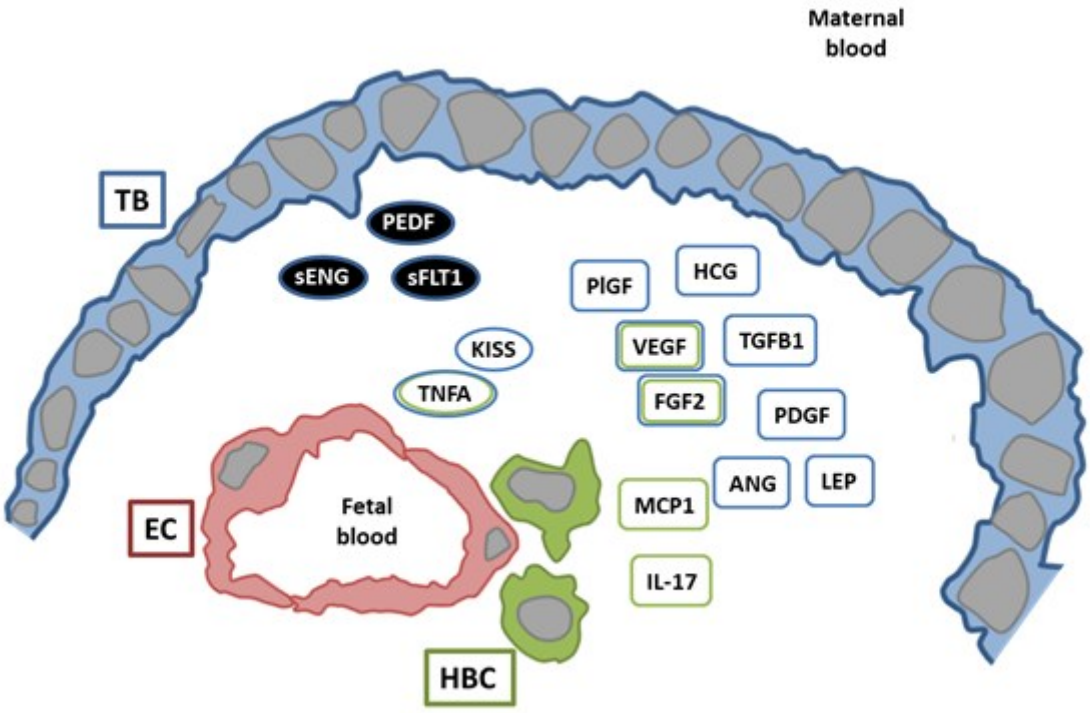

Figure 28. Angiogenesis modulating factors secreted by trophoblasts and Hofbauer cells in the third trimester placental villus. Endothelial cells (EC) in red, trophoblast cells (TB) in blue and Hofbauer cells (HBC) in green. Pro-angiogenic factors are in squares with white background, anti-angiogenic factors are in ovals with black background and molecules with both features are in ovals with white background. The colour of the frame indicates the source of the molecule, blue deriving from TB and green deriving from HBC.

There are more interesting investigations to be performed. For example, the direct interaction by cell to cell contact between placental endothelial cells and trophoblast and Hofbauer cells would give a deeper insight in the complex regulatory mechanisms of placental angiogenesis. Another interesting aspect would be to examine whether secreted factors by HBC and TB have an effect on one of these two cell types. Probably, VEGF or FGF2 released by HBC may stimulate TB differentiation or proliferation. Otherwise, TB derived sFlt1 or PEDF may regulate HBC migration or secretion of other molecules.

All these experiments were performed with healthy primary isolated cells from healthy pregnancies. Thus, investigations with cells complicated by GDM or IUGR may provide new aspects or characteristics of these gestational conditions.

6. References

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