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Regulatory factors controlling invasion of first trimester
human trophoblasts

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1. ABSTRACT

Invasion of the trophoblasts into the uterine wall and maternal spiral arteries is a crucial event during establishment and development of human pregnancy. After implantation of the blastocyst in decidua, cytotrophoblasts start to form placental villi. Progenitor cells remaining at the tip of the placental villi will further differentiate into specific trophoblastic cells playing a distinct role in placental development. Invasion of trophoblasts is a strictly controlled process. There are known many factors governing this fundamental process but still many mechanisms remain unknown.

During my PhD studies, I was investigating how different factors regulate the process of trophoblasts invasion. My first project dealt with the role of activating enhancer-binding protein-2 α in human trophoblasts invasion. It is known that loss of AP-2 α is associated with tumor progression; moreover AP-2 α is also involved in regulation of differentiation of human trophoblasts by controlling variety of invasion-associated genes. Therefore, it was interesting to study a role of AP-2 α in invasion of human trophoblasts. First, I performed descriptive studies to indicate the expression of AP-2 α in placental tissue, primary trophoblasts and trophoblast cell lines. Immunofluorescence (IF) and semiquantitative RT-PCR analyses revealed that AP-2 α and AP-2 γ are expressed in the villous trophoblasts, interestingly high level of AP-2 α expression was also detected in extravillous trophoblasts (EVTs). Next, I performed loss of function studies using small hairpin microRNA in the trophoblastic cell line SGHPL-5. Western Blot (WB) data indicated that the extent of AP2 α gene silencing was approximately 70% in both the SGHPL-5 cell pool and EVTs compared to non-targeting controls. Primary EVT cells were treated with small interfering RNA (siRNA) oligonucleotides targeting AP-2 α . Invasion assays with the knock-down cells were performed on fibronectin (EVTs) and matrigel (SGHPL-5 cells) coated transwells. Proliferation assays, were conducted by measuring cumulative cell numbers in SGHPL-5 cells deficient of AP-2 α by performing bromodeoxyuridine (BrdU) labeling in EVT cells knock-down cells. Additionally, the transfected cells were stimulated during functional assays with epidermal growth factor (EGF). EGF-dependent invasion of the AP-2 α knock-down cell pool in SGHPL-5 and

primary EVT_s respectively, was significantly decreased compared to controls, proliferation was unaffected.

One of the main goals was identification of AP-2 α target genes important for controlling trophoblasts invasiveness. Genes such as metalloprotease-2 (MMP-2), chorionic gonadotropin (CG), plasminogen activator inhibitors (PAIs) and urokinase plasminogen activator (uPA) were analyzed with WB in the absence or presence of EGF. Performed studies revealed upregulation of TIMP3 but downregulation of MMP2, MMP9, PAI-1, PAI-2, TIMP1, TIMP2, as well as the active form of uPA in the stable AP2 α knock-down pool. In transient AP2- α knock-down in EVT_s downregulation of MMP-2, and CG was observed. Knowing that AP-2 α regulates CG, I have shown that luciferase activity of CG β 5 was significantly decreased in the AP-2 α knock-down cells, this effect was compensated by transient overexpression of AP-2 α . Overexpression of AP-2 α in SGHPL-5 cells confirmed the previous experiments. After AP-2 α overexpression in SGHPL-5 cells the invasion on matrigel was increased approximately by 20%.

During my second project, I focused on the descriptive and functional studies of a disintegrin and metalloprotease 12 (ADAM-12) in first trimester trophoblasts invasion. The motility of human trophoblasts is a carefully regulated process where proteolytic enzymes are thought to play a fundamental role. First, I studied the expression pattern of ADAM-12, utilizing first and term placental tissues, differentiating primary trophoblasts and trophoblastic cell line SGHPL-5. IF staining, qRT-PCR and WB analyses revealed, that ADAM-12 is absent in proliferative cell column trophoblasts but its expression increases during EVT_s differentiation. In addition, in vitro differentiation of the EVT_s on fibronectin coated dishes indicated markedly increase of ADAM-12 expression during culture until 72h. Loss of function studies were performed in first trimester isolated EVT_s using siRNA oligonucleotides. WB data and qRT-PCR revealed that the extent of AP-2 α gene silencing was approximately 80% when compared to controls. Next, the functional studies, invasion on fibronectin-coated invasion inserts and proliferation using anti-Ki67 antibody staining of the generated knock-down in primary EVT_s and BrdU labeling were done. After ADAM-12 depletion invasion of primary EVT_s was decreased about 20% when compared to control. However, proliferation and adhesion assay revealed no significant changes when compared ADAM-12 knock-down EVT_s to controls. Additionally, I silenced ADAM-12 in first trimester placental explants cultivated on rat-tail collagen I. Knock-down of ADAM-12 in placental explants demonstrated decrease in

EVTs motility when compared to controls. Moreover, the cleavage assay of insulin-like growth factor binding protein 3 (IGFBP-3) was performed with supernatants from ADAM-12 knock-down cells. WB analysis has shown significant decrease of IGFBP-3 cleavage products in the supernatants of ADAM-12 deficient EVTs. Finally, I transiently overexpress ADAM-12L and -S respectively in the ADAM-12 negative SGHPL-5 cell line and performed invasion assay which revealed significant increase in cell invasiveness.

Summarizing, it was shown that AP-2 α controls proteases and inhibitors important for invasion of trophoblasts and therefore play important role in the control of growth dependent invasion. Studies of ADAM-12 revealed that ADAM-12 is strongly induced during primary trophoblasts differentiation and therefore is important for regulation of EVTs invasiveness.

2. ZUSAMMENFASSUNG

Die Einnistung der Blastozyste und die damit einhergehende Invasion der Trophoblasten in die Gebärmutterschleimhaut und Spiralarterien ist ein fundamentales Ereignis für die Entstehung einer erfolgreichen Schwangerschaft. In der Dezidua, dem durch den Einfluss von Östrogen und Progesteron umgewandelten Endometrium, beginnen Cytotrophoblasten mit der Formung plazentaler Villi, an deren Spitze durch Proliferation und Differenzierung invasive Trophoblasten hervorgehen. Die Invasion selbst ist ein streng kontrolliert ablaufender Prozess in dem viele Faktoren zusammenspielen, viele von ihnen aber noch unbekannt sind.

Während meiner Dissertation untersuchte ich wie unterschiedliche Faktoren den Prozess der Invasion regulieren und beeinflussen können. Mein erstes Projekt befasste sich mit der *activating enhancer-binding protein 2* (AP-2) Familie der Transkriptionsfaktoren, im speziellen mit AP-2 α . Es ist bekannt, dass ein Verlust dieses Faktors mit der Entstehung von Tumoren einhergeht und auch in der Differenzierung von Trophoblasten eine Rolle spielt. Des Weiteren wurde zuvor gezeigt, dass AP-2 α mehrere Gene deren Expression in Trophoblasten eine wichtige Rolle spielt reguliert. Zu Beginn zeigte ich die Expression von AP-2 α in plazentalem Gewebe, primären isolierten Trophoblasten und der Trophoblasten Zelllinie SGHPL-5. Mittels Immunofluoreszenz-Mikroskopie und semiquantitativer PCR Analyse konnte ich zeigen, dass AP-2 α und AP-2 γ sowohl in villösen als auch in extravillösen Trophoblasten (EVTs) exprimiert werden. Anschließend führte ich einen AP-2 α *knock-down* durch Transfektion mit *short hairpin RNA* in SGHPL-5 Zellen durch, welcher via Western Blot bestätigt wurde. In EVTs setzte ich siRNA Oligonukleotide zum *Gen-silencing* ein und verwendete *non-targeting* siRNA als Kontrolle. Funktionelle Auswirkungen des *Knock-downs* wurden dann in Bezug auf Invasion durch beschichtete Transwells (Matrigel für SGHPL-5 Zellen und Fibronektin für EVTs) und Proliferation durch Ermittlung der kumulativen Zellzahl (SGHPL-5 Zellen) beziehungsweise Bromodeoxyuridine (BrdU) Einbau (EVTs) ermittelt. Zusätzliche zur Transfektion wurden die Zellen mit *epidermal growth factor* (EGF) stimuliert, was eine Verringerung der Invasion, jedoch keine Veränderung der Proliferation zur Folge hatte. Des Weiteren war es von Interesse direkte Zielgene des

Transkriptionsfaktors AP-2 α zu identifizieren. Analysiert wurden die Gene von Metalloprotease-2 (MMP-2), chorionisches Gonadotropin (CG), Plasminogen Aktivator Inhibitor (PAI) und Urokinase Plasminogen Aktivator (uPA) mittels Western Blot (WB), mit und ohne Zugabe von EGF. Die Ergebnisse zeigen, dass TIMP3 hochreguliert, während MMP2, MMP9, PAI-1, PAI-2, TIMP1, TIMP2 und die aktive Form von uPA herunterreguliert wurden wenn AP-2 α dauerhaft heruntergeregelt worden war. Wenn AP-2 α in EVT's transient herunterreguliert wurde, konnte eine Abnahme von MMP-2 und CG beobachtet werden. Mit diesem Wissen führte ich einen *Luciferase Gene Reporter Assay* durch und konnte zeigen, dass die Aktivität von CG β 5 in der AP-2 α knock-down Kondition deutlich abnahm, während eine transiente Überexprimierung von AP-2 α diesen Effekt kompensierte. Eine Überexprimierung von AP-2 α in SGHPL-5 Zellen bestätigte diese Resultate, so nahm die Invasion hier um etwa 20% zu.

Mein zweites Projekt beschäftigte sich mit der deskriptiven und funktionellen Analyse von *a disintegrin and metalloprotease 12* (ADAM-12) während der Invasion von Trophoblasten des ersten Schwangerschaftstrimesters. Die Motilität dieser Trophoblasten ist ein genau regulierter Prozess in dem proteolytische Enzyme als wichtige Faktoren angesehen werden. Zu Beginn verglich ich das Expressionsmuster von ADAM-12 in Terminplazenten, Plazenten des ersten Trimesters, differenzierenden primären Trophoblasten und der Zelllinie SGHPL-5. IF Färbungen, qRT-PCR und Western Blot Analysen zeigten, dass ADAM-12 in proliferativen Zellsäulen Trophoblasten nicht detektierbar ist, jedoch mit der EVT Differenzierung zunimmt. In vitro Kultivierung von primären EVT's auf Fibronectin für 24 beziehungsweise 72 Stunden bestätigte dieses Ergebnis. Anschließend führte ich ADAM-12 *Gene-silencing* Experimente mit siRNA Oligonukleotiden durch, wobei mittels Western Blot und qRT-PCR ein Knock-down von etwa 80% festgestellt werden konnte. Daraufhin testete ich den Effekt des Knock-Downs in EVT's auf deren Invasion durch Fibronectin beschichtete *Transwells*, wobei eine Erniedrigung um 20% beobachtet wurde, während die durch Ki67 und BrdU Färbungen ermittelte Proliferation unverändert blieb. Auch die Adhäsion der Zellen wurde durch den ADAM-12 Knock-down nicht beeinflusst. Als weiteres Modell verwendete ich plazentäre Explantate des ersten Schwangerschaftstrimesters und kultivierte diese auf Rattenschwanzkollagen-1, mit und ohne Knock-down von ADAM-12. Hierbei wurde ein Abfall der Motilität der auswachsenden Trophoblasten festgestellt wenn ADAM-12 herunterreguliert war. Des Weiteren führte ich einen *cleavage assay* von IGFBP-3 mit

Überständen von ADAM-12 defizienten Zellen durch und konnte zeigen, dass in der Knock-down Kondition weniger IGFBP-3 Spaltprodukte vorhanden waren. Abschließend überexprimierte ich jeweils ADAM-12L und ADAM-12S transient in ADAM-12 negativen SGHPL-5 Zellen und konnte dadurch eine Erhöhung der Invasivität feststellen.

Zusammengefasst konnte durch meine Arbeit für meine Dissertation gezeigt werden, dass AP-2 α für die Invasion von Trophoblasten essentielle Proteasen und Inhibitoren reguliert und deshalb ein wichtiger Faktor in diesem System ist. Für ADAM-12 wurde bewiesen, dass dessen Expression mit fortlaufender Differenzierung der Trophoblasten induziert wird und dass es ebenfalls eine antagonistische Rolle in der Invasion von EVT's spielt.

3. INTRODUCTION

3.1 General introduction

3.1.1 Development of the human placenta

The human placenta is a unique, transient organ, which connects the mother with the developing embryo (**Fig.1**). Early development of the human placenta takes place under low oxygen conditions [1, 2]. The human placenta like in most of the mammals is an chorioallantoic organ [3]. Human placenta is discoidal in a shape, villous and hemochorial which means that the chorion is in direct contact with maternal blood. Placenta functions only for 9 months and only for pregnancy purpose, nevertheless the impact of this organ on the baby remains for the whole lifetime [4].

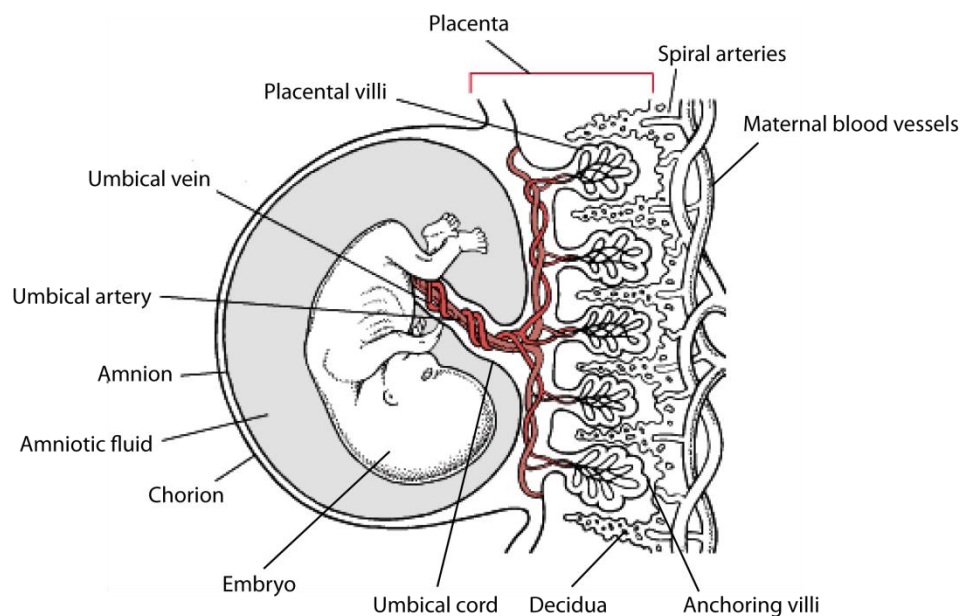


Figure 1. First trimester placenta with the developing embryo

The embryo is surrounded with the inner amnion membrane filled with amniotic fluid and outer membrane called chorion. The umbilical cord connects embryo with the placenta. The umbilical vein and the umbilical artery inside umbilical cord guarantee continuous exchange of gases and the delivery of the nutrients. From the Merck Manual Home Health Handbook, edited by Robert Porter. Copyright (November 2007) by Merck Sharp & Dohme Corp., a subsidiary of Merck & Co, Inc, Whitehouse Station, NJ. Available at <http://www.merckmanuals.com/home/>. Accessed (December 2012).

The influence of the placenta on the developing baby is enormous. The babies born after pregnancy with intrauterine growth restriction (IUGR) (see chapter 3.2, Page 14) have a higher risk to develop type 2 diabetes, hypertension and cardiovascular disease later in adult life [5-7]. The paradigm is called ‘fetal programming’ and can be caused mostly by mother malnutrition and reduced utero-placental blood flow [8]. Epigenetic modifications such as methylation status of the fetal genome can be as well a consequence of maternal nutrition [9]. Many imprinted genes are responsible for the fetal growth and most of them are of the parental origin e.g. insulin-growth factor (IGF-II) enhancing fetal growth [10].

The main function of the placenta is the delivery of nutrients and oxygen, as well as removal of metabolic wastes and gases as well as immunological protection of the child. Placenta guarantees immunological protection for the fetus providing a barrier for fetal infection [1]. Another important function of the placenta is immunological acceptance [11]. Human hemochorial placenta comes into direct contact with maternal blood. This requires immunological acceptance to protect new, foreign, developing organism against rejection by the mother immune system. The developing fetus is an allograft for the mother, containing paternal genes [12]. Important role in immunological acceptance of allogeneic fetus by the mother plays expression of MHC class I, histocompatibility antigen (HLA-G, HLA-C and HLA-E) by EVT. Interestingly, villous trophoblasts and syncytiotrophoblasts remaining in contact with mother blood do not express any of MHC molecules [13], HLA-G is elusively produced by the EVTs and present in the serum of pregnant women [12-14]. Recent studies indicate that secreted HLA-G1 suppress immune system by inducing apoptosis in CD4 positive T-cell proliferation. It was also shown that HLA-G1 might be expressed during viral infections or organ transplantation to protect tissue during inflammation by evolvment of Th2 response (anti-inflammatory response involving cytokines: IL-, -5,-13) [15, 16].

Beside HLA-G molecule, also uterine natural killer cells (uNK) play important role in acceptance of the fetus. uNK cells are a part of the innate immune system, they are found in both peripheral blood and decidua [14] uNK are the main compartment of the immune cells in the decidua, representing up to 70% all decidual leukocyte population [17]. It was shown that uNK may regulate trophoblasts invasion and arterial function [14]. Moreover, it was also shown that uNK cells produce various angiogenetic growth factors such as; angiopoietin-1, -2 (Ang-1, -2) and VEGF. In addition, uNK cells were indicated as producers of MMP-2 and MMP-9 which are known to regulate spiral artery remodeling

[18]. The number of uNK cells increases in decidua where invasion of trophoblasts takes place. One of the hypotheses is that uNK cells have capability to recognize paternal, trophoblasts ligands and therefore regulate extent of trophoblasts invasion. Furthermore, it was shown that uNK cells express receptors, such as killer-cell immunoglobulin-like receptors (KIRs), CD94/NKG2 and of HLA-G, HLA-C and HLA-E molecules [14].

One of the factors important for the trophoblasts differentiation and invasion is the oxygen level. The early placenta develops in a hypoxia (2% oxygen concentration) until 11th week of gestation, then oxygen concentration increases up to 8% [1]. It was shown that low level of oxygen keep trophoblasts in proliferative state whereas high oxygen level is required for invasive phenotype [2, 19]. Moreover, it was also shown that transcription factor hypoxia inducible factor 1 α (HIF-1 α) plays here important role in cellular regulation of hypoxia [20]. In addition, as published previously inhibition of HIF-1 expression inhibits expression of TGF β and therefore arrests cell proliferation, decreased α 5 integrin expression, gelatinase A activity and activate adhesive phenotype [2]. During first trimester of the pregnancy the uterine spiral arteries are plugged by the EVT's to limit the excess of the oxygen in developing placenta [21]. It was shown that low oxygen level is crucial for proper functionality of the placenta and reduces oxygen stress. Improper plugging of the maternal spiral arteries might be danger for placental development and therefore for the fetus, by causing oxygen mediated apoptosis [21]. The endovascular plugs disappear later when the pregnancy develops, to enable oxygen and nutrients transport to the fetus.

Placenta develops from the trophoblast which surrounds the blastocyst. The trophoblasts are the main cell type of the placenta; trophoblast (from Greek trephein- to feed and blastos- germinator). Briefly, after fertilization of the egg in the oviduct (**Fig.2**), so-called zygote is formed. Further cell divisions result in formation of the morula consisting of 32 cells. The blastocyst is formed 24h after morula stage and around 5 days after fertilization. The blastocyst is surrounded with an outer layer of epithelial cells, called trophoblast. The pluripotent cells called the inner cell mass are capsuled in the inner cavity of the blastocyst. The inner cell mass will give a rise to the three germ layers; endoderm, ectoderm and mesoderm and later form the embryo.

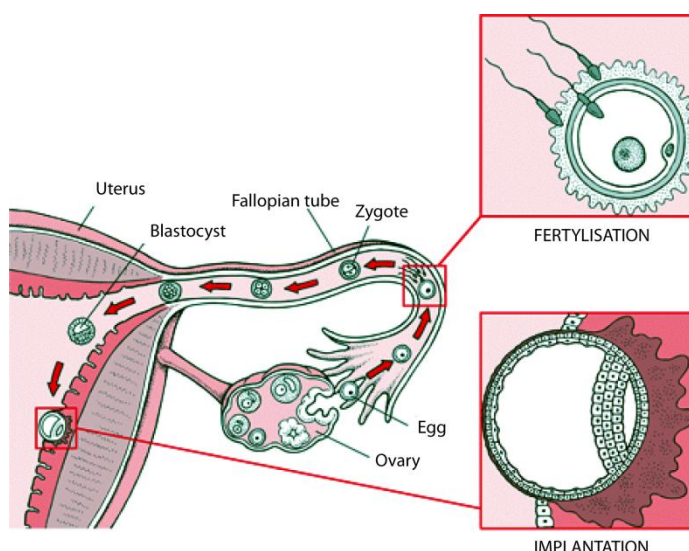


Figure 2. Process of the conception

Each month one egg leaves one of the ovaries. When the ovum meets the sperm the fertilization take place. The fertilized egg divides rapidly and extensively while moving along the oviduct. Finally, the blastocyst arrives at the uterus and implants into the endometrium. From the Merck Manual Home Health Handbook, edited by Robert Porter. Copyright (November 2007) by Merck Sharp & Dohme Corp., a subsidiary of Merck & Co, Inc, Whitehouse Station, NJ. Available at, <http://www.merckmanuals.com/home/>. Accessed (December 2012).

The implantation of the blastocyst into the decidua takes place around day 7-9 after fertilization and is strictly controlled by different factors [22]. Before the blastocyst attaches to the uterine wall, endometrial cells under control of rising progesterone levels proliferate and differentiate to form the decidua [23]. Decidualization occurs every month in the mid-secretory phase even when the fertilization did not take place and is mediated by estrogen and progesterone, two steroid hormones. Implantation occurs during the so-called 'implantation window' when decidualization process is accomplished and the decidualized endometrium is ready to accept the blastocyst [24]. Successful implantation is fundamental for initiation of pregnancy. Here, proper receptivity of the uterus is a critical factor allowing attachment of the blastocyst. As mentioned, the mechanism of implantation is regulated by many factors such as; hormones, cytokines, and growth factors. It is known, that cytokine interleukin-11, (IL-11), interleukin-1 β (IL-1 β) and leukemia inhibitor factor (LIF) are important factors regulating different implantation stages [25, 26]. The studies in vitro with LIF-null mutant mice showed failures in the blastocyst implantation [24], whereas blockage of the IL-1 receptor prevents implantation in mice [27]. Another molecule which is shown to play role in implantation is L-selectin, providing help for the blastocyst adhesion [28].

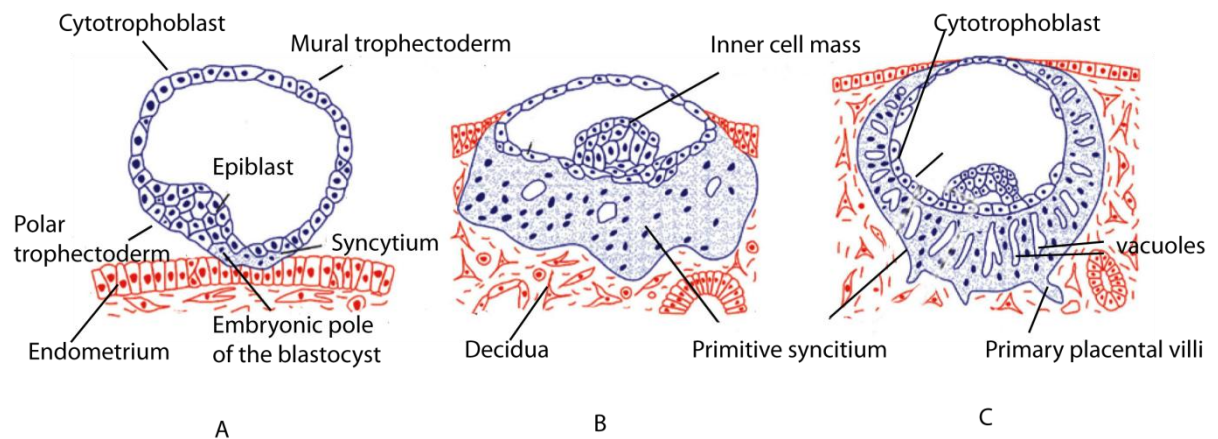


Figure 3. Implantation of the human blastocyst

A, B, prelacunar stage (apposition and adhesion), C, lacunar stage (invasion). Taken from and modified, "The Pathology of the human placenta" Kurt Benirschke, Peter Kaufmann

The implantation process is divided into three key steps; apposition, adhesion and invasion [29]. First, blastocyst is guided to a special area of the uterus (apposition). During adhesion blastocyst adhere to receptive endometrium with the so-called polar trophoblast (**Fig.3A**) which make up 1/3 of the whole trophoblastic layer and is the closest located trophoblast to the inner cell mass. The embryonic pole of the blastocyst is the side of the blastocysts which attaches to the decidua. Finally, primitive syncytium, the outermost layer of the polar trophoblast migrates into endometrial stroma (invasion) (**Fig.3B**) [30]. Primitive syncytium is responsible for production of digestion enzymes and therefore facilitating the invasion of the blastocyst. It probably exists only during the implantation time and later on is shed by the placenta. Subsequent to implantation the development and the differentiation of the different trophoblastic cell lineages occur. The differentiation and development of specific trophoblastic cell subtypes take place after implantation and is one the fundamental steps in the initiation and progression of pregnancy [31]. Around day 8-9 in the primitive syncytium appear vacuoles forming lacunae system (**Fig.3C**). Later on syncytial areas are invaded by cytotrophoblasts (CTBs) proliferating from the chorionic plate [32]. In the further stages villi develop into secondary and tertiary structured villi until the third week of the pregnancy.

3.1.2 Trophoblasts differentiation and invasion

3.1.2.1 Placental trophoblasts differentiation

The progenitor cells lying at the basement membrane of the placental villi give rise to specialized trophoblastic cells [31]. Basically, two types of placental villi are formed during development of the early placenta; the floating villi (FV), freely bathing in the maternal blood and anchoring villi (AV). The progenitor cells of the floating villi, so called villous CTBs (vCTBs) differentiate to form multinucleated syncytium (S). The main function of the syncytium is the production of pregnancy hormones; human CG and placental lactogen (PL) and control over placental-fetal interaction, namely regulation of oxygen and protein transport. The main function of the anchoring villi is the attachment to the maternal decidua and subsequently formation of the cell column (CC) (**Fig.4**). The progenitor CTBs at the tip of the anchoring villi proliferate extensively which results in formation of multilayered cell column of highly proliferative, CC CTBs. Upon attachment to the endometrium, the CC CTBs stop proliferation and gain an invasive phenotype. So-called, invasive, extravillous trophoblasts (EVTs) detach from the cell column and invade the decidua. [33]. EVT's differentiate into interstitial CTBs (iCTBs) which later differentiate into endovascular CTBs (eCTBs) and giant cells (GC) (**Fig.4**). The iCTBs invade decidua up to one third of the myometrium and next differentiate into GC. Exact function and differentiation process of GC remain still unknown. eCTBs are present in the lumen of uterine vessels and actively participate in remodeling of uterine spiral arteries. [33, 34]. They next replace endothelial cells, in the maternal blood vessels to make them wider. The maternal vessels are prepared for the higher pressure of blood delivering the oxygen and nutrients to the fetus and placenta and for transporting back to the mother the metabolic wastes and carbon dioxide [31].

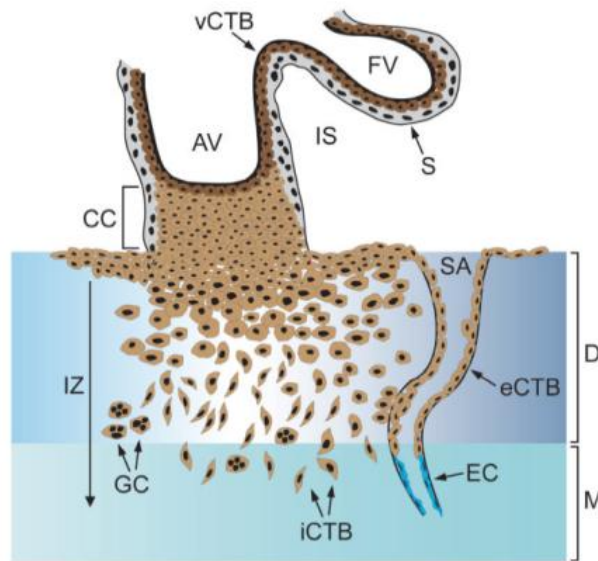


Figure 4. Attachment of the anchored villi (AV) to the maternal decidua (D)

The progenitor cells (vCTB) start to proliferate, forming cell column (CC). Whereas the vCTB in floating villi immersed in the maternal blood will fuse to form syncytium (S). The extravillous trophoblasts (EVTs) invade up to 1/3 of the mother myometrium (M). Endovascular trophoblasts (eCTB) remodel maternal spiral arteries (SA) by replacing endothelial cells (EC) and invade maternal decidua whereas interstitial cytotrophoblasts (iCTBs) penetrate the decidua and differentiate into giant cells (GC). Figure taken from [31].

3.1.2.2 Trophoblasts invasion

The invasion process of trophoblasts is complex and strictly controlled process by different forces and regulators. Different factors facilitate motility of trophoblasts being strictly controlled in time and distance. During early placentation, differentiating EVT's leave the CC, stop proliferation and invade extracellular matrix (ECM). The ECM consists mainly of collagens I and IV, fibronectin and fibrillin I. Additionally, trombospondin I, tenascin C, another collagens, glycoproteins and proteoglycans were detected [1]. It was shown that invasive cells interact with components of ECM by adhesion to its specific receptors [35]. The most crucial factors facilitating adhesion and invasion of trophoblasts are integrins, binding to most ECM proteins such as; collagens, laminins and fibronectin [36]. Normal trophoblasts invasive differentiation in vivo and in vitro is characterized by switching of the specific cellular markers. The undifferentiated CTBs residing at the placental villi membrane remain $\alpha 6 \beta 4$ and $\alpha 6 \beta 1$ integrin and E-cadherin positive. Whereas the growth arrested, invasive EVT's become $\alpha 5 \beta 1$ and $\alpha 1 \beta 1$ positive [36]. The expression of $\alpha 5 \beta 1$, a receptor for fibronectin is induced during

trophoblasts differentiation [37]. The invasive EVT's lose marker of polarized epithelium such as $\alpha 6 \beta 4$ and E-cadherin [37]. EVT's during their differentiation pathway express epithelial markers such as cytokeratin-7.

Another crucial factors playing role in invasion of human trophoblasts during early placentation are matrix metalloproteases which are widely described in chapter 3.3.3, of this thesis.

3.1.2.3 Epithelial mesenchymal transition

The physiological differentiation and invasion of the human trophoblasts shows some analogy with epithelial-mesenchymal transition (EMT) [31]. EMT occurs during metastasis but also during normal, developmental processes [38]. EMT is characterized by lose of cell-cell contact and cell polarity. Cells undergoing EMT gain invasive, mesenchymal phenotype [39]. Common characteristic for EMT and early placentation is an involvement of similar transcription factors, e.g. members T-cell factor (TCF) regulated by Wnt signaling and Snail [31]. The neoplastic cells similar to differentiating trophoblasts downregulate cell junction molecules such as E-cadherin. However, in contrast to EVT's remain vimentin (mesenchymal marker) negative, but cytokeratin-7 (epithelial marker) positive [40]. Another common characteristic of these two processes is expression of biochemical mediators of invasion by trophoblasts and metastatic cells, namely MMPs and their inhibitors TIMPs [41], widely described in chapter 3.3.3, of this thesis.

3.2 Pregnancy diseases

Proper trophoblasts invasion of the maternal decidua and remodeling of the uterine spiral arteries is a critical step in initiation and progression of the pregnancy. Pregnancy diseases caused by failures in trophoblast invasion take place when too shallow or too deep trophoblasts invasion into the myometrium takes place. Most of pregnancy disorders may cause life-threatening consequences for the mother and the embryo. Recurrent miscarriage affects 1-3% all pregnant women [24]. The main reason of this disease is still not known but oxidative stress, impaired decidualization and involvement of uNK cells may play a role. The role of uNK cells in recurrent miscarriage remain still unclear however, some studies reported increased number of CD56-positive uNK cells in such

pregnancies [42]. Another hypothesis is that in cases of recurrent miscarriages uNK cells show increased cytotoxicity to the implanting embryo [42]. In addition, oxidative stress could play fundamental role in recurrent miscarriage. Normally, it arises after earlier loosening of the trophoblasts plugs in the spiral arteries after 12th week of pregnancy. However, uncontrolled flow of the maternal blood into intervillous space, results in trophoblasts damage and may lead to pregnancy loss [21].

Superficial invasion of trophoblasts may lead to pregnancy disorder preeclampsia (PE). PE cases make up 3-8% of all pregnancies affecting in total about 8,5 million pregnancies worldwide. It is considered to be the most threatening pregnancy complication causing fetal and maternal death. PE is diagnosed by the presence of hypertension, proteinuria and edema in the mother [43]. Although, the mechanisms that cause PE remain still unclear. This severe pregnancy disorder may be caused by shallow trophoblasts invasion and inefficient remodeling of maternal spiral arteries [34]. One of the hypothesis might be that PE is caused by impaired expression of factors characteristic for invasive differentiation pathway of EVT_s [43]. It was found that in preeclamptic pregnancies expression of, integrin $\alpha 1\beta 1$, $\alpha 5\beta 3$, [44], VE-cadherin, vascular cell adhesion molecule (VCAM-1), and platelet cell adhesion molecule (PECAM-1) are diminish [43], interestingly expression of $\alpha 6\beta 4$ and E-cadherin [45] remain unaffected. This suggests that trophoblast start the invasive differentiation but do not have ability to accomplish this process. Moreover, the expression of matrix proteases MMP-9 [46] and HLA-G [13] in PE pregnancies was altered. Furthermore, it needs to be mentioned that immune maladaptation may lead to PE in pregnancy. uNK cells may be able to control the extent of the EVT_s invasion and remodeling of spiral arteries and therefore could play also important role in PE pathogenesis. Recently, the new clinical marker for detecting PE was found, an angiogenetic factor, namely placental growth factor (PlGF). It was shown that in pregnancies with PE complication PlGF was significantly decreased when compared to healthy ones.

PE in about 30% cases is associated with another pregnancy disorder, namely intrauterine growth restriction (IUGR). The IUGR can also be observed without PE in about 8-14% cases. The main complication of IUGR is very low weight of the new born baby. Both diseases are associated with inadequate remodeling of maternal spiral arteries, resulting in poor oxygen and nutrient transport to the placenta.

Conversely, excessive trophoblasts invasion leads to several gestational disorders like; placenta percreta, choriocarcinomas and hydatidiform mole placentae. Placenta percreta is the most severe kind of hyperinvasive placenta and is characterized by deep trophoblasts infiltration of the mother myometrium. Moreover, trophoblasts can migrate to other organs in the mother body e.g. urinary bladder [47].

The hydatidiform (HM), complete (CHM) and partial (PHM) moles and choriocarcinoma belong to a group of gestational neoplastic diseases (GTD)[48]. Clinically, HM is characterized by swelling of the chorionic villi and hyperproliferation of trophoblasts. It could be either classified as complete or partial depending on histopathological analysis and karyotype. CHM develops from fertilization of an anuclear ovum by haploid spermatozoa; therefore the final karyotype of CHM is diploid (46XX), without developed embryo. PHM develops when an anuclear ovum is fertilized by two spermatozoa, resulted in triploid zygote carrying 69 chromosomes. In case of PHM the unviable embryo may develop. An invasive mole is a type of HM which is formed of enlarged molar villi, penetrating deeply into the myometrium and producing metastatic lesions to surrounding organs. In contrast to choriocarcinoma invasive mole can regress spontaneously. Choriocarcinoma is characterized by absence of villi and high invasiveness of trophoblasts to surrounding tissues [48].

3.3 Factors regulating trophoblasts invasion in the human placenta

Trophoblasts invasion, maternal spiral arteries remodeling and development of the placenta are complex and precisely controlled processes. The mechanisms regulating trophoblasts motility develop from the trophoblasts itself or from the decidua. Among the factors playing a role in trophoblasts invasion are transcription factors, growth factors, metalloproteases, cytokines and pregnancy hormones.

3.3.1 Transcription factors

Transcription factors, proteins binding to specific DNA sequences are located in the cell nucleus where they bind to DNA and regulate expression of the target genes. Some transcription factors control processes in the human placenta development, as: proliferation, invasion, and formation of the syncytium [19, 49].

In mice, various transcription factors important during placentation were broadly described. One of such transcription factor Mash-2 belonging to achaete-scute family has been shown to be of special importance for murine trophoblasts as shown during studies with Mash-2 null mice [50]. The mice lacking Mash-2 die because of failure in the placental development [50]. It was shown that Mash-2 is responsible for maintenance and proliferation of spongiotrophoblasts. Interestingly, human variant of achaete-scute homologue 2 (Hash-2) was shown to be expressed in isolated second trimester CTBs and EVT_s in vivo [51], additionally was shown that its expression decreases during differentiation in vitro [52]. Another mice associated transcription factor playing role in placentation is heart and neural crest derivatives-expressed protein 1 (Hand1) belonging to a group of transcription factors basic helix-loop-helix (bHLH). Hand1 was indicated to be important for mice trophoblasts development, promoting differentiation of trophoblasts precursors to giant cells [53]. Furthermore, Hand1 was shown to increase during differentiation. However, in human Hand1 was only detected in the blastocyst and trophoctoderm, and additionally it's expressed in trophoblastic tumor cells [54, 55].

There are several transcription factors found to be important for regulation of human trophoblast invasion. Among the transcription factors controlling the proliferative state of the human trophoblast is earlier mentioned in chapter 3.1.1, HIF-1 α . Ubiquitously expressed HIF-1 α , is seems to be main regulator of the cellular response to the low oxygen concentration. HIF-1 α was shown to be upregulated under low oxygen condition (until 9th week of pregnancy) and downregulated during trophoblasts differentiation into invasive phenotype together with Tgf- β , an inhibitor of trophoblasts differentiation [2]. HIF-1 α was shown to play important role in pathogenesis of PE and IUGR [20]. T-cell factor 3 and 4 (TCF-3/4) are remarkably increased during EVT_s differentiation. It was found that TCF-3 and TCF-4 protein were weakly expressed in villous fibroblasts and term placenta but highly in first trimester placenta, primary extravillous trophoblasts. Furthermore, TCF-3 was shown to be produced in proliferative and invasive trophoblasts, whereas TCF-4 was shown to be highly expressed in noncycling p57KIP2 trophoblasts within the cell column [56, 57]. Presented data suggest that TCF-4 might be important for invasive differentiation of trophoblasts [57]. Interestingly, inhibitor of bHLH protein inhibitor of DNA binding protein 2 (Id-2) was indicated to play important role in human trophoblasts differentiation and motility [58]. Furthermore, as indicated in IF staining, expression of Id-2 was transiently downregulated during trophoblasts differentiation in

second trimester placenta. The studies on Id-2 have shown that upregulation of Id-2 was observed during inhibition of CTBs differentiation, in vitro by hypoxia and in vivo in preeclamptic pregnancies. This suggest that overexpression of Id-2 may lead to altered invasion of trophoblasts.

The STAT3, a family member of signal transducers and activators of transcription was discovered as regulatory factor in invasive, metastatic tumor cells [59]. Moreover, STAT3 was detected in trophoblast cell line Jeg-3 and first trimester trophoblasts [31]. Later, it was demonstrated that STAT3 is highly phosphorylated in first trimester placenta whereas in term placenta remains unchanged [60]. In addition, proinvasive effect on primary EVTs was shown to be triggered by IL-11 via activation of STAT3 [61].

3.3.1.1 AP-2 α transcription factor

Activating enhancer-binding protein 2 (AP-2) family of transcription factors consists of five isoforms: AP-2 α , AP-2 β , AP-2 γ , AP-2 δ and AP-2 ϵ . They are encoded by separate genes and show high homology. The whole AP-2 family is involved in developmental biological processes such as differentiation, proliferation and cell motility. The isoforms possess highly conserved helix-loop-helix motif, carboxyl terminus and proline, glutamine rich domain with PY motif at the amino terminus, which is absent only in the AP-2 δ homologue (**Fig.5**) [62]. AP-2 α , a retinoic acid transcription factor seems to be the most often studied homologue in physiological processes and pathology.

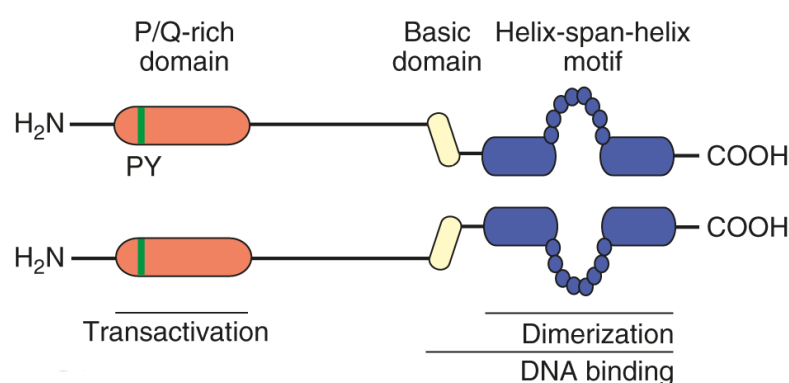


Figure 5. The structure of AP-2 α dimer

On the left hand side amino terminus with proline- and glutamine rich domain, next the basic domain and finally helix-loop-helix domain allowing binding of the protein to the DNA. Figure taken from [62]

Whereas AP-2 α , AP-2 β , AP-2 γ are expressed in various tissues in the body, AP-2 δ and AP-2 ϵ are mainly expressed in neuronal cells. AP-2 δ is specifically expressed in the mitral cell layer of the developing olfactory bulb what suggest its role in forebrain development [63]. Mice express only three homologues: AP-2 α , AP-2 β , AP-2 γ . As shown in the in vitro studies in mice AP-2 α knockout mice develop defects in the cranial neural crest, the limbs, the facial and skull, in the effect they die at about day 9 postcoitum [64]. AP-2 γ knockout mice die soon after implantation due to the failures in trophoctoderm development. Trophoblasts in AP-2 γ deficient mice are not able to proliferate, and this leads to problems of the placenta development [65]. In humans AP-2 family is not only restricted to control physiological processes but also they seem to play role in cancer. It was shown that AP-2 α , is strongly upregulated in some types of cancer e.g. breast cancer, ovarian cancer [66]. Nevertheless, there are types of the cancer where AP-2 α is downregulated e.g. colon cancer [67] and in the melanoma [68]. AP-2 α controls various target genes, some of AP-2 α target genes are trophoblasts-associated and play role during early placenta development. Among the AP-2 α target genes found to be the most important for invasion process are MMP-2 and its inhibitor (TIMP-2), urokinase plasminogen activator (uPa), plasminogen activator inhibitors (PAIs) [69]. AP-2 α was also shown to control pregnancy hormones like human placental lactogen (hPL) but also human chorionic gonadotropin (hCG). Previously, hCG was shown to be essential for pregnancy establishment and crucial for implantation, decidualization and development of the human placenta [70-72].

3.3.2 Growth factors and their inhibitors

There are large numbers of the various growth factors and their inhibitors demonstrated to play a role during placental development. Basically growth factors which regulate placental development can be divided according to their source of secretion. Paracrine factors of trophoblasts invasion origin from uterus. These factors are crucial for implantation of the blastocyst, and early placental development. However, during invasive trophoblasts invasion, decidual cells and glands produce growth factors and inhibitors to govern the invasion and remodeling of the spiral arteries. Autocrine factors are produced by trophoblasts itself [73]. During differentiation of trophoblasts and placental development insulin growth factors II (IGF II) and EGF as well as placental growth factor (PlGF) and vascular endothelial growth factor (VEGF) are secreted in

autocrine and paracrine manner to facilitate trophoblasts proliferation, differentiation and invasion [33, 73].

3.3.2.1 Endostatin

Endostatin, the proteolytic product of collagen XVIII was first found in hemangioendothelioma cells as a factor of an anti-angiogenic property [74, 75]. Angiogenesis is the process of new blood vessels formation, especially important during tumor pathogenesis. Endostatin was shown to suppress endothelial proliferation and motility. Moreover, endostatin was described to reduce tumor induced angiogenesis and therefore presenting very strong anti-tumor properties in many tumor mice models [74, 75]. Endostatin was found to inhibit invasion associated protease, as MMP-2 [74] and downregulate urokinase plasminogen activator (uPA) [76], therefore it could play a role in regulation of trophoblast motility. Endostatin together with angiostatin inhibits endothelial cell migration in response to fibroblast growth factors (FGF) and VEGF. As there is some analogy between the tumor progression and invasive trophoblasts differentiation, it was interesting to investigate whether endostatin might play a role during invasive differentiation of EVT and if endostatin interacts with signaling pathways controlling trophoblasts invasiveness [77].

3.3.2.2 EGF and IGF

The significance of EGF and IGF I/II was very broadly studied in the field of trophoblast biology. The members of the EGF family proteins (14 members); EGF and heparin-binding EGF (HBEGF) are produced as transmembrane proteins as pro-forms and by action of the metalloproteinases shed and released, allowing them binding to the ERBB receptors. They are secreted by decidual glands and other decidual cell types and were demonstrated as one of the most critical mediators of the implantation [78]. EGF polypeptide was first isolated from the salivary glands and it was shown that it stimulates epithelial growth and differentiation, by injecting EGF to newborn mice [79]. It was found that EGF is expressed in the endometrium but its expression increases in decidual cells. Additionally, EGF was detected to be expressed by all CTBs, probably by massive expression of EGF receptors on the surface of CTBs. In the trophoblasts cells EGF is known to stimulate secretion of MMP-2 and MMP-9, PAIs and uPA and therefore suggested to be important for regulating invasiveness [69, 80]. Another growth factors

Insulin-like growth factor I and II (IGF-I and II) was shown to be secreted by various placental cell types, particularly by the mesenchymal and endothelial cells [81]. Whereas IGF-II is produced mainly by vCTBs and EVT's of first trimester and term trimester placenta [81, 82], IGF-I is expressed in syncytiotrophoblasts and CTBs during the whole gestation. Moreover, the level of IGFs and EGF correlates with the fetal growth, as shown in mice and humans [83]. Studies with mice have shown that IGF-II is also important for mice placentation [84] since IGF-II regulates the development of the diffusional exchange. The mice deficient in IGF-II developed thicker placenta with decreased diffusion capacity, as a consequence the weight of mice during embryonic life was lower when compared to the healthy ones.

3.3.3 Metalloproteases

The process of trophoblasts invasion through the decidua and myometrium involves breakdown of ECM. Metalloproteases (MMPs), also called matrixins, are a big family of proteolytic, zinc-dependent enzymes. MMPs are produced by invading cells in order to allow them digestion of the extracellular matrix and penetration of the surrounding tissue. Metalloproteases are produced as inactive zymogens (pro-MMPs) and next activated by other active MMP or serine proteinases [85]. They are known to take part in the biological and pathological processes such as; wound healing, tumorigenesis, embryogenesis. Tissue inhibitors of metalloproteases (TIMPs), produced by decidual cells, are they natural specific inhibitors, controlling and inhibiting their action. Inhibition of MMPs by TIMPs occurs by binding to highly conserved zinc binding site of active MMPs [73]. The MMPs are divided into: collagenases, gelatinases, stromelysins, matrilysins and membrane type MMPs. The MMPs are upregulated in almost every type of the cancer. In the placenta research the most widely investigated are gelatinases MMP-2, MMP-9 and stromelysin MMP-3 [86]. Gelatinases degrade mostly denatured collagens (gelatins) and native collagen IV, whereas stromelysins digest; fibronectin, laminin, collagens III, IV, V and elastin [87]. It is known that MMP-2 is expressed in EVT's during the first trimester, whereas MMP-9 is expressed mainly in villous CTBs [87]. Therefore, MMP-2 might be suggested to be the key metalloprotease facilitating trophoblasts invasion [80]. Besides MMPs, uPA and PAIs play important role in regulation of early placentation. It was found, that trophoblasts cells treated with TGF β show reduced

secretion of uPA and increased PAI-1 secretion [88]. Moreover, it was shown that TNF α induced inhibition of trophoblasts invasiveness was caused by upregulation of PAI [89].

3.3.3.1 ADAM-12

ADAM-12 is one of 40 already identified ADAMs, but only 12 of them are active proteases [90]. ADAM-12 is produced in the rough endoplasmic reticulum and matures in the Golgi compartment [91]. ADAM-12 as a metalloprotease is synthesized in the form of an inactive enzyme and therefore requires furin like proprotein for prodomain removal and activation of enzyme. The mature, active form of ADAM-12 is delivered to the extracellular space of the cell. [92, 93]. ADAM-12 exists in two isoforms, produced due to differential splicing. ADAM-12 was discovered as the protein important during myoblasts fusion [94]. ADAM-12 deficient mice die short after birth for unknown reasons, exhibiting little muscle phenotype [95]. The expression of both ADAM-12 splice variants is restricted to developing and regenerating tissue as well as certain cancer types [96]. As I mentioned already, ADAM-12 has two splice variants, a transmembrane, longer form so-called ADAM-12L and soluble, shorter isoform so-called ADAM-12S (Fig.6) [93].

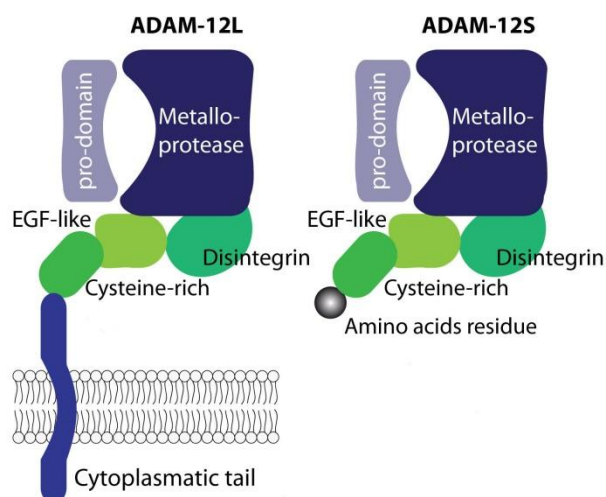


Figure 6. The structure of both ADAM-12 isoforms

The longer, membrane bound ADAM-12L, containing cytoplasmic tail, and shorter ADAM-12 S containing small residue in the C-terminus. Both isoforms contain prodomain, metalloprotease, disintegrin-like, cysteine-rich and EGF-like domain. The figure prepared by J.Pollheimer.

ADAM-12L possesses a cytoplasmatic tail allowing bounding to the cell membrane. The secreted isoform ADAM-12S instead of the cytoplasmatic tail possess small 33 amino acids residue. Both ADAM-12L and ADAM-12S isoforms are active proteolytic enzymes and are inhibited for instance by TIMP-3 [97]. Cleavage properties allow release of active proteins which can bind to the receptors and activate signaling pathways, controlling processes such as: cell growth and motility. ADAM-12L main function is ectodomain shedding, including activation of growth factors and cytokines. The proteins are proteotically cleaved and converted to soluble factors. ADAM-12L is known to mediate shedding of; pro heparin binding epidermal growth factor (proHBEGF) and Delta-like ligand 1, (DLL1), a critical ligand of Notch signaling [91]. ADAM-12S mediates cleavage of insulin-like growth factor binding protein -3 and -5 (IGFBP-3 and -5) which could suggest its control over insulin-like growth factor I and II (IGFI and II) [97]. Both IGFI and II are growth factors playing role in EVT's invasive differentiation [98] Both It is well known that ADAM-12L and ADAM-12S mediate cell adhesion, via their disintegrin domain and cysteine-rich domain. Moreover; both isoforms were shown to interact with integrins and syndecans and therefore regulate cell adhesion [99]. It was previously shown that ADAM-12 interacts with $\alpha 9\beta 1$, $\alpha 7\beta 1$, $\alpha 5\beta 1$ integrins [100, 101]. Moreover, ADAM-12 also plays a role during cancer progression. ADAM-12 is strongly upregulated in various cancer types and its high expression correlates with tumor metastasis and bad prognosis [102]. Furthermore, ADAM-12 plays a crucial role during pregnancy development. Both isoforms are shown to be expressed in the human first and term placenta [93, 103]. Our unpublished data (chapter RESULTS) show that ADAM-12 is strongly expressed by EVT's and its expression increases in vitro during the invasive differentiation pathway. It is important to mention that the healthy pregnancy serum contains high level of soluble ADAM-12S and its decrease have been reported in association with fetal trisomy 21 or 18 and in cases of PE and IUGR [104].

4. MODEL SYSTEMS

4.1 Trophoblast cell lines

Currently, there are many models available to study differentiation and invasion of human trophoblasts. The most common in the placenta research field are trophoblastic cell lines. Generally the cell lines can be divide into the ones generated from normal, healthy, tissue and cell lines generated from chorion carcinomas with some characteristics of trophoblast differentiation [105]. In our laboratory the most commonly use cell lines are; SGHPL-5 and HTR-8/SVneo.

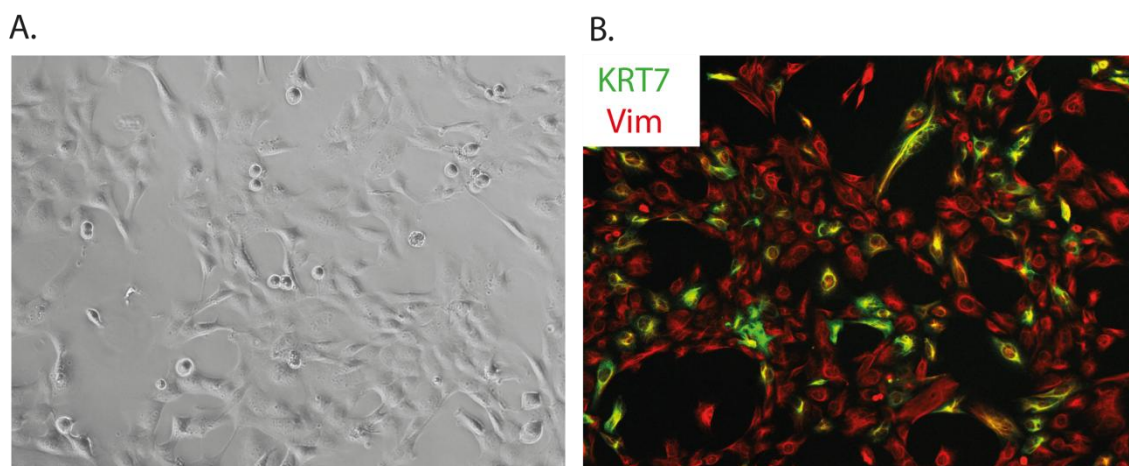


Figure 7. SGHPL-5 trophoblast cells

A, Light microscope picture of SGHPL-5 cells (200x magnification). B, Immunofluorescence of SGHPL-5 cells, KRT7, cytokeratin-7 in green, vim, vimentin staining in red (100x magnification)

SGHPL-5 is a cell line produced from the minced first trimester human placentae, followed by trypsinization and gradient separation and afterwards transfection with SV40 large T antigen (**Fig. 7**) [106, 107]. The HTR-8 cell line were generated by plating the placental villus and afterwards collecting the outgrowing cells, later on collected cells were transfected with SV40 large T antigen. Whereas transfected HTR-8 so-called HTR-8/SVneo exhibit unlimited lifespan in vitro and are stable cell line, SGHPL-5 cells exhibit only an extended lifespan. Both cell lines express vimentin, and are partially positive for

cytokeratin-7, additionally HTR-8/SVneo express chorionic gonadotropin (CG) and become cytokeratin-7 positive when cultivated on matrigel [108]. Both cell lines similarly to primary EVT_s have invasive phenotype. Recently, there were many speculations considering similarity of the trophoblast cell lines with primary EVT_s and advantage of using cell lines as in vitro model for cell differentiation and invasion. As shown by Gene Chip data analysis comparing different trophoblast cell lines with primary EVT_s and CTBs, there are considerable differences between primary cells and cell lines in gene expression. After performed analysis of gene expression, EVT_s were shown to be more related to CTBs as to SGHPL-5 or HTR-8/SVneo [109].

4.2 Primary cells

Considering that the cell lines are not the best model to use for performing the functional studies, the researchers developed methods to establish isolation and cultivation of primary cells isolated from first and term placentae [110, 111]. The placental tissue of early pregnancy (7th to 12th week of gestation) are obtained after legal abortions of healthy pregnancies, after approval of ethical committee of Medical University of Vienna. In our laboratory two methods are used for isolation of primary cells. Firstly trophoblasts are isolated according to so-called Kliman protocol [111] where heterogeneous primary cell preparation is obtained from human first and term trimester placentae. The obtained primary cells are a mixture of different trophoblasts subtypes (EVT_s, CC CTBs, vCTBs) with mostly villous CTBs. The isolation steps include a Percoll gradient centrifugation step and standard trypsin-DNase dispersion. The most used in our laboratory protocol for isolation of the mixed culture of CTBs, CC CTBs and EVT_s is so-called Tarrade protocol, a method of isolation modified from original Tarrade A. et al, protocol [112]

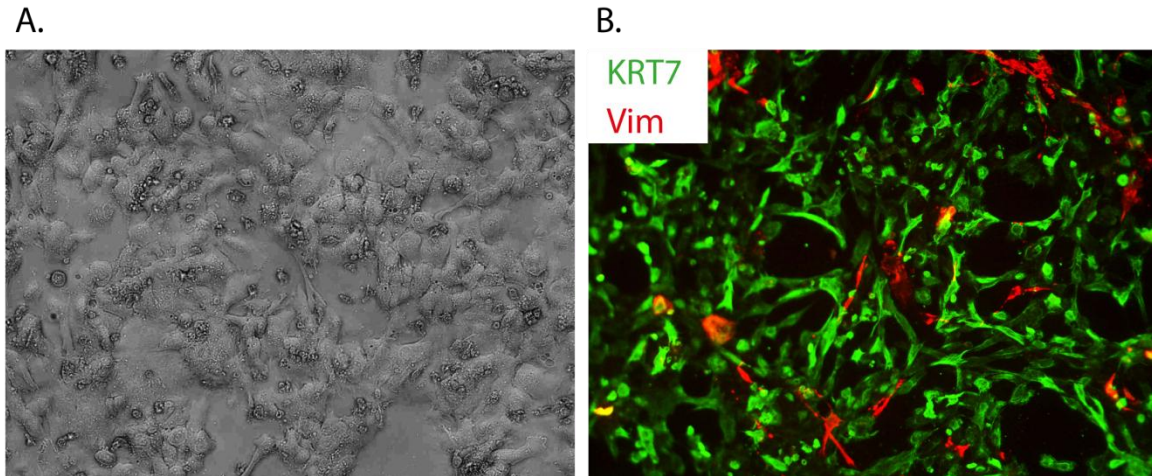


Figure 8. Primary EVTs

A, Light microscope picture of the primary EVTs. (100x magnification), 24h after isolation, cultivated of fibronectin. B, Immunofluorescence staining of the primary EVTs (100x magnification) cells, cytokeratin-7, KRT7 in green, vim, vimentin staining in red. The cells were isolated according to modified Tarrade et al [112]

The isolation method consists of digestion step with trypsin and DNase, Percoll density gradient centrifugation, lysis of erythrocytes with and removal of fibroblasts by sedimentation and adherence of placental fibroblasts to plastic tissue culture. The obtained primary cell culture is routinely checked for trophoblastic markers expression such as; cytokeratin-7 and vimentin to visualize the percentage of non-trophoblastic cells within the population (**Fig.8**). After isolation primary cells require ECM coated dishes and medium with 10% serum to live in the culture. Primary trophoblasts can be cultivated on matrigel a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm mouse sarcoma cells (BD Biosciences). However, in our lab we prefer to use for primary EVTs cultivation fibronectin (0,02 mg/ml). Fibronectin is a glycoprotein and a component of natural endometrial extracellular matrix [1] and it seems to be more neutral than matrigel. Matrigel in contrast to fibronectin contains additional growth factors which may influence the results of some studies. Moreover, according to our own observations EVTs cells are more viable (**Fig.9**) when cultivated of fibronectin, less apoptotic cells were visible when compared to cells cultivated on matrigel. Isolated cells after seeding on fibronectin tend to adhere and undergo invasive differentiation pathway.

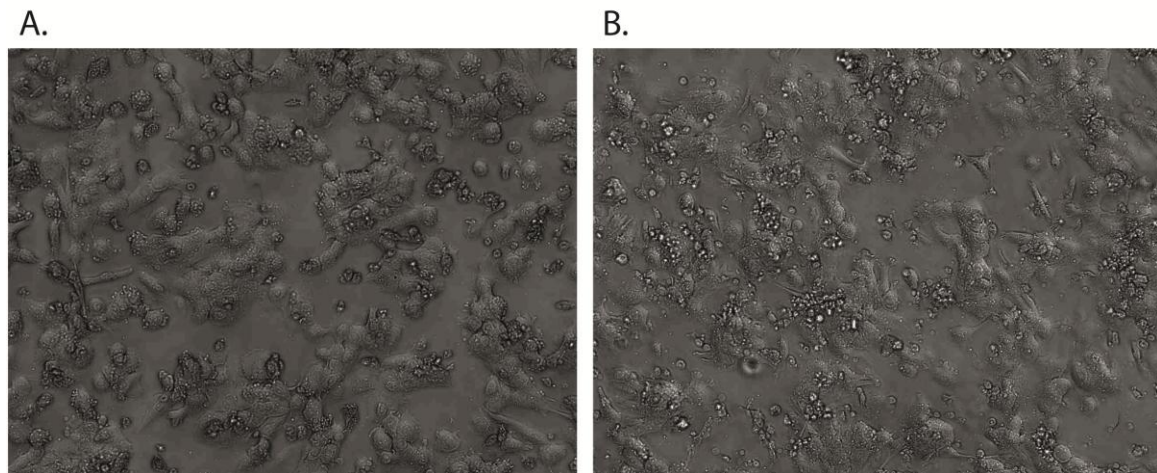


Figure 9. Primary EVTs cultivated on different ECM

A, Light microscope picture of the primary EVTs. (100x magnification), 24h after isolation, cultivated on fibronectin B, Light microscope picture of the primary EVTs. (100x magnification), 24h after isolation, cultivated on Matrigel. The cells were isolated according to modified Tarrade et al [112]

Integrin $\alpha 6$ expression is diminishing, 11% cells after 72h cultivation on fibronectin are $\alpha 6$ positive. However, expression of $\alpha 5\beta 1$ and $\alpha 1$ drastically increases, after 72h cultivation on fibronectin, 92% are $\alpha 1$ positive and 88% are $\alpha 5\beta 1$ positive [77].

4.3 Primary explant culture

Another adequate model for studying invasion, migration, differentiation of human CTBs in vitro is the villous explant culture system [110, 113]. The big advantage of this in vitro model is its well-kept, intact structure of the placental villi. Placental explant model allows investigation of trophoblast outgrowth, EVTs motility and mimics events which occur in anchoring villi during the first trimester placenta development [114]. Placental villi are dissected from first trimester placenta under the light microscope and seeded either on Matrigel or rat-tail collagen I [110]. Once the placental villus is seeded on ECM it forms anchoring site and form the cell column with actively proliferating CTBs. The EVTs outgrowing from the placental explant seeded on Matrigel invade underneath the anchoring site. In contrast, placental explants cultivated on collagen migrate on the surface of gel and merged to form a shell [37] (**Fig.10**).

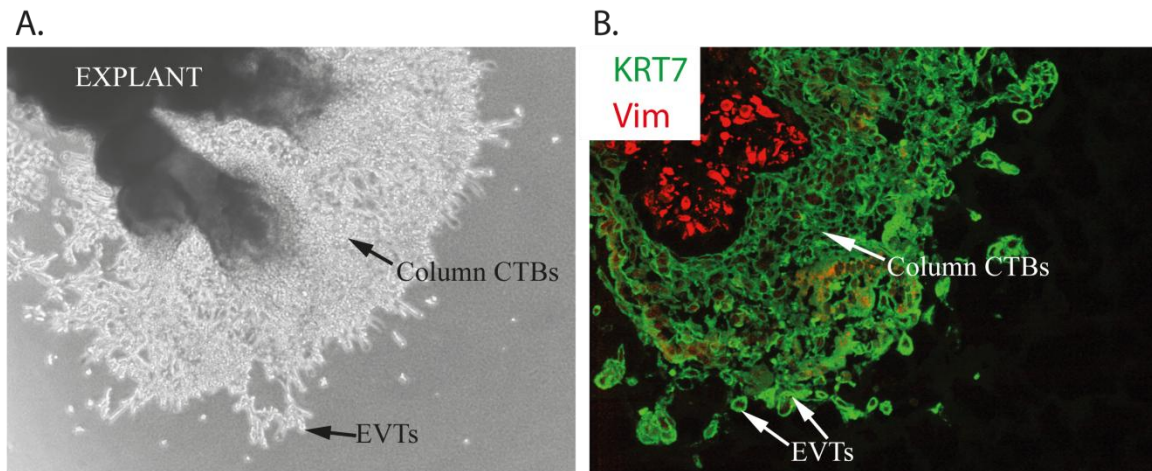


Figure 10. First trimester placental villous explant

A, Light microscope picture of outgrowing placental explant, after 24h cultivation on rat-tail collagen I (200x magnification). B, IF staining of outgrowing placental explant after 72h cultivation on matrigel (200x magnification). EVT, extravillous trophoblast, KRT7, cytokeratin-7, vim, vimentin.

The villous explant culture is the most adequate one to mimic EVT differentiation and invasion in vivo. This model allows wide range of techniques to be used for conducting functional studies; like inhibition and stimulation of proliferation, invasion and transfection of outgrowing cells.

5. AIM OF THE WORK

During my PhD studies, I was focused on studying the role of different important factors controlling invasion of the human trophoblasts. The aim of my work was to study the role of transcription factor AP-2 α and the proteolytic enzyme ADAM-12 during early placental development. It is known that all three factors are multifunctional and are not only important for physiological processes like cell differentiation, migration, proliferation but also play role during pathological processes including tumorigenesis.

The role of AP-2 α , ADAM-12 in controlling the motility of first trimester human trophoblasts remained until now unknown. Interestingly, the role of AP-2 α was already shown in villous trophoblast differentiation of term placentae [115], but the expression and functional role of the AP-2 α in early placenta development needed to be investigated. One of the main reason to study role of AP-2 α during trophoblasts invasion was that AP-2 α was shown to control target genes important for trophoblasts invasion like MMP-2, hCG, uPA, PAIs [69]. In our lab was found, that AP-2 α enhances transcription of important pregnancy hormone CG β 5, in differentiating term trophoblasts [116]. Another important argument, why AP-2 α might be important for trophoblast invasion is the analogy between EVTs invasion and EMT process. AP-2 α was shown to control some tumor related genes such as; MUC 18 (melanoma associated protein), KAI1 (prostate cancer anti-metastatic gene1) and MMP-2 [117]. Moreover, it was indicated that AP-2 α is upregulated in many cancer types [118, 119].

ADAM-12 was shown to be a crucial player in proteolytic cleavage and ectodomain shedding and therefore activation of many growth factors important for regulation of trophoblasts motility, such as HBEGF, IGFBP-3 or TNF α [120]. Altered serum level of ADAM-12 was also described in pregnancy disorders like ectopic pregnancy [121], PE [122], IUGR and some genetic disorders of the embryo, where ADAM-12 expression was compared to serum of healthy pregnancies. Moreover, it was shown that ADAM-12 is strongly upregulated in many cancer types [91, 123] and correlates always with bad prognosis. Taken together, all above arguments ADAM-12 might be considered as an important candidate for regulator of trophoblasts invasion.

First I performed descriptive studies and later on set-up functional studies to study a role of AP-2 α and ADAM-12 in trophoblasts biology. At the beginning, I needed to investigate expression and localization of these proteins within the placenta tissue and in the trophoblast cell lines. My next goal was to perform loss of function studies of AP-2 α and ADAM-12 and subsequently perform functional studies with the knock-down cells. Subsequently, the functional studies of AP-2 α and ADAM-12 knock-down were performed to investigate the role of these two factors in regulating motility of trophoblasts. Knowing that EGF is triggering invasion process [124], I used this stimulant in the functional studies with AP-2 α . Both AP-2 α and ADAM-12 were needed to be transiently overexpressed to observe the effect of upregulation of these proteins in trophoblast cell line SGHPL-5. Next, I performed functional studies including invasion assay. One of the goals of ADAM-12 investigations was generation of the knock-down in the outgrowing placental explants cultivated on matrigel and collagen. I was also interested to perform cleavage assay confirming ADAM-12S cleavage properties of IGFBP-3.

6. RESULTS

6.1 The role of AP-2 α in human trophoblasts invasion

6.1.1 Transcription factor AP-2 α promotes EGF-dependent invasion of human trophoblasts. Biadasiewicz K, Sonderegger S, Haslinger P, Haider S, Saleh L, Fiala C, Pollheimer J, Knöfler M. Endocrinology. 2011 Apr; 152(4):1458-69. Epub 2011 Feb 8.

Transcription Factor AP-2 α Promotes EGF-Dependent Invasion of Human Trophoblast

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The basic helix-span-helix transcription factor activating protein (AP)-2 α is critically involved in cell-specific hormone expression of syncytializing human trophoblasts. Its role in invasive trophoblast differentiation, however, remains largely elusive. Using RT-PCR, Western blotting, and immunofluorescence of first-trimester placentae, we here show that AP-2 α is expressed in extravillous trophoblasts (EVTs) both *in situ* and *in vitro* as well as in invasive trophoblast cell lines. Its protein expression was increased upon supplementation of epidermal growth factor (EGF) both in primary EVTs and trophoblastic SGHPL-5 cells. Gene silencing of AP-2 α using small hairpin microRNA (shRNAmir) did not affect basal invasion of SGHPL-5 cells through Matrigel-coated filters but reduced EGF-stimulated invasion. Similarly, treatment of primary EVTs with AP-2 α small interfering RNA decreased EGF-dependent invasion. Proliferation of SGHPL-5 cells and primary EVTs, measured by cumulative cell numbers and 5-bromo-2'-deoxyuridine labeling, respectively, were not affected on loss of AP-2 α . EGF-dependent induction of matrix metalloproteinase (MMP)-2, pro- and active form of urokinase plasminogen activator, and chorionic gonadotropin (CG)- β was noticed in shRNAmir-control cells, whereas these genes were suppressed in EGF-treated shRNAmir-AP-2 α cells. Similarly, EGF-stimulated MMP-2 and CG β protein expression was reduced in AP-2 α small interfering RNA-treated primary EVTs. Knockdown of AP-2 α also decreased luciferase activity of the CG β 5 promoter in SGHPL-5 cells, which was compensated upon transient overexpression of AP-2 α cDNA. In conclusion, we show that AP-2 α expression positively affects human trophoblast invasion under EGF-stimulated conditions, likely by inducing critical invasion-promoting genes such MMP-2, urokinase plasminogen activator, and CG. (*Endocrinology* 152: 1458–1469, 2011)

Invasion of human placental trophoblasts into maternal uterine tissues is critical for normal placental development and successful pregnancy. Invasive extravillous trophoblasts (EVTs) develop from anchoring villi and migrate into the uterine stroma as well as into maternal spiral arteries where they adopt a vascular phenotype (1). Remodeling of the maternal arteries by trophoblasts is associated with vessel dilation ensuring adequate delivery of nutrients and oxygen to the placenta and the developing fetus. Failures in vascular remodeling, noticed by the lack of endovascular trophoblasts, is thought to result in limited placental blood supply eventually provoking gestational diseases such as preeclampsia or severe forms of intrauterine growth restriction (2, 3).

Hence, a better understanding of the endogenous differentiation program of EVTs as well as their complex interactions with various maternal uterine cell types might improve our understanding of the pathogenesis of gestation diseases.

Trophoblast invasion involves integrin-mediated adhesion (4) and the activation of different protease systems (5, 6) such as matrix metalloproteinase (MMP) and urokinase plasminogen activator (uPA) and their respective inhibitors, tissue inhibitor of metalloproteinases (TIMPs) and plasminogen activator inhibitors

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Abbreviations: AP-2, Activating protein-2; BrdU, 5-bromo-2'-deoxyuridine; CG, chorionic gonadotropin; 4 DAPI, 4',6'-diamino-2-phenylindole; EGF, epidermal growth factor; EVT, extravillous trophoblast; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; miRNA, microRNA; MMP, matrix metalloproteinase; PAI, plasminogen activator inhibitor; shRNAmir, small hairpin microRNA; siRNA, small interfering RNA; TBST, Tris-buffered saline and Tween 20; TIMP, tissue inhibitor of metalloproteinase; uPA, urokinase plasminogen activator; Wnt, wingless.

(PAIs). The secretion and activation of the proteases is provoked by numerous growth and angiogenic factors produced at the fetal-maternal interface (7). Many of these soluble ligands signal through ERKs and phosphatidylinositol 3-kinase/Akt, suggesting that activation of these signaling pathways are critical for human placental and trophoblast differentiation (8).

Although novel regulatory factors and signaling pathways are being unraveled, transcription factors controlling invasive trophoblast differentiation are less well understood (9). Previous studies indicated that, for example, signal transducer and activator of transcription-3 (10), wingless (Wnt)-dependent T cell factor (11), peroxisome proliferator-activated receptor- γ (12), and glial cells missing 1 (13), are expressed in EVT's and control the differentiation/invasion process.

Transcription factors of the activating protein-2 (AP-2) protein family play diverse roles during development (14) and have also been implicated in placental function and differentiation. Murine AP-2 γ was shown to control placental adenosine deaminase expression (15), and AP-2 γ -deficient mice display failures in trophoblast and placenta formation (16). Along those lines, a recent study indicated that AP-2 γ can induce trophoblast formation in embryonic stem cells and cooperates with Cdx2 caudal type homeobox 2 to maintain trophoblast cell fate (17). In humans, AP-2 factors are dynamically regulated during trophoblast cell fusion. Whereas AP-2 γ decreases, AP-2 α protein and binding activity increase during syncytialization of term trophoblasts (18, 19). In the differentiated cells, AP-2 α was shown to be important for transcription of pregnancy-specific hormones. Not only placental lactogen and CRH are controlled by AP-2 α (20, 21), but the transcription factor is also required for the coordinate, cell-specific expression of chorionic gonadotropin (CG)- α and - β subunits in both choriocarcinoma cells and primary trophoblasts (18, 22, 23). Overexpression of dominant-negative AP-2 α in term trophoblasts provoked changes in mRNA levels of AP-2 α target genes such as different hormones but did not affect cell fusion or syncytin expression (24). Hence, the main role of AP-2 α in villous trophoblasts could be maintenance of the differentiated phenotype rather than regulation of cell fusion *per se*.

Given that fact that AP-2 α controls a considerable number of genes important for trophoblast function (24), we here sought to investigate the role of AP-2 α in trophoblast invasion. Using gene silencing of AP-2 α in trophoblastic SGHPL-5 cells and primary EVT's, we demonstrate that the transcription factor positively affects epidermal growth factor (EGF)-dependent trophoblast invasion. This is likely achieved by EGF-induced AP-2 α expression

provoking activation of the invasion-promoting genes such as MMP-2, uPA, and CG β .

Materials and Methods

Collection of placental tissues

Placental tissues of early pregnancy ($n = 65$) between the seventh and 12th weeks of gestation were obtained from legal abortions of uncomplicated pregnancies. The use of tissues was approved by the Ethical Committee of the Medical University of Vienna.

Cultivation of SGHPL-5 cells

SGHPL-5 cells exhibiting features of invasive trophoblasts cells such as HLA-G and cytokeratin 7 expression were cultivated in DMEM/Ham's F-12 (PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% fetal calf serum (FCS; Biochrom AG, Berlin, Germany) and 1:200 gentamicin (Invitrogen Life Technologies, Inc., Life Technologies, Paisley, UK) as described (25). HTR-8/SVneo were grown in RPMI 1640 (Gibco BRL, Life Technologies, Paisley, UK) supplemented with 5% fetal bovine serum (Biochrom), 2% penicillin/streptomycin (Gibco BRL), and 2 mM glutamine (Gibco BRL) as described (26).

Cell culture of primary extravillous cytotrophoblasts

Primary EVT's of pooled first-trimester placentae ($n = 60$) between 7 and 12 wk gestation were isolated by adapted enzymatic dispersion and Percoll density gradient centrifugation (10–70%; Pharmacia, Uppsala, Sweden) as described (27). Briefly, placentae were washed with ice-cold PBS and kept overnight in DMEM/Ham's F-12 supplemented with 0.05 mg/ml gentamicin (Life Technologies) and 2.5 μ g/ml Fungizone (Life Technologies, Invitrogen). The villous tips were scraped with a scalpel blade and digested for 30 min in 0.125% Trypsin (Life Technologies) and 12.5 mg/ml deoxyribonuclease I (Sigma, Castle Hill, Australia) in 10% Hanks' balanced salt solution-Mg/Ca free (Sigma, Vienna, Austria). Next, the cells were filtered through a 70- μ m cell strainer (Greiner Bio-One GmbH, Frickenhausen, Germany). Cell suspension was poured onto a Percoll gradient and centrifuged. Collected cells from 35–50% Percoll layer were seeded on 0.025 mg/ml diluted Matrigel (Becton Dickinson, Schwechat, Austria) in cold DMEM/Ham's F-12. After 48 h, isolated trophoblasts were routinely stained against cytokeratin 7 (clone OV-TL 12/30, 8.3 μ g/ml; Dako, Vienna, Austria), vimentin (clone Vim 3B, 1.2 μ g/ml; Dako), α 5 β 1-integrin (clone JBS51; Chemicon, Temecula, CA; 1:50), α 1-integrin (clone FB12; Millipore, Vienna, Austria; 2 μ g/ml) as described recently (28). The primary cells were positive for cytokeratin 7 (>96%) and largely negative for vimentin (<4%). The cytokeratin 7-positive cells expressed α 5 β 1-integrin (100%) and α 1-integrin (100%), suggesting that the trophoblast population consists mainly of EVT's. The isolated cells were seeded in 24-well plates at a density of 2×10^5 and cultivated in 500 μ l DMEM/Ham's F-12 supplemented with 10% FCS and 0.05 mg/ml gentamicin.

Construction of AP-2 α knockdown in SGHPL-5 cells

Stable knockdown of AP-2 α in SGHPL-5 cells was generated by transduction with the microRNA (miRNA)-adapted retroviral vector LMP (Open Biosystems, Huntsville, AL) as mentioned recently (26). Briefly, miRNA-adapted short hairpin RNA against human AP-2 α obtained in pSM2 vector (Open Biosystems) was subcloned into the LMP vector with *Xho*I and *Eco*RI (Invitrogen Corp., Carlsbad, CA) restriction enzymes. To produce murine stem cell virus particles, human embryonic kidney 293FT cells (Invitrogen) were transiently cotransfected with a vector containing necessary viral proteins (gag, pol), a vector containing VSV-g, and plasmids harboring either nontargeting small hairpin microRNA (shRNA_{mir}) (control) or shRNA_{mir} AP-2 α (miRNA against human AP-2 α). Lipofectamine 2000 (Invitrogen) reagent was used for transfection. Forty-eight hours after transfection, viral supernatants were collected, supplemented with 8 μ g/ml Polybrene (Sigma), and used to infect SGHPL-5 cells. Stable integrants were selected with puromycin (5 ng/ml) over a period of 2 wk. For growth factor stimulation and subsequent Western blot analyses, 5×10^5 shRNA_{mir} cells were seeded in 12-well plates. After attachment cells were washed with PBS and incubated with serum/antibiotic-free culture medium in the absence or presence of 5 ng/ml EGF (Pepro-Tech, Inc., Rocky Hill, NJ). After 72 h supernatants were collected, spun down for 5 min to remove cell debris, and concentrated using Ultrafree-MC filter tubes (Millipore, Billerica, MA) according to the manufacturer's instructions. Cells of each well were trypsinized for 5 min (0.25% Trypsin-EDTA; Life Technologies, Inc., Invitrogen) and counted using a multi-channel electronic cell counter (CASY-I; Schärfe Systems, Reutlingen, Germany), and protein lysates were prepared. For Western blot analyses, supernatants were normalized to cell numbers.

Small interfering RNA (siRNA) transfection of primary EVT

Primary extravillous trophoblasts (1×10^5) were plated in 48-well plates (Iwaki; Lennox Laboratory Supplies, Dublin, Ireland) coated for 30 min (37 C) with 0.025 mg/ml Matrigel (BD Biosciences, Franklin Lakes, NJ) diluted in DMEM/Ham's F-12. After 24 h cells were washed three times with PBS to remove cell debris. In separate tubes 2.5 μ l of either siRNA nontargeting control or siRNA AP-2 α (each 100 μ M, Accell; Dharmacon Inc., Lafayette, CO) were mixed with 200 μ l delivery medium. siRNA AP-2 α SMART pool (Dharmacon) contains a set of the following target sequences: CCCUGUUGAGCAUGUUA, GCAUUGAUUGAGUUGUGC, CUGGUAUUCUGUAUUUUA, and UUUUGAAACUUGUGGUU. After addition of siRNA oligos, EVTs were incubated for 72 h at 37 C. Subsequently transfection medium was removed, cells were washed with PBS, and 150 μ l of serum/antibiotics-free DMEM/Ham's F12 medium without or without 5 ng/ml EGF was added. After an additional 24 h, the supernatants and cells were collected for Western blot analyses.

RNA extraction and semiquantitative RT-PCR

Total RNA extraction and semiquantitative RT-PCR from placental tissues, primary trophoblast cells, and trophoblastic cell lines HTR-8/SVneo and SGHPL-5 were prepared as previously described (11). Briefly, RNA was isolated with Tri-Reagent peqGOLD TriFast (PEQLAB, Erlangen, Germany). Two micrograms of total RNA were reverse transcribed in a total of 20 μ l using 1 μ l reverse transcriptase Moloney murine leukemia virus (200 U/ μ l;

Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. PCR conditions were 45 sec at 96 C, 1 min at annealing temperature, and 1 min at 72 C. Annealing temperature, cycle numbers, and Mg²⁺ concentrations of individual PCR reactions were as follows: AP-2 α , 55 C, 30 cycles, 1.5 mM MgCl₂; AP-2 β , 52 C, 40 cycles, 1 mM MgCl₂; AP-2 γ , 52 C, 40 cycles, 1.5 mM MgCl₂; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 55 C, 23 cycles, 1.5 mM MgCl₂. The following sequences of forward and reverse primers were: AP-2 α -sense, 5'-TACTCCCACGT-CAACGAC-3', AP-2 α -antisense, 5'-GAACAGAAGACTTC GT-TGG-3'; AP-2 β -sense, 5'-GGCTCGGTG TCCCAAGGA-3', AP-2 β -antisense, 5'-GAAGTCACCGATTGGA-3'; AP-2 γ -sense, 5'-GGAGCAGCAATGGGAATC-3', AP-2 γ -antisense, 5'-AG-GAGAGTCACAT GAGCG-3'; and GAPDH-sense, 5'-GAAGGCT GGGGCTCAATTTGC-3', GAPDH-antisense, 5'-CTGACACGTTGGCAGTG GGG-3'. PCR products were analyzed on 1.5% agarose gels containing ethidium bromide and digitally photographed under UV.

Preparation of protein lysates and Western blot analyses

Protein extraction and Western blot analyses were performed using standard protocols as recently described (28). Briefly, equal amounts of protein lysate (20 μ g) were separated on 10% sodium dodecyl sulfate/PAA gels and transferred onto polyvinylidene difluoride membranes (Hybond-P; Amersham Pharmacia Biotech, Piscataway, NJ). After blocking in 5% nonfat milk in Tris-buffered saline and Tween 20 (TBST), cells were incubated overnight at 4 C in 5% BSA in TBST with the following primary antibodies: AP-2 α (rabbit, C83E10, 1:1000; Cell Signaling, Beverly, MA; 48 kDa), AP-2 β (rabbit, 1:500, Cell Signaling; 49 kDa), AP-2 γ (mouse, 6E4/4, 0.4 μ g/ml, Santa Cruz Biotechnology Inc., Santa Cruz, CA; 48 kDa) MMP-2 (rabbit, 1:1000; Cell Signaling; 72 kDa, proform), TIMP-1 (rabbit, 0.4 μ g/ml H-150; Santa Cruz Biotechnology; 22 kDa), TIMP-2 (rabbit, 0.4 μ g/ml, H-140; Santa Cruz Biotechnology; 21 kDa), TIMP-3 (mouse, 2 μ g/ml; Millipore; 26 kDa), uPA (mouse, 1 μ g/ml; R&D Systems Inc., Minneapolis, MN; 55 kDa, precursor, 33 kDa, active enzyme), PAI-1 (1 μ g/ml; Calbiochem, San Diego, CA; 50 kDa), PAI-2 (mouse, 2 μ g/ml; American Diagnostica, Stamford, CT; 46 kDa), CG β (rabbit, 1:500; Dako; 40 kDa). After incubation in blocking solution (5% nonfat milk/TBST), membranes were incubated with secondary antibodies (antimouse or antirabbit Ig horseradish peroxidase linked, 132 ng/ml; Sigma). Signals were developed for 5 min by using an ECL Plus Western blotting detection system (Amersham, Piscataway, NJ). As a molecular size marker PageRuler (Fermentas) was used. Quantification of signals on films was done by densitometrical scanning using α EaserFC software (Alpha Innotech, San Leandro, CA).

Immunofluorescence of first-trimester placenta

First-trimester placental tissues were embedded in paraffin and sectioned on a microtome as described (23). Briefly, after fixation in 4% paraformaldehyde for 24 h at 4 C, samples were dehydrated and embedded in paraffin (Merck, Darmstadt, Germany). Serial sections were prepared (5 μ m), deparaffinized, and heated in the microwave (2 $\times$ 5 min, 850 W). Blocking procedures, antibody stainings, and sample analyses using immunofluorescence were performed as mentioned (11). The following antibodies were used: AP-2 α (rabbit, clone H-79, 2 μ g/ml; Santa Cruz Biotechnology), AP-2 β (rabbit, clone H-87, 2 μ g/ml; Santa Cruz Biotechnology),

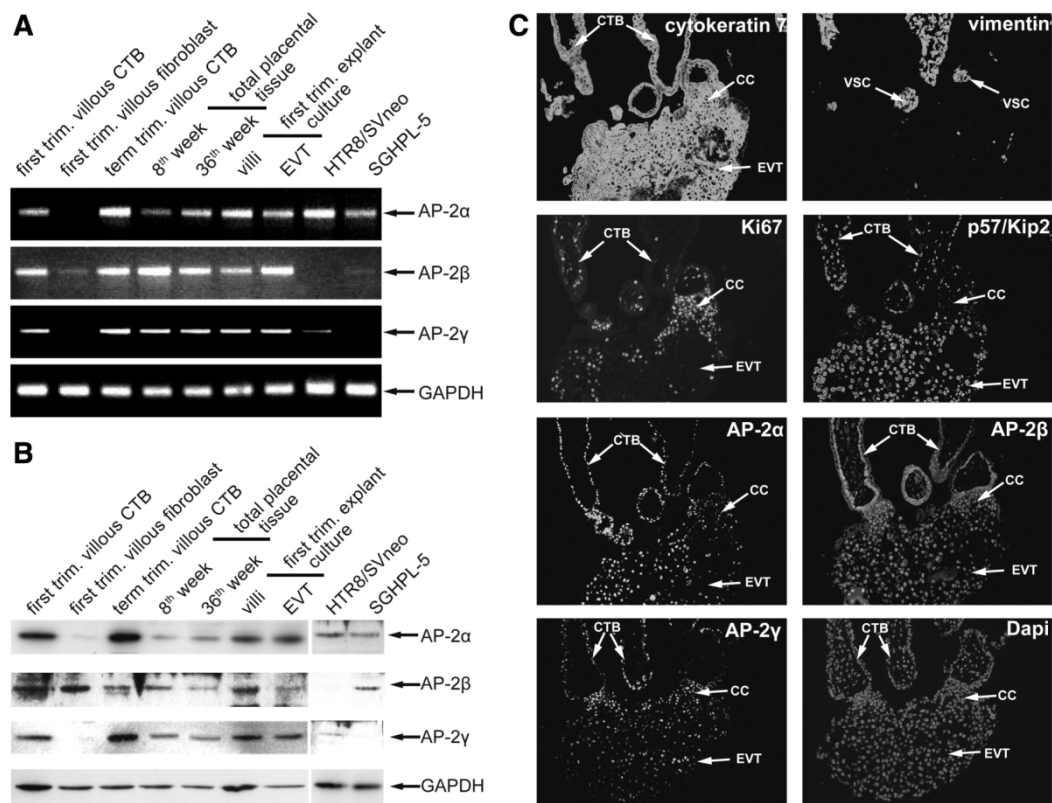


FIG. 1. Expression analyses of AP-2 family members in placental tissues and trophoblast model systems. RNA and protein lysates were prepared from placental tissues, first-trimester villous explant cultures, primary trophoblasts and fibroblasts, and trophoblastic cell lines. RT-PCR, Western blot, and immunofluorescence were performed as described in *Materials and Methods*. Representative examples are shown. Specific signals are indicated by arrows. A, Semiquantitative RT-PCR analyses detecting cDNA fragments of AP-2α (354 bp), AP-2β (500 bp), and AP-2γ (843 bp) and GAPDH (185 bp) as a housekeeping reference gene. B, Western blot analyses. For immunodetection of proteins, specific antibodies recognizing AP-2α (48 kDa), AP-2β (49 kDa), AP-2γ (48 kDa), and GAPDH (37 kDa, loading control) were used. Specific signals are marked by arrows. C, Immunofluorescence of serial sections prepared from 10th week placental tissue. Representative pictures taken from one of three different first-trimester placentae analyzed are shown. CC, Cell column; CTB, cytotrophoblast; VSC, villous stromal cell.

AP-2γ (mouse, clone 6E4/4, 2 μg/ml; Santa Cruz Biotechnology), cytokeratin 7 (mouse, 8.3 μg/ml clone OV-TL 12/30; Dako), vimentin (clone Vim 3B, 1.2 μg/ml; Dako), Ki67 (mouse, clone Ki-S5, 5 μg/ml; Chemicon), p57/Kip2 (rabbit, 2 μg/ml, c-20; Santa Cruz Biotechnology). Next, samples were incubated with secondary antibodies Alexa Fluor 488 (antimouse) and Alexa Fluor 568 (anti-rabbit), both at a concentration of 4 μg/ml (Molecular Probes, Eugene, OR), stained with 4',6'-diamino-2-phenylindole (DAPI; 1 μg/ml; Roche, Mannheim, Germany), and embedded using fluoromount G (Soubio, Birmingham, AL). Stained sections were analyzed by fluorescence microscopy (Olympus BX50; Olympus, Hamburg, Germany) and digitally photographed at a 200-fold magnification.

Invasion assays

Invasion of shRNA^{AP-2α} knockdown cells and controls through Matrigel-coated transwells (BD Biosciences) was performed as recently described (29). Briefly, 750 μl DMEM/Ham's F-12 medium supplemented with 10% FCS and 0.05 mg/ml gentamicin were added to the lower chamber. The cells (50,000) were seeded in the upper chamber using the same medium (500 μl). To determine the effect of EGF on AP-2α knockdown cells, 5 ng/ml EGF was added to both chambers. After 24 h cells were

washed three times with PBS, fixed for 10 min in ice-cold methanol, again washed three times with PBS, and subsequently incubated with DAPI (1 μg/ml; Roche) for 10 min. After 24 h noninvaded cells on the upper side of the inserts were removed by a cotton swap. Membranes were cut out with a scalpel blade, placed on glass slides, and embedded with Fluoromount G (Soubio, Birmingham, AL). Nuclei of cells on the underside of membranes were counted under fluorescence using Olympus cell-imaging software. Experiments were done in triplicates, and of every membrane five digital pictures were taken. To determine invasive properties of AP-2α siRNA-treated primary EVTs, invasion assays through fibronectin-coated transwells were performed. Millicell invasion inserts (12 μm pore size; Millipore) were coated on top with 25 μl fibronectin (BD Biosciences; 0.5 μg/μl) in PBS and left in the incubator for 30 min (37°C). Coated invasion inserts were placed in 24-well plates, each containing 300 μl of Accell delivery medium (Dharmacon) supplemented with 10% serum. The 60,000 isolated primary EVTs were mixed with 300 μl Accell delivery medium containing 1.25 μM of Accell siRNA oligos and seeded into the invasion chamber. In case of EGF treatment, 5 ng/ml of the growth factor was added to both chambers. After 48 h cells were fixed with 4% paraformaldehyde (10 min), washed three times in PBS, and incubated for 5 min

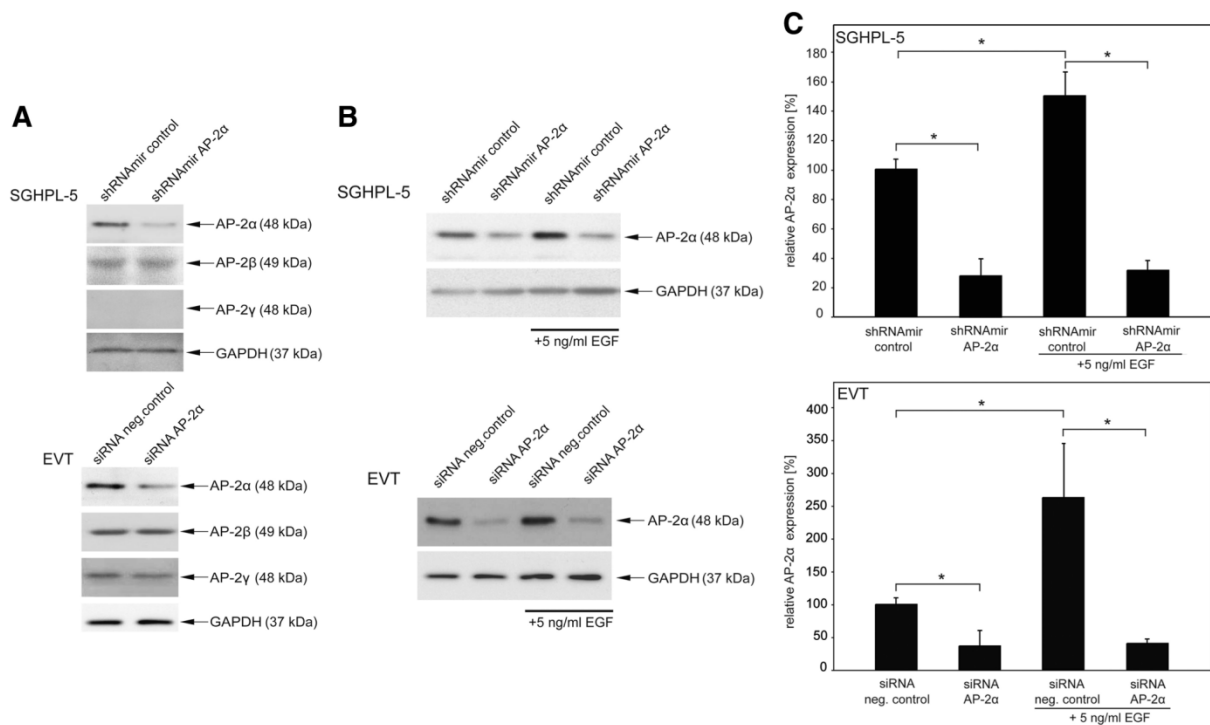


FIG. 2. Gene silencing of AP-2 α in SGHPL-5 cells and primary EVTs in the absence or presence of EGF. Different cell pools constitutively expressing AP-2 α shRNA and AP-2 α siRNA-treated primary EVTs were prepared as described above and analyzed by Western blotting (representative examples shown). Quantification of blots was done by densitometrical scanning, and shRNA and siRNA controls were arbitrarily set at 100%. A, Western blots showing shRNA- and siRNA-mediated down-regulation of AP-2 α in SGHPL-5 cells and primary EVTs, respectively. Specific signals (kilodaltons) of AP-2 family members and GAPDH (loading control) are marked by arrows. B, Western blot analyses of AP-2 α shRNA gene-silenced SGHPL-5 cell pool and AP-2 α siRNA-treated EVTs in the absence or presence of EGF. C, Densitometrical quantification of AP-2 α down-regulation in controls and EGF-treated SGHPL-5 cells and primary EVTs. Bar graphs show mean values $\pm$ SD of each ($n = 3$) different gene-silenced SGHPL-5 cell pools and primary EVTs. *, Significance with $P < 0.05$ compared with controls, respectively.

with 0.1% Triton X-100/PBS at 4°C. Next, invasion inserts were blocked with 0.05% gelatin diluted in PBS (30 min). Again, non-invaded cells on top of the membrane were removed with a cotton swab. Membranes were then costained with cytokeratin 7 (clone OV-TL 12/30, 8.3 μ g/ml; Dako) and vimentin (clone Vim 3B4, 1.2 μ g/ml; Dako) (1 h at 37°C) to evaluate trophoblast and fibroblast content, respectively. After staining with secondary antibodies, Alexa Fluor 488, antimouse, and Alexa Fluor 568, antirabbit (4 μ g/ml; Molecular Probes) filters were counterstained with DAPI and embedded on microscope slides using fluoromount G (Soubio). Cytokeratin 7-positive cells on the underside of filters were counted as described above. Four independent experiments (EVTs pooled from two to three placentae each) were performed in duplicates.

Proliferation assays

Proliferation of shRNA-transfected SGHPL-5 cell pools was determined by counting cumulative cell numbers. The 1.3×10^4 cells/cm² were seeded in duplicates in the absence or presence of 5 ng/ml EGF. Cells were counted after 24, 48, and 72 h using a multichannel electronic cell counter (CASY-I; Schärfe Systems). The proliferation of siRNA-treated primary EVTs was performed with 5-bromo-2'-deoxyuridine (BrdU) immunofluorescent assay according to the manufacturer's instructions (Roche Diagnostics, Vienna, Austria). Briefly, 1.2×10^5 cells/cm² were seeded in chamber slides (Lab-Tec; NUNC, Glostrup,

Denmark) coated with fibronectin (20 μ g/ml). Subsequently Accell oligos (1.25 μ M; Dharmacon), and BrdU (10 μ M) diluted in Accell delivery medium with or without 5 ng/ml EGF were added. After 48 h cells were costained with mouse monoclonal antibody against BrdU (10 mg/ml) and cytokeratin-wide spectrum antibody (rabbit, 1:100; GeneTex, Inc., San Antonio, TX), the latter detecting only trophoblast in EVT preparations (data not shown). After staining with secondary antibodies (Alexa Fluor 488, antimouse, and Alexa Fluor 568, antirabbit, 1:50,000; Molecular Probes, Invitrogen) slides were counterstained with DAPI. Chambers were photographed under fluorescence and analyzed with Olympus cell-imaging software. Cytokeratin-positive trophoblasts were counted and the percentage of BrdU-positive cells was determined.

Luciferase assay

Stable SGHPL-5 cell pools (7.5×10^4 cells/cm²) harboring either shRNA AP-2 α or shRNA control were cotransfected with 1.5 μ g of a luciferase reporter containing the proximal wild-type CG β 5 promoter (23), 0.5 μ g pCMV- β -galactosidase (normalization control) and 1 μ g of plasmid expressing AP-2 α (pAAV-IRES-hrGFP-AP-2 α) using 2 μ l Lipofectamine 2000 (Invitrogen). A pcDNA3 vector (Invitrogen) encoding AP-2 α was kindly provided by F. Domann (University of Iowa) (30). To evaluate numbers of AP-2 α -expressing cells, its cDNA was subcloned into pAAV-IRES-hrGFP (Stratagene, La Jolla,

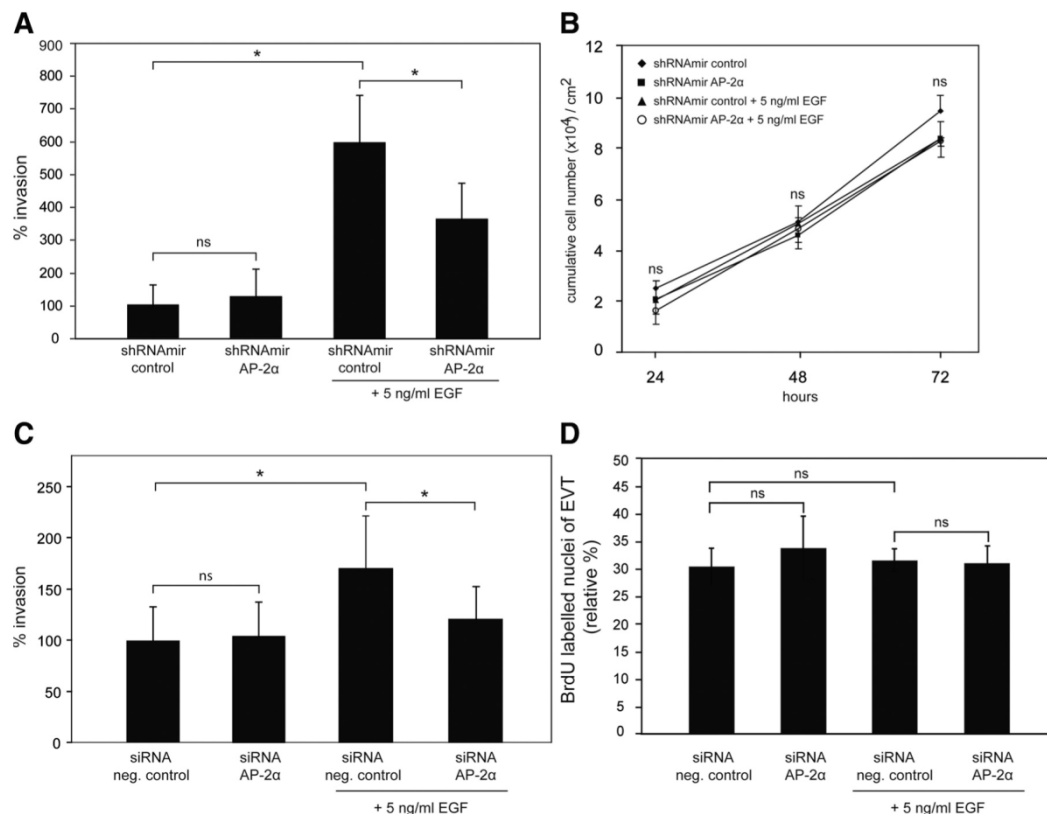


FIG. 3. EGF-dependent invasion and proliferation of AP-2α gene-silenced SGHPL-5 cells and primary EVTs. Matrigel invasion and proliferation assays with AP-2α shRNA-expressing SGHPL-5 cells or AP-2α siRNA-treated EVTs were performed as described in *Materials and Methods*. A, Invasion of AP-2α shRNA-expressing SGHPL-5 cells. Bars represent mean values $\pm$ SD of each of four different shRNA controls and knockdown cell pools measured in triplicates. Mean value of shRNA control was arbitrarily set to 100%. *, $P < 0.05$. ns, Not significant compared with the respective controls. B, Cumulative cell numbers of AP-2α gene-silenced SGHPL-5 cells in the absence or presence of EGF. Bars show mean values $\pm$ SD of three independent experiments per cell pools performed in duplicates. ns, Not significant compared with shRNA control. C, Invasion of AP-2α siRNA-treated primary EVTs. Bars represent mean values $\pm$ SD of four independent siRNA transfections/trophoblast preparations performed in duplicates. *, $P < 0.05$. ns, Not significant compared with the respective controls. D, BrdU labeling of primary EVTs after AP-2α siRNA treatment and EGF stimulation. Bars represent mean values $\pm$ SD of three independent siRNA transfections/trophoblast preparations performed in duplicates. ns, Not significant compared with controls, respectively.

CA) using *HindIII* and *XhoI* restriction enzymes (Roche Diagnostics). For negative control empty pAAV-IRES-hrGFP plasmids were used. Luciferase activity was measured on a luminometer (Lumat LB 9507; EG&G Berthold, Bad Wildbad, Germany) using a luciferase assay system (Promega, Madison, WI). Activity of β -galactosidase was quantitated on a photometer by determining the conversion of the chromogenic substrate chlorophenol red- β -D-galactopyranoside (Roche Diagnostics) at 570 nm as described (28). Luciferase values were normalized to β -galactosidase values.

Transient AP-2α overexpression in SGHPL-5 cells

SGHPL-5 cells (6.4×10^4 cells/cm²) were seeded. After attachment (4 h), cells were washed with PBS and transfected with each 4 μ g of pAAV-IRES-hrGFP-AP-2α or empty plasmids (pAAV-IRES-hrGFP, mock control) using Lipofectamine 2000 (Invitrogen). Transfection rate was determined by analyzing green fluorescent protein (GFP)-positive cells (~30%) under the fluorescent microscope. Twenty-four hours after transfection, cells were used for Western blot analyses and Matrigel invasion assays as described above.

Statistical analyses

For comparison of two conditions of interest, statistical analyses were performed with Student's paired *t* test using SPSS 14 (SPSS Inc., Chicago, IL). Gaussian distribution and equality of variances were examined with Kolmogorov-Smirnov test and Levene's test, respectively. A *t* test $P < 0.05$ was considered statistically significant.

Results

Expression pattern of AP-2 family members in trophoblasts

Descriptive studies were performed to analyze expression of AP-2 family members in placental tissues, primary trophoblasts, and EVT cell lines. Semiquantitative RT-PCR analyses revealed expression AP-2α, β - and γ -mRNAs in first-trimester and term villous cytotrophoblasts, total placental tissues of early and late pregnancy, and

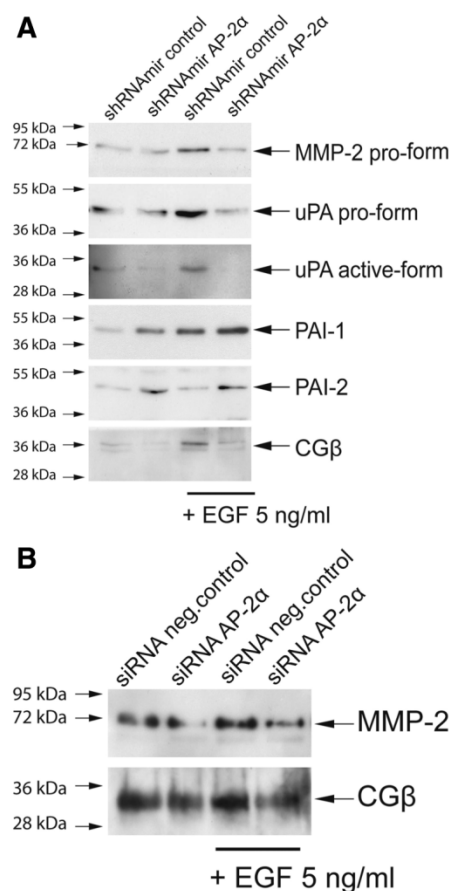


FIG. 4. Western blot analyses and quantification of invasion-associated genes in AP-2 α gene-silenced SGHPL-5 cells and primary EVTs. EGF stimulation and Western blotting of supernatants were performed as described in *Materials and Methods*. Representative examples are shown, and specific protein signals and marker bands are indicated. A, Western blot of supernatants of AP-2 α shRNA-mir-expressing and shRNA-mir control SGHPL-5 cells in the absence or presence of EGF. B, Western blot of supernatants of AP-2 α siRNA- and control siRNA-treated primary EVTs in the absence or presence of EGF.

EVTs isolated from differentiating villous explant cultures (Fig. 1A). First-trimester villous fibroblasts weakly express AP-2 β mRNA, whereas trophoblastic HTR-8/SVneo cells lack AP-2 β transcripts. SGHPL-5 cells, on the other hand, do not express AP-2 γ mRNA. A similar expression pattern could be observed in Western blot analyses (Fig. 1B). Both HTR-8/SVneo cells and SGHPL-5 produce AP-2 α protein, whereas AP-2 β protein was detectable only in SGHPL-5 cells. AP-2 γ protein was absent from SGHPL-5 cells and weakly expressed in HTR-8/SVneo cells. Immunofluorescence of tissue sections also confirmed expression of AP-2 protein family members in first-trimester placenta (Fig. 1C). AP-2 α , - β , and - γ were detectable in villous trophoblasts and proliferative, Ki67-positive cell column trophoblasts as well as p57/Kip2-positive, differentiated EVTs.

Gene silencing of AP-2 α in SGHPL-5 cells and primary EVTs

To analyze the functional role of AP-2 α in trophoblast invasion, gene silencing in SGHPL-5 cells and primary EVTs was performed. In SGHPL-5 cells constitutive down-regulation of AP-2 α was achieved upon retroviral transduction with shRNA-mir against AP-2 α and puromycin selection. Western blot analyses revealed reduction of AP-2 α protein in the stable cell pools (Fig. 2A, *top panel*). Densitometrical scanning indicated that basal AP-2 α levels significantly decreased to 28% compared with shRNA-mir controls (100%; Fig. 2C). Similarly, siRNA treatment of primary EVTs decreased AP-2 α to 37% compared with siRNA controls (Fig. 2A, *bottom panel*; Fig. 2C). Western blot analyses also indicated that AP-2 β and - γ protein levels were not affected upon down-regulation of AP-2 α in SGHPL-5 cells or primary EVTs.

Recently EGF, a positive regulator of trophoblast invasiveness, was shown to up-regulate AP-2 α in choriocarcinoma cells (31). Hence, the effect of the growth factor on AP-2 α expression in controls and AP-2 α gene-silenced SGHPL-5 cells and primary EVTs, respectively, was investigated. EGF induced AP-2 α in both culture systems, and differences in AP-2 α protein levels between controls and knock-down cells were stronger in the presence of the growth factor (Fig. 2B). Compared with untreated cultures (100%), AP-2 α increased to 150% in SGHPL-5 cells and to 260% in primary trophoblasts upon EGF treatment (Fig. 2C). Densitometrical quantification of the EGF-supplemented cultures also revealed that AP-2 α was significantly reduced in shRNA-mir-treated SGHPL-5 cells and siRNA-treated primary EVTs, respectively (Fig. 2C).

AP-2 α is a positive regulator of trophoblast invasion

To investigate the role of AP-2 α in trophoblast invasion, SGHPL-5 cells harboring the constitutive, shRNA-mir-mediated AP-2 α knockdown were analyzed in Matrigel transwell assays (Fig. 3A). Quantification of invasion revealed that gene silencing of AP-2 α did not affect basal invasion but significantly reduced EGF-induced motility. Proliferation of the cells was not influenced upon AP-2 α gene silencing up to 72 h and also was not significantly altered upon EGF treatment (Fig. 3B). Similarly, AP-2 α siRNA treatment of primary EVTs did not alter basal invasion through Matrigel but decreased EGF-stimulated invasion (Fig. 3C). Again, basal and EGF-mediated proliferation of EVTs, measured by BrdU labeling, was not changed upon incubation with AP-2 α siRNA (Fig. 3D).

TABLE 1. Quantification of protein expression in supernatants of shRNAmir-treated SGHPL-5 cell pools and siRNA-treated primary EVT

SGHPL-5	shRNAmir-control = A	shRNAmir-AP2 α = B	shRNAmir-control + EGF = C	shRNAmir-AP2 α + EGF = D
MMP-2 proform	100 $\pm$ 36	84 $\pm$ 31 ns compared with A	220 $\pm$ 49 Compared with A ^a	63 $\pm$ 10 Compared with C ^a ns compared with A
uPA proform	100 $\pm$ 45	104 $\pm$ 33 ns compared with A	238 $\pm$ 21 Compared with A ^a	87 $\pm$ 64 Compared with C ^a ns compared with A
uPA active form	100 $\pm$ 30	84 $\pm$ 27 ns compared with A	305 $\pm$ 46 Compared with A ^a	19 $\pm$ 28 Compared with C ^a Compared with A ^a
PAI-1	100 $\pm$ 62	243 $\pm$ 60 Compared with A ^a	356 $\pm$ 100 Compared with A ^a	483 $\pm$ 19 Compared with C ^a Compared with A ^a
PAI-2	100 $\pm$ 10	345 $\pm$ 97 Compared with A ^a	110 $\pm$ 28 ns compared with A	391 $\pm$ 27 Compared with C ^a Compared with A ^a
CG β	100 $\pm$ 26	34 $\pm$ 14 Compared with A ^a	238 $\pm$ 67 Compared with A ^a	11 $\pm$ 5 Compared with C ^a Compared with A ^a
EVT	siRNA control = A	siRNA AP2 α = B	siRNA control + EGF = C	siRNA AP2 α + EGF = D
MMP-2 proform	100 $\pm$ 39	118 $\pm$ 51 ns compared with A	270 $\pm$ 54 Compared with A ^a	116 $\pm$ 52 Compared with C ^a ns compared with A
CG β	100 $\pm$ 23	68 $\pm$ 11 Compared with A ^a	152 $\pm$ 32 Compared with A ^a	70 $\pm$ 26 Compared with C ^a ns compared with A

Densitometrical scans of Western blots depicted in Fig. 4, A and B, were analyzed as described in *Materials and Methods*. Mean values (percentage) $\pm$ SD of three Western blots each are shown. For normalization, mean values of shRNAmir control and siRNA control were arbitrarily set to 100%. ns, Not significant.

^a $P < 0.05$.

AP-2 α controls expression of proteases and their inhibitors

To study genes potentially promoting AP-2 α -dependent invasion, different trophoblast-derived proteases as well as their inhibitors were analyzed in supernatants of controls and AP-2 α shRNAmir-treated SGHPL-5 cells in the absence or presence of EGF (Fig. 4A). Pro-MMP-2, and the pro- and active forms of uPA increased under EGF stimulation and were found to be reduced in AP-2 α knockdown cell pools. Similarly, PAI-1 and PAI-2 were elevated in shRNAmir-control and shRNAmir-AP-2 α cells, suggesting that the transcription factor may suppress the particular inhibitors. However, protein expression of TIMP1, -2, and -3 was not affected upon AP-2 α knockdown or EGF stimulation (data not shown). Moreover, CG β increased in the presence of EGF but was found to be suppressed upon loss of AP-2 α . Due to the limited amounts of cells, only a few potential target genes of AP-2 α could be tested in primary EVTs. Similar to SGHPL-5 cells, the EGF-stimulated MMP-2 and CG β expression was reduced in supernatants of

AP-2 α siRNA-treated EVTs (Fig. 4B). Quantification of protein expressions depicted in Fig. 4, A and B, are presented in Table 1.

AP-2 α transactivates the proximal CG β 5 promoter in SGHPL-5 cells

AP-2 α is a well-known regulator of CG genes in villous trophoblasts (18, 23); however, its role in the CG transcription of EVTs has not been tested. Hence, the effects of AP-2 α gene silencing and the overexpression on CG β gene transcription were analyzed with a luciferase reporter harboring the most active CG β promoter in trophoblasts, the CG β 5 promoter (23) (Fig. 5). Western blot analyses revealed that overexpression of a AP-2 α cDNA compensated shRNAmir-mediated down-regulation of the factor in SGHPL-5 cells (Fig. 5A). CG β 5 promoter analyses indicated that knockdown of AP-2 α decreased luciferase expression to 68% compared with shRNAmir-controls (100%) (Fig. 5B). The overexpression of a AP-2 α cDNA, however, transactivated the CG β 5 wild-type promoter

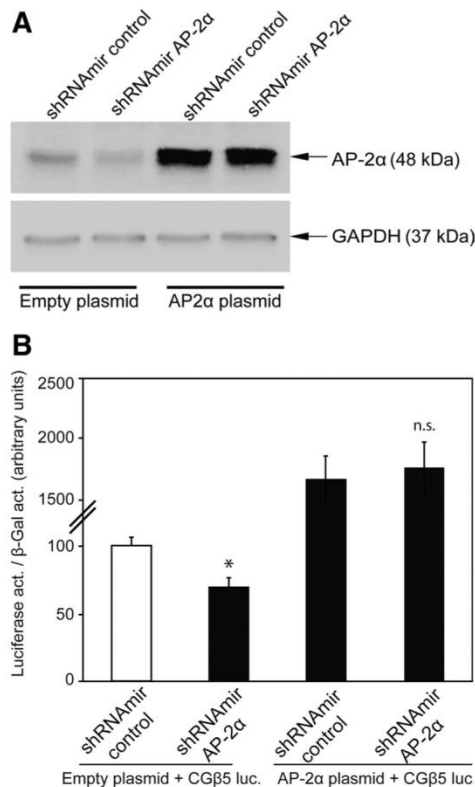


FIG. 5. Effects of overexpression of AP-2 α on luciferase activity of the CG β 5 promoter in AP-2 α shRNA-expressing SGHPL-5 cells and shRNA controls. A, Western blot analysis showing AP-2 α protein levels after overexpression in shRNA-AP-2 α cells and shRNA control cells. GAPDH (37 kDa) was used as a loading control. B, Luciferase activity of the CG β 5 promoter in AP-2 α shRNA-expressing SGHPL-5 cells and shRNA controls in the absence or presence of AP-2 α expression plasmids. Cotransfection of shRNA-expressing SGHPL-5 cell pools with the proximal CG β 5 wild-type promoter, the AP-2 α expression plasmid, and CMV- β -galactosidase plasmids for normalization were done as described in *Materials and Methods*. Mean values $\pm$ SD of three experiments/different cell pools performed in duplicates are shown. Normalized value of shRNA control cells transfected with empty plasmids was arbitrarily set at 100%. ns, Not significant. *, $P < 0.05$ compared with the respective shRNA control.

and reverted the effect of AP-2 α gene silencing on luciferase expression.

Overexpression of AP-2 α modestly affects basal trophoblast invasion

Finally, invasion of SGHPL-5 cells was tested after overexpression of AP-2 α cDNA (Fig. 6). Western blot analyses revealed abundant amounts of AP-2 α protein in SGHPL-5 cells after transient transfection of the AP-2 α expression plasmid (Fig. 6A). Analyses of Matrigel transwells assays suggested that exogenous expression of AP-2 α only weakly increased invasion to 125% compared with cells transfected with empty plasmids (100%) (Fig. 6B).

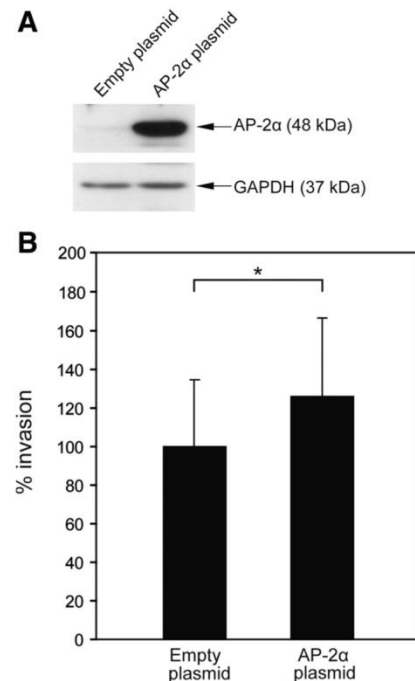


FIG. 6. Overexpression of AP-2 α modestly affects SGHPL-5 cell invasion. Transient transfection of SGHPL-5 cells with AP-2 α expression plasmids and invasion assay were performed as described above. A, Western blot showing AP-2 α (48 kDa) overexpression in SGHPL-5 cells. Specific bands are marked by arrows. GAPDH (37 kDa) was used as a loading control. B, Matrigel invasion assay of empty plasmid-transfected and AP-2 α cDNA-transfected SGHPL-5 cells. Bars represent mean values of $\pm$ SD of three transfections performed in duplicates. For normalization mean value of empty plasmid-transfected control was arbitrarily set to 100%. *, $P < 0.05$.

Discussion

Transcription factor AP-2 α plays a pivotal role in cell proliferation, migration, and invasion. Previous *in vitro* analyses of cancer cells suggested that the protein may act as an inhibitor of these processes. For example, loss of AP-2 α decreased epithelial E-cadherin and increased proliferation as well expression of MMPs and other invasion-associated genes such as protease-activated receptor 1 (32, 33). Accordingly, AP-2 α likely acts as a tumor suppressor in different types of cancers such as breast carcinomas, melanomas, or gliomas (34–36). On the other hand, numerous reports suggest that overexpression of AP-2 α provokes tumorigenesis (37–39). In HER2-positive cancers, elevated levels of AP-2 were noticed and estrogen was shown to regulate HER2 expression via AP-2 (40, 41). Along those lines, AP-2 α was demonstrated to enhance tumor, neural crest, and endothelial cell migration by promoting secretion of soluble factors such as CXC chemokine ligand 2 and expression of MMP-2 (42–44). Hence, the role of the transcription factor family likely depends on

the cell type, the degree of cellular transformation, and the distribution of different AP-2 isoforms.

In accordance with previous studies on physiological cell migration, our analyses suggest a positive role of AP-2 α in trophoblast invasion. Gene silencing in both trophoblastic SGHPL-5 cell and primary EVT_s did not affect basal invasion but significantly reduced EGF-induced cell movement. Indeed, the growth factor increased AP-2 α expression in SGHPL-5 cell and EVT_s, which has also been previously noticed in trophoblastic JAR cells (31). Hence, the data suggest that EGF-induced invasiveness is at least partly mediated through elevated AP-2 α expression. In tumor cells AP-2 α was shown to control transcription of numerous genes involved in cell cycle regulation, migration, and apoptosis. In particular, down-regulation of AP-2 α was shown to increase proliferation by affecting cyclins and cyclin-dependent kinase inhibitors such as p21 (43, 45). Here we could not detect changes in cellular growth because gene silencing of AP-2 α altered neither cumulative SGHPL-5 cell numbers nor DNA synthesis of primary EVT_s in the absence or presence of EGF. First, the data suggest that the invasion-promoting effects of EGF cannot be explained by possible elevation of cell proliferation. Second, we also may conclude that AP-2 α is dispensable for trophoblast proliferation. However, neither SV40 large T-transformed SGHPL-5 cells nor matrix-attached EVT_s may represent ideal systems to study trophoblast growth. Although not intended in this study, additional experiments such as gene silencing in outgrowing villous explant cultures would be necessary to delineate the role of AP-2 α in trophoblast proliferation.

In agreement with its positive role in trophoblast invasion, Western blot analyses of controls and AP-2 α knock-down cells showed that the transcription factor regulates EGF-dependent expression of proteases and inhibitors involved in trophoblast cell motility. MMP-2 and uPA could be critical target genes because their EGF-stimulated expression was reduced upon AP-2 α gene silencing. Indeed, AP-2 α was shown to control MMP-2 transcription under basal as well as EGF-stimulated conditions (31, 46). Also, interactions of AP-2 α with transcription factors p53 and YB-1, which were shown to be critical for MMP-2 promoter activity (47), might be disturbed upon down-regulation of AP-2 α . Although the uPA gene promoter also contains AP-2 binding sites (48), EGF-dependent induction was shown to depend on E26 and AP-1 recognition sequences (49). Hence, AP-2 α -dependent expression of uPA in the presence of EGF may involve indirect mechanisms such as secretion, processing, or protein stability, although a promoter-regulatory role in trophoblast cannot be excluded. Gene silencing of AP-2 α increased PAI-1 and PAI-2 levels in SGHPL-5 cells, suggesting that

binding of the factor to cognate sequences present in the PAI-1 and PAI-2 gene promoters (50, 51) may exert inhibitory effects in trophoblasts. In accordance with previous studies (52, 53), however, EGF also induced PAI-1 in shRNA_{mir}-control cells. Hence, we suspect that the overall ratio of uPA to its inhibitors (PAI-1 and PAI-2), which was highest in EGF-treated shRNA_{mir}-control cells, could be critical.

Numerous studies indicated that AP-2 α is an important transcriptional regulator of CG subunit expression both in choriocarcinoma cells and primary villous trophoblasts (18, 22, 23, 54). Here Western blot analyses of secreted CG β as well as experiments using the CG β 5 promoter suggest that AP-2 α also induces CG β gene transcription in EVT_s. Because CG was shown to promote trophoblast invasion through different mechanisms such as MMP-2 activation and increased IGF-II binding (29, 55, 56), the suggestion that AP-2 α positively influences EGF-stimulated trophoblast motility is further strengthened.

This, however, is in contrast to a recent publication suggesting that AP-2 α expression reduces trophoblast invasiveness upon overexpression in HTR-8/SVneo cells (57). The reason for this discrepancy is currently unknown; however, several possibilities have to be considered. Whereas data presented here are based on combined evidence from nonimmortalized, trophoblastic SGHPL-5 cells and primary EVT_s, the sole use of the immortalized cell line HTR-8/SVneo may not be sufficient to analyze the role of AP-2 α in trophoblast invasion. Moreover, passage number of HTR-8/SVneo cells might be critical, poten-

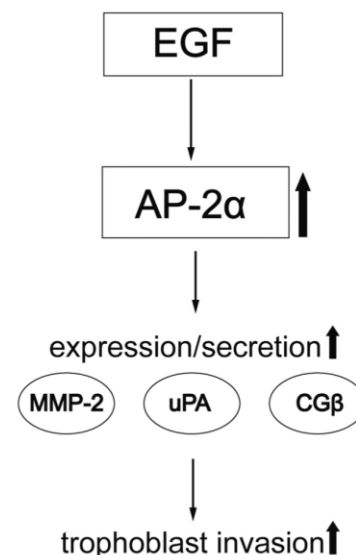


FIG. 7. Current model system showing the putative role of AP-2 α in human trophoblast invasion. EGF stimulation increases AP-2 α expression in EVT_s. The elevated AP-2 α transcriptionally activates invasion-promoting genes such as MMP-2, uPA, and CG β .

tially affecting AP-2 α levels. The notion that AP-2 α is only weakly expressed in trophoblastic cells and primary EVT_s (57) is not in agreement with our data obtained in Western blots and immunofluorescent analyses of placental tissues. Here gene silencing and overexpression of AP-2 α in the absence of EGF was not sufficient to provoke considerable changes in trophoblast invasion. This could be due to the fact that only approximately 30% of transiently transfected SGHPL-5 cells used in invasion assays overexpressed AP-2 α . On the other hand, besides increasing AP-2 α levels, EGF may exert additional effects on trophoblast invasion such as recruitment of coactivators facilitating AP-2-dependent transcription and gene expression (14). Moreover, it is likely that additional target genes of AP-2 α , which have not been identified/studied in trophoblasts so far, contribute to EGF-dependent trophoblast invasion.

In conclusion, the data suggest that EGF-dependent expression of AP-2 α plays a role in trophoblast invasion. The positive role of the transcription factor in EGF-stimulated trophoblast motility could be explained by induction of proteases such as MMP-2 either through direct activation and/or indirectly through stimulation of CG expression (Fig. 7). Moreover, the data provide evidence that AP-2 α may contribute to EGF-induced uPA expression but suppress its inhibitors PAI-1 and PAI-2, thereby presumably shifting the balance toward an invasive phenotype.

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6.1.2. Declaration of the authorship responsibility and contribution to published manuscript

Transcription factor AP-2 α promotes EGF-dependent invasion of human trophoblasts.

Here, I confirm that as a first author of this manuscript I was responsible for research design and I provide the data on which the manuscript is based. Additionally, I was mainly responsible for statistical analysis of the data and partially for drafting the manuscript. I was also responsible for preparing of all the figures for the manuscript.

Precisely, I was responsible for:

1. Cultivation of the trophoblast cell line SGHPL-5.
2. Isolation and cultivation of the first trimester primary EVTs.
3. Helping during generation of stable microRNA mediated knock-down in SGHPL-5 cell line and further evaluation of the knock-down with Western Blot analyses.
4. Generation of transient AP-2 α knock-down in primary EVTs and further analysis of the knock-down using Western Blot analysis.
5. Identification of invasion related target genes of AP-2 α in stable shRNA^{mir} AP-2 α knock-down in cell lysates and supernatants by Western Blot analysis.
6. Invasion assays on matrigel coated transwell invasion inserts with stable shRNA AP-2 α knock-down in SGHPL-5 cell line.
7. Establishment of the invasion assay on fibronectin coated transwell invasion inserts with primary EVTs.
8. Analyses of the invasion assays using fluorescent microscope.
9. Proliferation assay performed by measuring cumulative cell number in SGHPL-5 and BrdU labeling in EVTs.
10. Luciferase assay with shRNA AP-2 α knock-down in SGHPL-5 cell line with CG β 5 construct.
11. Transient AP-2 α overexpression in shRNA AP-2 α knock-down in SGHPL-5 cell line for luciferase assay purpose.
12. Cloning of AP-2 α constructs for transient transfection of SGHPL-5 cell line.

13. Transient overexpression using above mentioned construct containing AP-2 α with Lipofectamine 2000.

I was responsible for providing data, statistical analysis and final preparation of the following figures in the manuscript: 2A, 2B, 2C, 3A, 3B, 3C, 3D, 4A, 4B, 5B, 6A, 6B, 7 and Table 1.

6.2 The role of the endostatin in the human trophoblast invasion

6.2.1 Endostatin suppresses IGF-II-mediated signaling and invasion of human extravillous trophoblasts. Pollheimer J, Haslinger P, Fock V, Prast J, Saleh L, Biadasiewicz K, Jetne-Edelmann R, Haraldsen G, Haider S, Hirtenlehner-Ferber K, Knöfler M. Endocrinology. 2011 Nov; 152(11):4431-42. Epub 2011 Sep 20.

Endostatin Suppresses IGF-II-Mediated Signaling and Invasion of Human Extravillous Trophoblasts

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Endostatin, a biological active fragment of the extracellular matrix protein collagen XVIII, is known to interfere with cellular motility in the context of pathological angiogenesis. However, the physiological role of endostatin remains largely elusive. Recent evidence suggested that the inhibitor is produced in human decidual cells of early pregnancy, indicating that endostatin could be involved in diverse reproductive processes, such as implantation and/or placental differentiation. To gain more insights into the role of endostatin, we here analyzed its effects on trophoblast motility, proliferation, and signaling using purified primary trophoblasts, first-trimester villous explant cultures, and trophoblastic SGHPL-5 cells. *In vitro* Transwell assays demonstrated that purified endostatin inhibited both basal and IGF-II-induced migration and invasion as well as outgrowth from villous explant cultures. In contrast, basal and IGF-II-stimulated proliferation was unaffected upon addition of the inhibitor. Analyses of IGF-II-associated downstream signaling events showed that endostatin interfered with activation of various signaling kinases such as ERK1/2, protein kinase B (Akt)/mammalian target of rapamycin/p70 S6 kinase, and focal adhesion kinase. Furthermore, virus-mediated, stable gene silencing of Akt1 in SGHPL-5 cells using a micro-RNA-adapted short hairpin RNA-expressing plasmid revealed that endostatin-mediated inhibition of IGF-II-induced Akt phosphorylation was critically dependent on the expression of the particular isoform. In conclusion, the data suggest that endostatin could be a physiological inhibitor of IGF-II-dependent trophoblast cell motility by suppressing focal adhesion kinase/Akt/mammalian target of rapamycin/p70 S6 kinase signaling. (*Endocrinology* 152: 4431–4442, 2011)

During pregnancy, the so-called extravillous trophoblast (EVT) establishes the uteroplacental circulation by invading into the maternal decidua and spiral arteries (1). These processes are pivotal for the support of the growing embryo with nutrients and oxygen involving a not yet fully understood network of regulatory mechanisms (2). Trophoblast invasion is characterized by a multistep differentiation process starting at the placental villous by the formation of a proximally proliferating and distally growth-arrested cell column anchoring to the epithelial surface of the maternal uterus (3). In a next step, EVT detach from the placental villous cell column and

migrate into the uterine stroma and vessels, ensuring continuous blood flow to the placenta and the developing embryo (1). There is good evidence that EVT invasion is orchestrated by pro- and antimigratory stimuli, the latter restricting the invasive capacity of EVT in a temporally and spatially controlled manner (4). Indeed, different studies showed that either uterine stromal cells or EVT produce antiinvasive factors, including TGF- β 1-3, tissue inhibitor of metalloproteinases (TIMP), and plasminogen-activator inhibitors type 1 and 2 (5–8). Furthermore, there is also evidence that a shift in this balance toward an antiinvasive environment might con-

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Abbreviations: Akt, Akt/protein kinase B; DAPI, 4',6-diamidino-2-phenylindole; EVT, extravillous trophoblast; FAK, focal adhesion kinase; FCS, fetal calf serum; miRNA, micro-RNA; MMP, matrix metalloproteinase; mTOR, mammalian target of rapamycin; shRNA, miRNA-adapted short hairpin RNA; S6K, p70 S6 kinase; TORC2, mTOR complex 2; uPA, urokinase plasminogen activator.

tribute to the pathogenesis of pregnancy-related disorders, such as preeclampsia (8).

It is well described that invading cells interact with surrounding host extracellular matrix proteins, resulting in a protease-dependent release of breakdown products. In this context, collagen XVIII was the first extracellular matrix protein shown to harbor a bioactive proteolytic cleavage product called endostatin, at its C-terminal end (9). Endostatin was demonstrated to bind several cell surface molecules including $\alpha 5 \beta 1$, $\alpha v \beta 3$ integrins (10, 11) and shown to inhibit cancer-associated angiogenesis and tumor growth in mice (12). Furthermore, it impairs endothelial, epithelial, and cancer cell function via blockage of growth factor-dependent proliferation and motility (12–14). Endostatin was further noticed to inhibit activation of cellular signaling components such as focal adhesion kinase (FAK) (11) and ERK (11). Although the inhibitor was excessively studied in the context of cancer therapy, very little is known about its physiological role (15). Along those lines, collagen XVIII/endostatin was shown to alter eye development (16) and wound healing in mice (17) as well as suppression of axon guidance in *Caenorhabditis elegans* (18). Moreover, both collagen XVIII and endostatin are highly expressed in different organs (9), and endostatin was detected in human serum (19), suggesting that endostatin may exert a physiological role in humans. Interestingly, we have previously demonstrated that collagen XVIII and its proteolytic fragment endostatin are abundantly expressed at the human fetal-maternal interface (20). Moreover, trophoblasts were shown to secrete proteases, which cleave endostatin from its precursor, the NC1 domain of collagen XVIII (21). Consistently, we and others were also able to correlate severity of preeclampsia with increased serum levels of endostatin (22–24).

To gain a better understanding of the putative function of endostatin during pregnancy, we here investigated its functional properties in trophoblast invasion and migration. Considering that endostatin has profound effects on cellular signaling, we speculated that the inhibitor might interfere with signaling pathways critically involved in trophoblast invasion (25, 26). Using primary trophoblast model systems and a trophoblast cell line, we show that endostatin inhibited both unstimulated and IGF-II-mediated trophoblast cell motility. Further analyses of downstream signaling components in trophoblasts revealed that endostatin suppressed IGF-II-induced phosphorylation of FAK, Akt/protein kinase B (Akt)/mammalian target of rapamycin (mTOR), and p70 S6 kinase (S6K) and, to a lesser extent, activation of ERK1/2. Finally, a virus-mediated knockdown approach demonstrated that endostatin mainly exerts its antiinvasive properties via inhibition of Akt1 signaling.

Materials and Methods

Tissue collection

Placental tissues of early pregnancy (between the seventh and 12th week of gestation, $n = 90$) were obtained from legal abortions of uncomplicated pregnancies. Use of tissues was approved by the ethical committee of the Medical University of Vienna.

Reagents

Recombinant human IGF-II was purchased from Sigma-Aldrich (St. Louis, MO), and VEGF₁₆₅ was obtained from R&D Systems (Minneapolis, MN). Human plasma-derived fibronectin and rat tail collagen-I were purchased from BD Biosciences (San Jose, CA).

Flag-tagged purified human endostatin was produced as previously described (13). Briefly, human embryonic kidney cells (293-EBNA) were used for transfection of C-terminal end FLAG-tagged endostatin (hES-Cflag) cloned into a pCEP-Pu vector (kind gift from B. Olsen, Harvard Medical School, Boston, MA). Transfected cells were selected with 0.5 μ g/ml puromycin, and serum-free conditioned medium was collected. The conditioned medium was dialyzed against 0.05 M Tris-HCl pH 7.5, 0.15 M NaCl and applied to an anti-flag antibody column using Anti-Flag M2 affinity gel (Sigma). Flag fusion protein was eluted by competition with flag peptide (Sigma). Purified proteins were dialyzed against 0.05 M Tris-HCl (pH 7.5), 0.15 M NaCl overnight and then stored at -80°C . Produced and purified endostatin was tested in a tube-formation assay using human umbilical cord vein endothelial cells. Briefly, human umbilical cord vein endothelial cells (4500 cells/cm²) were preincubated with purified endostatin (37 $^{\circ}\text{C}$ for 30 min) and seeded into 96-well plates coated with growth factor-reduced Matrigel (BD Biosciences) in the presence or absence of 10 ng/ml VEGF₁₆₅. After 6–8 h, cells were fixed in methanol and photographed, and tube formation was determined by counting numbers of tubular circles per photographed area. VEGF-mediated tube formation was fully blocked by the addition of 100 ng/ml purified endostatin as previously shown (13, 27).

Cell culture of primary EVT and SGHPL-5 cells

Primary EVT of pooled first-trimester placentae ($n = 8$ –12) between the seventh and 12th week of gestation were isolated by adapted enzymatic dispersion and Percoll density gradient centrifugation (10–70%; Pharmacia, Uppsala, Sweden) as described (28). Briefly, placentae were washed with ice-cold PBS and kept overnight in DMEM/Ham's F-12, supplemented with 0.05 mg/ml gentamicin (Life Technologies, Carlsbad, CA) and 2.5 μ g/ml fungizone (Life Technologies). Villous tips were scraped with a scalpel blade and digested for 30 min in 0.125% Trypsin (Life Technologies, Inc.) and 12.5 mg/ml deoxyribonuclease I (Sigma) in 10% Hanks' balanced salt solution-Mg/Ca-free medium (Sigma). Next, cells were filtered through a 70- μ m cell strainer (Greiner Bio-One GmbH, Frickenhausen, Germany). Cell suspension was separated by a Percoll gradient and subsequent centrifugation. Isolated cells (from 35–50% Percoll layer) were cultivated on fibronectin (20 μ g/ml) at a density of $2.5 \times 10^5/\text{cm}^2$ and cultivated in DMEM/Ham's F-12, supplemented with 10% fetal calf serum (FCS) and 0.05 mg/ml gentamicin. Cells were characterized on fibronectin-coated Lab-Tek chamber slides (Nalge Nunc International, Hereford, UK)

using immunofluorescence with antibodies against cytokeratin-7 (clone OV-TL 12/30, 8.3 μ g/ml; Dako, Glostrup, Denmark), vimentin (rabbit polyclonal, 20 μ g/ml; GeneTex Inc., Irvine, CA), α 5 β 1 integrin (clone JBS5, 1 μ g/ml; Chemicon, Temecula, CA), α 1 integrin (clone FB, 12.2 μ g/ml; Chemicon), and α 6 integrin (clone 4F10, 2 μ g/ml; Chemicon). Isolated cells were mostly positive for cytokeratin-7 (97%) and largely negative for vimentin (Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>).

The trophoblast cell line SGHPL-5 was cultivated in a 1:1 mixture of DMEM and Ham's F-12, respectively, supplemented with 10% FCS, 2 mmol/liter glutamine, and 0.05 mg/ml gentamicin (all purchased from GibcoBRL Life Technologies) as recently mentioned (29). As previously published, SGHPL-5 cells were described to exhibit features of invasive EVT such as expression of histocompatibility antigen class I G and cytokeratin-7 (30). All experiments using SGHPL-5 cells were performed between passages five and ten.

Protein extraction and Western blot analyses

SGHPL-5 cells or primary trophoblasts were seeded into uncoated or fibronectin-coated 24-well plates, respectively, and serum starved overnight. Cells were then pretreated with different concentrations of endostatin (as indicated) and cultivated for another 15 min in the absence or presence of IGF-II (100 ng/ml). Total protein was harvested from cells by lysing them in 2 $\times$ Laemmli buffer (4% sodium dodecyl sulfate, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 mol/liter Tris-HCl) containing protease inhibitor cocktail (P-8340, 1:100; Sigma-Aldrich), diluting the lysates 1:1 in PBS, and subsequent boiling for 10 min. Protein lysates were blotted onto nitrocellulose membranes (Hybond-ECL, RPN303D; Amersham Biosciences, Piscataway, NJ), and protein loading was controlled by staining with Ponceau S solution [0.1% (wt/vol) Ponceau S, 5% acetic acid in double-distilled H₂O]. After blocking in 5% milk/PBS-Tween (0.05%), rabbit or mouse antihuman antibodies directed against phosphorylated forms of Akt (rabbit, 1:1000, Ser374 and Thr308; Cell Signaling, Boston, MA), FAK (rabbit, 0.25 μ g/ml, Y397; BD Biosciences), mTOR (rabbit, 1:1000, Ser2448 and Ser2441; Cell Signaling), S6K (mouse, 1:1000, Thr389; Cell Signaling), and ERK1/2 (rabbit, 1:1000, Thr202/Tyr204; Cell Signaling) were used. Briefly, growth factor-mediated activation of Akt is mainly mediated by activation of its amino acid residues Ser473 and Thr308 (31). Here, phosphorylation of Ser473 was shown to be dependent on the mTOR complex 2 (TORC2), whereas phosphorylation of Thr308 is TORC2 independent (31). Moreover, to further discriminate between the two mTOR complexes (TORC1/2), we used mTOR phosphospecific antibodies recognizing Thr2481 (predominantly TORC2) and Thr2248 (predominantly TORC1) (32, 33). In addition, phosphorylation of the TORC1-specific substrate S6K (31) was analyzed. To control for equal protein loading, the respective blots were reincubated with rabbit antihuman antibodies against total forms of Akt (1:1000; Cell Signaling), Akt1 (1:1000; Cell Signaling), FAK (1 μ g/ml, A-17; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and glyceraldehyde-3-phosphate dehydrogenase (1:1000; Cell Signaling). Horseradish peroxidase-conjugated goat antirabbit IgG (NA934V, 1:10,000; Amersham Biosciences) or antimouse IgG (1:1000, NA931; Amersham Biosciences) antibodies were used as sec-

ondary reagent. Horseradish peroxidase signal was then detected by enhanced chemiluminescence substrate (ECL Plus Western Blotting Detection System; GE Healthcare, Piscataway, NJ) and visualized on an Amersham Hyperfilm MP (GE Healthcare). The intensity of protein bands was quantified using AlphaEaseFC software (Alpha Innotech, San Leandro, CA).

Immunocytochemistry

Primary trophoblasts were seeded on fibronectin-coated chamber slides as described above and cultivated for 24, 48, and 72 h. Cells were fixed in methanol for 10 min, air dried for 10 min, and subsequently incubated with primary antibodies diluted in 0.05% fish skin gelatin overnight at 4 C, followed by secondary antibodies including goat antimouse Alexa Fluor 488 or goat antirabbit Alexa Fluor 568 (both 4 μ g/ml; Molecular Probes, Eugene, OR) for 1.5 h at room temperature. Irrelevant concentration-matched primary antibodies were used as negative controls. Double staining with mouse and rabbit reagents was performed by mixing the primary antibodies and mixing fluorescein isothiocyanate-conjugated reagents, respectively. Microscopy was performed with a Nikon (Tokyo, Japan) Eclipse E-800 fluorescence microscope equipped with Nikon Plan-Fluor objective lenses and an F-VIEW digital camera controlled by Analysis version 3.2 software (Soft Imaging System, Münster, Germany).

Construction of a stable Akt1 knockdown in SGHPL-5 cells

Stable knockdown of Akt1 in SGHPL-5 cells was generated by transduction with the micro-RNA (miRNA) adapted retroviral vector LMP (Open Biosystems, Huntsville, AL) as mentioned recently (34). Briefly, miRNA-adapted short hairpin RNA (shRNA_{mir}) against human Akt1 obtained in a pSM2 vector (Open Biosystems) was subcloned into the LMP vector with *Xho*I and *Eco*RI (Invitrogen Corp., Carlsbad, CA) restriction enzymes. To produce murine stem cell virus particles, HEK293FT cells (Invitrogen) were transiently cotransfected with a vector containing the viral packaging proteins (gag, pol, and env), a vector containing VSV-G, and plasmids harboring either nontargeting shRNA_{mir}-control or shRNA_{mir}-Akt (miRNA against human Akt1). Lipofectamine 2000 (Invitrogen) reagent was used for transfection. Forty-eight hours after transfection, viral supernatants were collected, supplemented with 8 μ g/ml Polybrene (Sigma), and used to infect SGHPL-5 cells. Stable integrants were selected with puromycin (5 ng/ml) over a period of 2 wk. Knockdown efficacy was confirmed by Western blot analyses.

Proliferation assay

Proliferative capacity of SGHPL-5 cells was studied by measuring cumulative cell numbers. Cells (2600/cm²) were seeded in 24-well dishes in the absence or presence of 100 ng/ml recombinant IGF-II and 100 ng/ml purified endostatin and counted after 24, 48, 72, 96, and 120 h using a multichannel electronic cell counter (CASY-I; Schärfe Systems, Reutlingen, Germany). Medium (containing endostatin and/or IGF-II) was changed every second day.

First-trimester villous explant cultures

Preparation and cultivation of villous explants of different first-trimester placentae (n = 15) between the seventh and 12th

week of gestation were performed as described elsewhere (29, 35). Briefly, anchoring villi were carefully dissected under the microscope and transferred to collagen-I-coated (collagen-I was mixed with 10× DMEM and 7.5% sodium bicarbonate) 48-well plates. Placental explants were then allowed to adhere to the matrix (4–7 h), subsequently covered with DMEM/Ham's F-12, supplemented with 0.05 mg/ml gentamicin, and further incubated for 48 h. Where indicated, recombinant human IGF-II (100 ng/ml) and/or endostatin (100 ng/ml) was added to the culture medium. To analyze the migratory capacity of placental explants, the area of outgrowth was digitally photographed and analyzed using the imaging software CellP (Olympus, Hamburg, Germany).

Invasion and migration assays

Invasion and migration assays involving SGHPL-5 cells were performed using Matrigel-coated (BD Biosciences) or noncoated (Costar, Corning, NY) 8-μm-pore Transwells, respectively, as recently described (29). Both migration and invasion assays were performed by adding growth medium containing 10% FCS to the lower compartment (500 μl) of the Transwell chamber and 50,000 cells into the upper chamber containing the same medium (200 μl). To test the effect of endostatin on trophoblast invasion/migration, cells were preincubated with different concentrations

of endostatin (1–1000 ng/ml) at 37°C for 30 min as described previously (13). To study the effect of endostatin on growth factor-induced motility, 100 ng/ml IGF-II was added to both chambers. After 24 h, cells were washed three times with PBS, fixed for 10 min in ice-cold methanol, again washed three times with PBS, and subsequently incubated with 4',6-diamidino-2-phenylindole (DAPI) (1 μg/ml; Roche, Indianapolis, IN) for 10 min. Finally, the noninvaded/migrated cells on the upper side of the Transwell insert were removed by a cotton swab and membranes were cut out with a scalpel blade, placed on glass slides, and embedded with Fluoromount G (Soubio, Birmingham, AL). Nuclei of cells on the underside of membranes were counted under fluorescence using Olympus cell imaging software.

To study the effect of endostatin on primary EVT, invasion assays using fibronectin-coated 12-μm-pore Transwells (Millipore, Billerica, MA) were performed. Fibronectin (0.5 μg/ml) was added to the upper side of the Transwells, incubated for 30 min at 37°C, and subsequently placed into 24-well plates containing 300 μl EVT medium containing 10% FCS as described above. About 150,000 primary EVT, resuspended in EVT medium and supplemented with 0.1% FCS, were incubated with endostatin as described above and subjected to fibronectin-coated Transwells. Where indicated, IGF-II (100 ng/ml) was added to both chambers. After 48 h incubation, cells were fixed with ice-cold

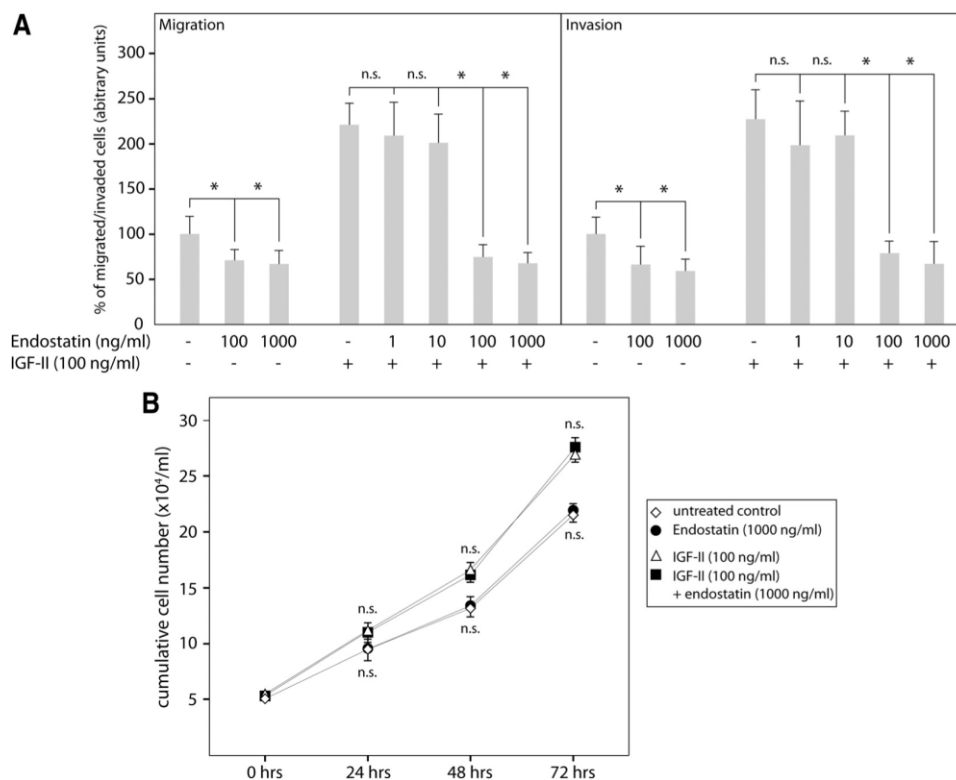


FIG. 1. Effect of purified endostatin on basal and growth factor-triggered SGHPL-5 cell migration/invasion and proliferation. **A**, Unstimulated and IGF-II-stimulated (100 ng/ml) SGHPL-5 cells were subjected to coated and noncoated Transwell assays in the absence or presence of endostatin (1–1000 ng/ml). Migration/invasion was quantified as the percentage of traversed endostatin-treated cells in comparison with respective controls (untreated or IGF-II treated). Untreated control was set to 100%. *, $P < 0.01$; n.s., not significant. Each value is a mean $\pm$ SD from three independent experiments performed in duplicate. **B**, Proliferation of IGF-II-treated (100 ng/ml) SGHPL-5 cells in the absence or presence of endostatin (1000 ng/ml), measured by cumulative cell numbers. Absolute cell numbers are indicated; each value is a mean $\pm$ SD from at least three independent experiments performed in triplicate. Nontreated control was set to 100%. *, $P < 0.01$; n.s., not significant.

TABLE 1. Immunofluorescent evaluation of integrin expression pattern of isolated EVT

	Cytokeratin-7	Integrin $\alpha 1$	Integrin $\alpha 5\beta 1$	Integrin $\alpha 6$
24 h	97 $\pm$ 6	42 $\pm$ 5	41 $\pm$ 2	22 $\pm$ 6
48 h	94 $\pm$ 9	88 $\pm$ 4	65 $\pm$ 10	15 $\pm$ 2
72 h	88 $\pm$ 11	92 $\pm$ 3	88 $\pm$ 8	11 $\pm$ 4

The percentage of specific immunofluorescent signal was compared with total amount of cells by counting of DAPI-positive cell nuclei (100%). Numbers represent percentages $\pm$ SD of four independent experiments performed in duplicate.

methanol (10 min), and membranes were treated as described for SGHPL-5 cells. In a next step, filters were costained with cytokeratin-7 and vimentin as described above. Subsequent to staining with secondary antibodies (goat antimouse Alexa Fluor 488 and goat antirabbit Alexa Fluor 568), cell nuclei were visualized by DAPI staining and embedded, and cytokeratin-7-positive cells were counted and digitally analyzed.

Statistics

Statistical analyses were performed using SPSS version 18 (SPSS Inc., Chicago, IL) using Student's paired *t* test. Gaussian

distribution and equality of variances were examined with Kolmogorov-Smirnov test and Levene's test, respectively. A *P* value <0.01 was considered statistically significant.

Results

Endostatin suppresses IGF-II-induced migration and invasion of SGHPL-5 cells

To investigate the role of endostatin in trophoblast motility, SGHPL-5 cells were analyzed in both uncoated and Matrigel-coated Transwell assays. Compared with untreated control (set to 100 $\pm$ 22% SD), 100 and 1000 ng/ml endostatin significantly suppressed basal migration rates of SGHPL-5 cells to 74% ($\pm$ 18% SD) and 69% ($\pm$ 20% SD), respectively (Fig. 1A). Similarly, basal invasion of the cell line was reduced in the presence of the inhibitor (Fig. 1A). These findings confirmed previously published data obtained by use of commercially available endostatin produced in *Pichia pastoris* (21). Of note, purified endostatin produced in 293FT cells was about 10-fold more potent as

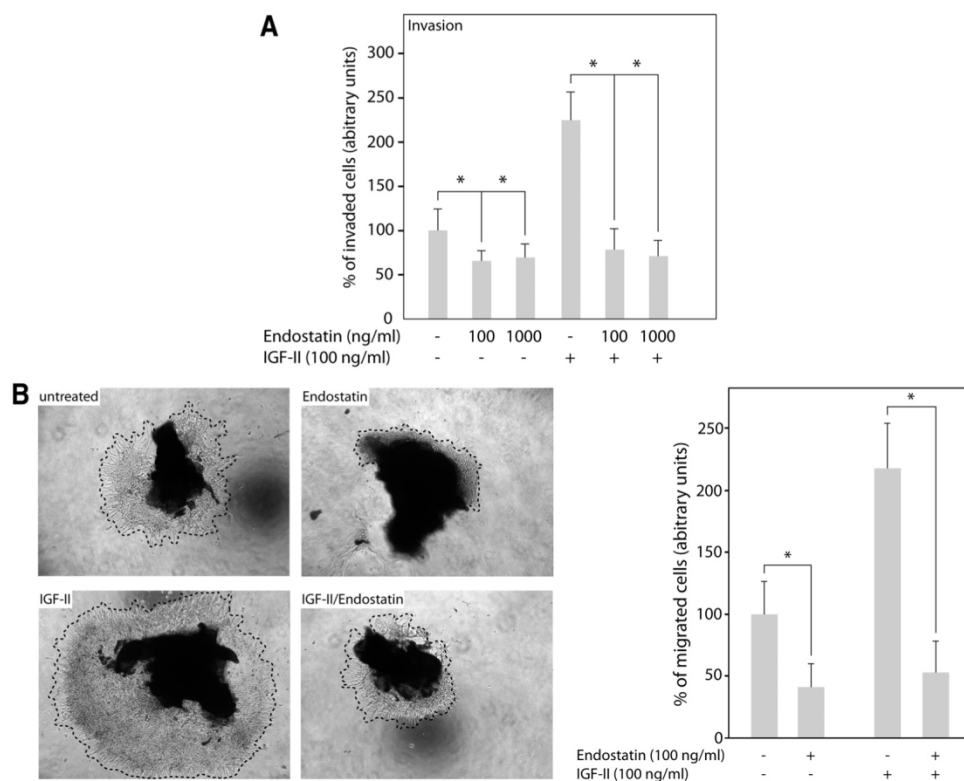


FIG. 2. Endostatin-mediated effects on basal and IGF-II-induced motility of primary trophoblasts. **A**, Primary trophoblasts were isolated and subsequently seeded onto fibronectin-coated Transwell inserts and treated as indicated in *Materials and Methods*. Invasion was evaluated as the percentage of invaded cells compared with untreated (100%) or IGF-II-induced control. *, *P* < 0.01 . Mean values $\pm$ SD of three independent experiments performed in triplicate are shown. **B**, First-trimester anchoring villi were cultivated on collagen-I and stimulated with or without IGF-II (100 ng/ml) in the absence or presence of purified endostatin (100 ng/ml). Representative examples photographed at $\times 10$ magnification are shown. Area of outgrowing trophoblasts is indicated by a dotted line. Quantification is presented in mean values (four explants per condition originating from four different placentae) $\pm$ SD.

the recombinant *P. pastoris*-produced protein (data not shown) (21), possibly reflecting differences in posttranslational modification and/or stability, as suggested by others (36). Furthermore, IGF-II was shown to be a main regulator of trophoblast invasion by activating critical signaling pathways, such as Akt and ERK (37, 38). Consequently, to test whether endostatin interferes with growth factor-mediated motility, we exposed IGF-II-treated SGHPL-5 cells to endostatin. Evaluation of Transwell assays revealed that 100 and 1000 ng/ml of the inhibitor specifically blocked IGF-II-induced cell migration and invasion, whereas lower concentrations were ineffective (Fig. 1A). Additional experiments determining cumulative cells num-

bers in the absence or presence of IGF-II revealed that endostatin did neither alter basal nor IGF-II-induced proliferation of SGHPL-5 cells (Fig. 1B).

Endostatin abolishes IGF-II-induced invasion and migration in primary trophoblast model systems

To test whether endostatin also interferes with motility of primary trophoblasts, we first established an invasion assay using isolated primary EVT according to a previously published protocol (28). Immunofluorescent analyses of isolated trophoblasts cultured on fibronectin revealed that the vast majority of cells gained features of EVT in a time-dependent manner (Table 1 and

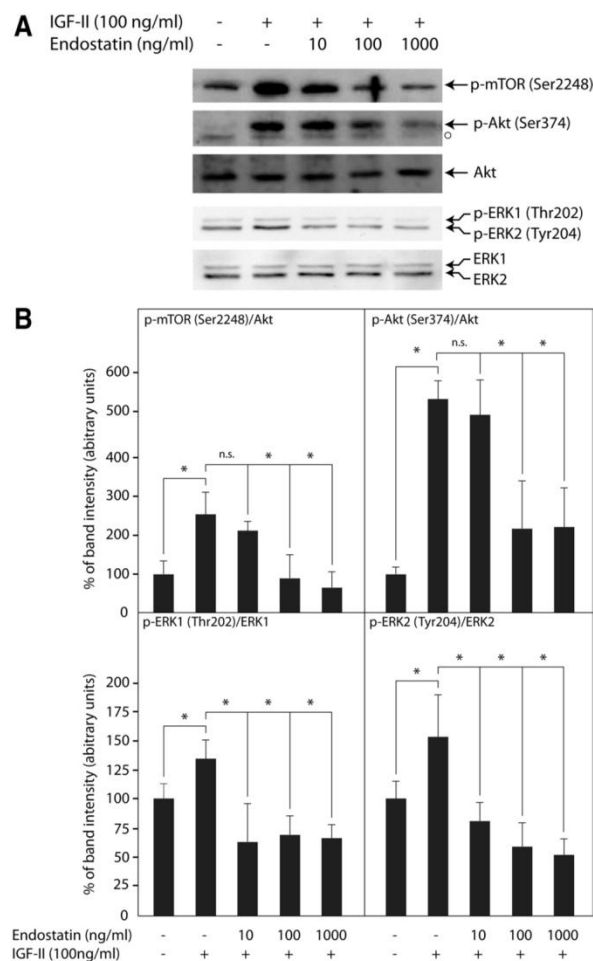


FIG. 3. Western blot analyses showing endostatin-dependent changes in phosphorylation of IGF-II-activated kinases. A, SGHPL-5 cells were seeded in 24-well plates and treated as described in *Materials and Methods*. A representative Western blot of three independent experiments is shown. B, Quantification of Western blot analyses representing the percentage of band intensity (mean value of three independent experiments $\pm$ SD) in relation to untreated control (100%) and/or IGF-II-treated cells (as indicated). *, $P < 0.01$; n.s., not significant. Nonspecific signals are depicted by open circles.

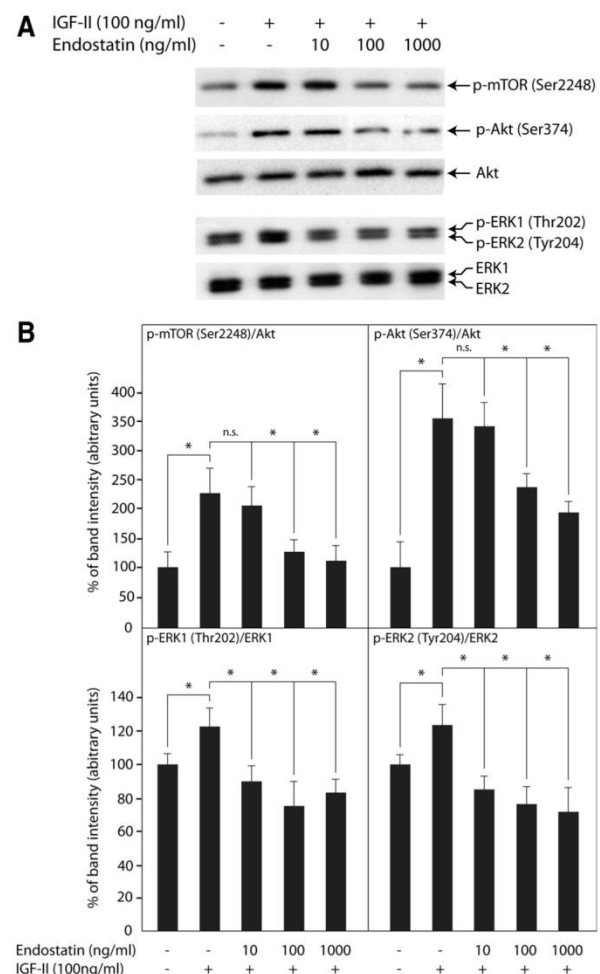


FIG. 4. Endostatin affects IGF-II-mediated downstream signaling in EVT. A, Primary trophoblasts were isolated, cultivated, and treated as described in *Materials and Methods*. A representative experiment of three independent experiments is shown. B, Bars (mean $\pm$ SD) represent percentages relative to untreated control (100%). Quantification was performed by analyzing three Western blots. Statistical analysis was performed as indicated by connecting lines. *, $P < 0.01$; n.s., not significant at $P \geq 0.05$.

Supplemental Fig. 1). In detail, the villous cytotrophoblast marker integrin $\alpha 6$ was noticed in 22% ($\pm 6\%$ SD) of cultivated trophoblasts at d 1 and decreased to 15% ($\pm 2\%$ SD) and 11% ($\pm 4\%$ SD) at d 2 and 3, respectively (Table 1). Moreover, the EVT-specific integrins $\alpha 1$ and $\alpha 5\beta 1$, a putative endostatin receptor, were found to be highly expressed at 72 h of cultivation ($92 \pm 3\%$ SD and $88 \pm 6\%$ SD, respectively), suggesting that the isolated trophoblasts differentiate toward an EVT phenotype *in vitro*.

Subsequently, isolated trophoblasts were subjected to fibronectin-coated Transwell chambers in the absence or presence of endostatin (100 and 1000 ng/ml). Counting of DAPI-stained nuclei of cytokeratin-7-positive invaded cells revealed that endostatin interferes with both basal and IGF-II-induced invasion of primary trophoblasts in a similar fashion compared with SGHPL-5 cells (Fig. 2A). Addition of 100 and 1000 ng/ml endostatin reduced basal invasion rates of primary trophoblasts to $60 \pm 12\%$ SD and $68 \pm 14\%$ SD, respectively, compared with untreated controls ($100 \pm 23\%$ SD). Moreover, IGF-II-triggered invasion ($229 \pm 33\%$ SD) was abolished in the presence of 100 and 1000 ng/ml endostatin. Because EVT did not attach to noncoated Transwell inserts (data not shown), primary trophoblast migration was studied by using first-trimester villous explant cultures seeded on collagen I. After 48 h cultivation, both basal and IGF-II-induced migration was significantly suppressed upon addition of 100 ng/ml endostatin (Fig. 2B).

Endostatin impairs IGF-II-dependent signaling in SGHPL-5 cells and primary trophoblasts

To further analyze the suppressive effects of endostatin on trophoblast invasion, IGF-II-induced activation of Akt (Ser473) and ERK (Thr202/Tyr204) was investigated in the absence or presence of the inhibitor. Western blot analyses revealed that IGF-II treatment increased phospho-Akt to $557 \pm 45\%$ SD and $368 \pm 112\%$ SD in SGHPL-5 cells (Fig. 3A) and primary trophoblasts (Fig. 4A), respectively. However, IGF-II-mediated phosphorylation of ERK1/2 was less pronounced in SGHPL-5 cells (Fig. 3A) and primary trophoblasts (Fig. 4A), suggesting that activation of that particular kinase might be of minor importance for IGF-II-dependent invasion. Furthermore, purified endostatin abolished IGF-II-mediated Akt phosphorylation in both SGHPL-5 cells and primary trophoblasts. Densitometric analyses showed a 2.5-fold endostatin-dependent (100 ng/ml) decrease of IGF-II-induced Akt phosphorylation in SGHPL-5 cells (Fig. 3B) and a 1.9-fold decrease in primary cell cultures (Fig. 4B). Concisely, IGF-II-induced phosphorylation of mTOR (Ser2248), a downstream target of Akt (see *Materials and Methods*), was also suppressed upon addition of

endostatin to SGHPL-5 cells (Fig. 3A) or to primary trophoblasts (Fig. 4A). Moreover, endostatin also down-regulated phosphorylation of ERK1/2 in SGHPL-5 cells (Fig. 3A) and primary trophoblasts (Fig. 4A).

The inhibitory effect of endostatin is critically dependent on Akt1 expression

To assess whether the inhibitory effect of endostatin on IGF-II-triggered Akt phosphorylation is isoform specific, the Akt1 gene was stably silenced in SGHPL-5 cells after murine stem cell virus-mediated transduction of a shRNAir-

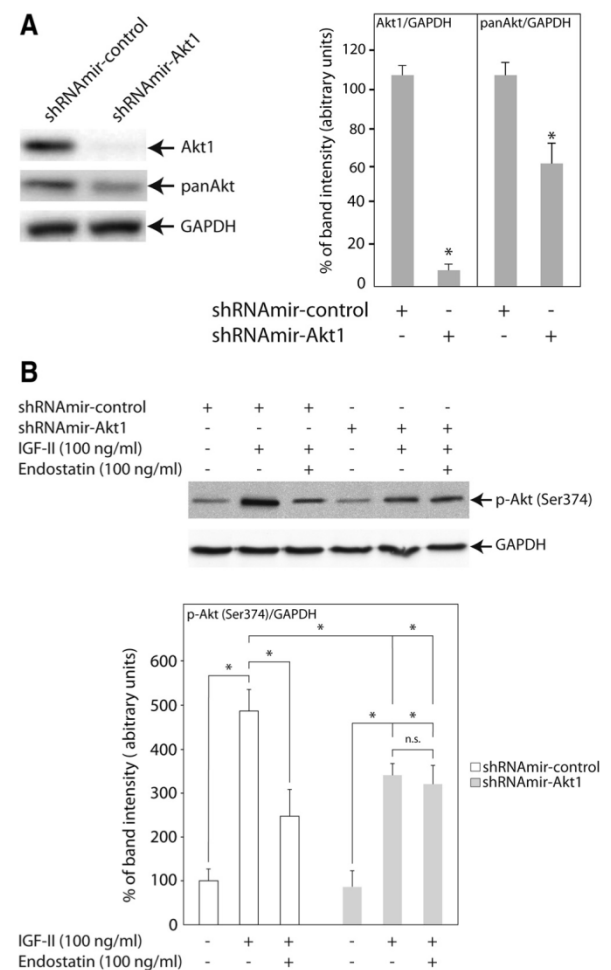


FIG. 5. Virus-mediated knockdown of Akt1 abolishes the inhibitory effect of endostatin in SGHPL-5 cells. **A**, Evaluation of total Akt1 and total Akt expression in SGHPL-5 cells clones stably expressing shRNAir-Akt1 or control. *, $P < 0.01$. **B**, Basal and IGF-II-induced Akt phosphorylation in SGHPL-5 cells stably expressing shRNAir-Akt1 or shRNAir-control was analyzed in the absence or presence of endostatin. SGHPL-5 cells constitutively expressing respective shRNAir (as indicated) were treated as described in *Materials and Methods*. Graph shows percentages of mean values $\pm$ SD of three independent experiments performed in triplicate (compared with respective controls; untreated control was set to 100%). *, $P < 0.01$; n.s., not significant.

Akt1 construct and puromycin selection as previously described (34). Western blot analyses of the cell pool expressing shRNA^{mir}-Akt1 revealed efficient knockdown of the kinase to $7 \pm 2\%$ SD compared with controls (non-targeting shRNA^{mir}-control, 100%) (Fig. 5A). However, 60% of total Akt was unaffected by the knockdown, most likely reflecting residual Akt2 and Akt3 expression (Fig. 5A). Importantly, proliferation and survival assays showed that Akt1 knockdown was not sufficient to alter cell growth or induce apoptosis (data not shown). Subsequently, shRNA^{mir}-Akt1- and shRNA^{mir}-control-expressing cells were evaluated by Western blot in the absence or presence of endostatin and IGF-II (Fig. 5B). As expected, IGF-II-dependent Akt phosphorylation was strongly inducible in shRNA^{mir}-control cells ($486 \pm 45.45\%$ SD compared with untreated control,

100%). On the other hand, Akt1 knockdown resulted in a decrease but not complete down-regulation of IGF-II-inducible total Akt phosphorylation ($338 \pm 26.82\%$ SD compared with untreated control, 100%), suggesting that the remaining Akt isoforms are also activated by IGF-II. Subsequent experiments revealed that whereas endostatin treatment (100 ng/ml) inhibited IGF-II-triggered Akt phosphorylation in shRNA^{mir}-control cells, the inhibitor was ineffective in Akt1-deficient cells. This suggests that the negative effects of endostatin on IGF-II-induced Akt phosphorylation could be mainly exerted through inhibition of Akt1.

Endostatin exerts a broad effect on IGF-II/Akt-related signaling events

To refine the impact of endostatin on IGF-II-mediated

Akt signaling, we sought to evaluate both upstream activators and downstream effectors of the particular kinase (see *Materials and Methods*). First, the phosphorylation status of FAK, an upstream activator of Akt and well-described mediator of trophoblast invasion (39, 40), at site Y397 was investigated. In agreement with the existing literature (41), IGF-II activated FAK in primary EVT (Fig. 6). Moreover, preincubation with endostatin markedly reduced IGF-II-dependent activation of FAK in a dose-dependent manner (100–1000 ng/ml) (Fig. 6A). To further analyze the impact of endostatin on mTOR signaling complexes, we tested IGF-II-induced phosphorylation of TORC1-mediated activation of S6K and induction of the TORC2-associated phosphorylation residue phospho-Ser2481 in the presence or absence of endostatin. Interestingly, endostatin (100–1000 ng/ml) (Fig. 6B) inhibited IGF-II-dependent phosphorylation of both proteins tested. In addition, the inhibitor suppressed IGF-II-mediated phosphorylation of Akt at Thr308 (Fig. 6B).

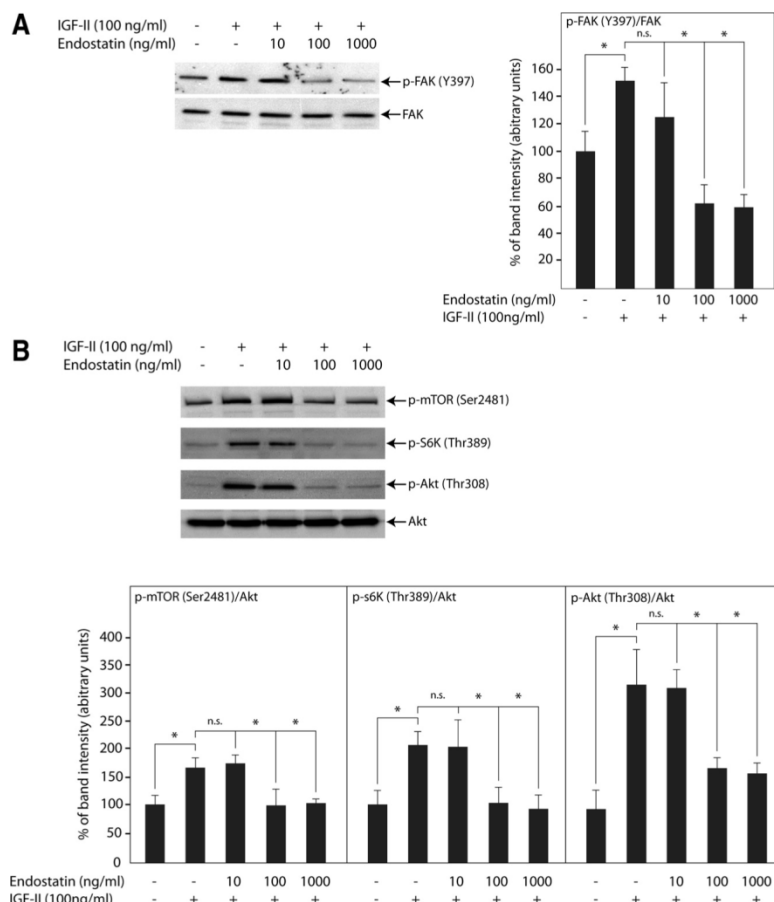


FIG. 6. Further evaluation of endostatin-affected signaling cascades in EVT. A, Phosphorylation of FAK phosphorylation (Y397) in IGF-II-stimulated primary EVT in the presence or absence of endostatin. One representative Western blot of three independent experiments is shown. Changes relative to untreated control or IGF-II-treated cells (as indicated) are shown in percentages ($\pm$ SD, $n = 3$). *, $P < 0.01$; n.s., not significant. B, Influence of endostatin treatment on IGF-II-dependent activation of mTOR (Thr2481), S6K (Thr389), and Akt (Thr308). Bars (mean $\pm$ SD) show changes of three independent experiments in percentage relative to control (100%).

Discussion

On the basis of our previous studies demonstrating that collagen XVIII and endostatin are abundantly expressed at the fetal maternal interface (20) and,

second, that trophoblast-derived proteases are able to cleave endostatin from its respective collagen XVIII precursor region (21), we aimed at exploring the potential role of endostatin in trophoblast invasion. Although endostatin has mostly been studied in the context of angiogenesis, it is reasonable to assume that the inhibitor may influence epithelial cell function as demonstrated by its interference with renal epithelial cell branching (14) and inhibition of epithelial repair mechanisms in the lung (42). Endostatin was further shown to bind several trophoblast-associated cell surface proteins (10, 11, 43–45) including heparan sulfates (43) and integrins (10). In particular, various studies suggest binding of endostatin to $\alpha 5 \beta 1$ integrin (10, 11, 43, 46), a well-described marker of invasive trophoblasts. Accordingly, we found the latter to be highly expressed in primary isolated trophoblasts (Table 1 and Supplemental Fig. 1), suggesting that endostatin could bind to trophoblasts via integrin $\alpha 5 \beta 1$. In addition, we confirmed that these cells gain features of EVT *in vivo*, demonstrated by high expression of integrin $\alpha 1$ /cytokeratin-7 and low levels of integrin $\alpha 6$ /vimentin. To carefully dissect the effect of endostatin on EVT, we first substantiated our previous results obtained in SGHPL-5 cells (21) by demonstrating that purified endostatin also inhibits basal motility of primary trophoblasts. Trophoblast invasion/migration has been suggested to depend on the activation of a proteolytic machinery involving matrix metalloproteinase (MMP)-2/9 and the urokinase plasminogen activator (uPA) system (25). Surprisingly, we previously noticed an increased expression of MMP-2 (20) and unchanged uPA/uPA receptor/plasminogen activator inhibitor-1/2 levels in the presence of endostatin (data not shown), indicating that endostatin does not negatively interfere with the proteolytic system in trophoblasts. In this context, an induction of MMP-2 secretion may indicate counteractive/protective mechanisms to overcome the inhibitory effect of endostatin as suggested previously (20). We next studied whether endostatin may influence growth factor-dependent motility and proliferation of both SGHPL-5 cells and primary trophoblasts. Interestingly, we found that endostatin interferes with IGF-II-induced trophoblast migration and invasion. IGF-II is pivotal for placental development in mice (47), and it was shown to regulate various crucial steps of EVT differentiation in human trophoblasts (48). For instance, this particular growth factor mediates proliferation of primary villous cytotrophoblasts via ERK, whereas IGF-II-mediated Akt activation does not affect cell cycle but rather survival (48). Additional studies using trophoblast cell lines or choriocarcinoma cells revealed that IGF-II-induced migration or invasion is mediated via ERK (38), ras homology (Rho) GTPases (49), and Akt (50) phos-

phorylation. In the present study, IGF-II-dependent activation of Akt and, to a lesser extent, of ERK was observed in both SGHPL-5 cells and primary EVT. These data imply that Akt could be one of the main signaling mediators of this particular growth factor in these trophoblast models. Moreover, Akt was shown to be a major regulator of trophoblast invasion (25). Therefore, we tested whether endostatin would interfere with IGF-II-induced Akt phosphorylation. Western blot analyses revealed that, indeed, endostatin suppressed IGF-II-induced Akt activation in SGHPL-5 cells and primary EVT. Moreover, we found that endostatin also interferes with activation of mTOR, a well-described mediator of trophoblast invasion (51, 52). In humans, mTOR exists in two protein complexes, TORC1 and TORC2 (53). The latter, lying upstream of Akt, is described to activate Akt at Ser473, whereas the TORC1 is a downstream target of Akt that upon phosphorylation leads to activation of various downstream effectors such as S6K (31). Here, we found that endostatin interferes with both mTOR complexes because the inhibitor suppressed activation of the TORC2-specific phosphorylation residue of mTOR at Ser2481 and Akt at Ser374 as well as phosphorylation of S6K (TORC1 mediated). Additional experiments revealed that endostatin

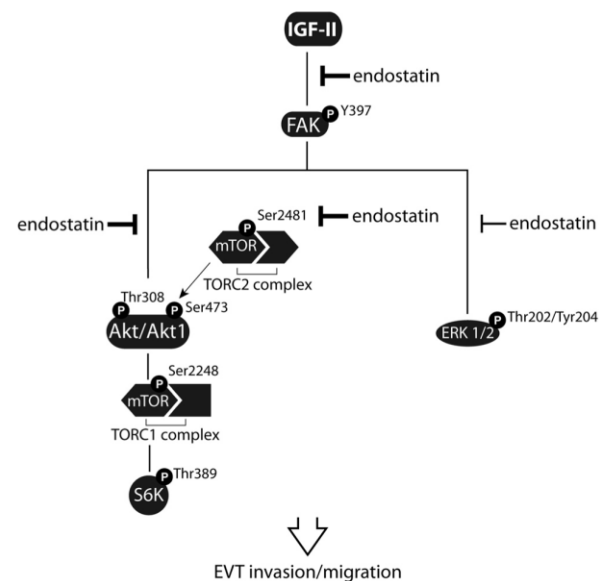


FIG. 7. Proposed model of endostatin-mediated suppression of trophoblast motility. Endostatin exerts broad effects on IGF-II-mediated signaling in human trophoblasts by affecting phosphorylation of FAK/Akt/mTOR/S6K and to a lesser extent ERK1/2. Of note, endostatin interferes with both mTOR complexes, TORC1 and TORC2, thereby affecting activation as well as downstream signaling of Akt. However, suppression of the Akt phosphorylated residue at Thr308 suggests additional endostatin targets upstream of Akt, such as FAK. Finally, its main suppressive role in the context of trophoblast invasion was found to be dependent on the expression of Akt1.

also affected the TORC2-independent Akt phosphorylation residue Thr308 (31), suggesting additional endostatin targets upstream of Akt. In this context, we demonstrated that endostatin also interferes with phosphorylation of FAK in primary trophoblasts. Interestingly, endostatin has already been described to interfere with phosphorylation of FAK via its receptor $\alpha 5 \beta 1$ integrin (11). Moreover, FAK is a critical mediator of trophoblast invasion (39, 40) and functionally linked to IGF-II signaling because it was shown to be phosphorylated upon activation of IGF-I receptor (54). However, due to the broad and complex signaling network controlling the activation of Akt, endostatin may interfere with additional upstream regulators of the respective kinase.

The Akt family comprises the three isoforms, Akt1, Akt2, and Akt3, of which Akt1 is believed to be the most ubiquitously expressed member in mammalian cells. Because Akt1 was shown to be a positive regulator of non-malignant cell migration (55), we sought to establish a shRNA-Akt1 knockdown in SGHPL-5 cells. First analyses revealed that knockdown of the particular isoform was not sufficient to induce apoptosis (data not shown), confirming a previous study reporting that knockdown of all three isoforms of the kinase is necessary to affect cell proliferation and survival (56). Moreover, we found that Akt1 accounts for approximately 45% of total Akt expression in trophoblasts and that its knockdown negatively affects IGF-II-dependent Akt phosphorylation in SGHPL-5 cells. In detail, IGF-II-dependent activation of Akt was about 5-fold increased in the shRNAmir-control cells, whereas the shRNAmir-Akt1 knockdown cells showed only a 3.3-fold response to IGF-II in comparison with the respective untreated controls. Interestingly, the reduced growth factor-triggered Akt phosphorylation in the Akt1-deficient cells was not affected by the addition of endostatin. This implicates that expression of Akt1 is crucial for endostatin-mediated blockage of IGF-II-dependent Akt phosphorylation in trophoblasts. In summary, we demonstrate that Akt is a major target of endostatin in human trophoblasts. However, a role of ERK cannot be excluded because a modest but significant impact of endostatin on ERK phosphorylation was noticed. This confirms previous studies demonstrating endostatin-dependent suppression of ERK activation in endothelial cells (11, 57). Additional experiments determining cumulative cell numbers revealed that endostatin does not interfere with trophoblast proliferation. The finding that endostatin does not suppress basal and growth factor-induced proliferation is largely in accordance with the existing literature (11, 13), although some studies describe an inhibitory effect on endothelial cell proliferation (58). More-

over, IGF-II was demonstrated to regulate trophoblast proliferation independently of Akt (48).

Taken together, we propose that endostatin interferes with IGF-II-activated signaling in human trophoblasts by inhibiting phosphorylation of FAK/Akt/mTOR/s6K and ERK. In addition, our data suggest that endostatin-mediated inhibition of trophoblast invasion is critically dependent on Akt1 expression (Fig. 7).

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6.2.2. Declaration of the authorship responsibility and contribution to published manuscript

In the publication; *Endostatin suppresses IGF-II-mediated signaling and invasion of human extravillous trophoblasts*. I, as a coauthor contributed to the following publication by providing the material, mainly isolation and cultivation of primary EVT_s. In the following publication the invasion assay with primary isolated EVT_s on fibronectin coated invasion assay transwells were used, which I responsible to establish for the purpose of previous publication *Transcription factor AP-2 α promotes EGF-dependent invasion of human trophoblasts*. The technique was described in above mentioned publication.

7. UNPUBLISHED DATA

7.1 Descriptive and functional analysis of ADAM-12 during extravillous trophoblasts invasion

7.1.1 Expression pattern of ADAM-12 in the first trimester placental tissue and SGHPL-5 trophoblast cell line

According to previously published Gene Chip Data ADAM-12 was among other genes strongly upregulated and specifically expressed in EVT_s [56]. Moreover, the role of ADAM-12 was investigated in different cancer types [91, 123], and in pregnancy disorders [125-127]. The importance of ADAM-12 in invasion of first trimester human trophoblasts remained still unknown. Therefore it was important to investigate the role of ADAM-12 in early placenta development.

First, I evaluated the expression pattern of ADAM-12 in first trimester placenta, placental fibroblasts, differentiating EVT_s and in trophoblast cell line SGHPL-5 with real-time quantitative PCR (**Fig. 11**). Quantitative Real-Time PCR analyses revealed that both isoform ADAM-12L and -S are strongly expressed in EVT_s. The expression of both ADAM-12 splice variants increase in time during invasive differentiation of EVT_s in vitro on fibronectin. The analysis revealed also that placental fibroblasts are weakly positive for ADAM-12L, whereas negative for ADAM-12S. Both ADAMs -L and -S are absent in SGHPL-5 cell line.

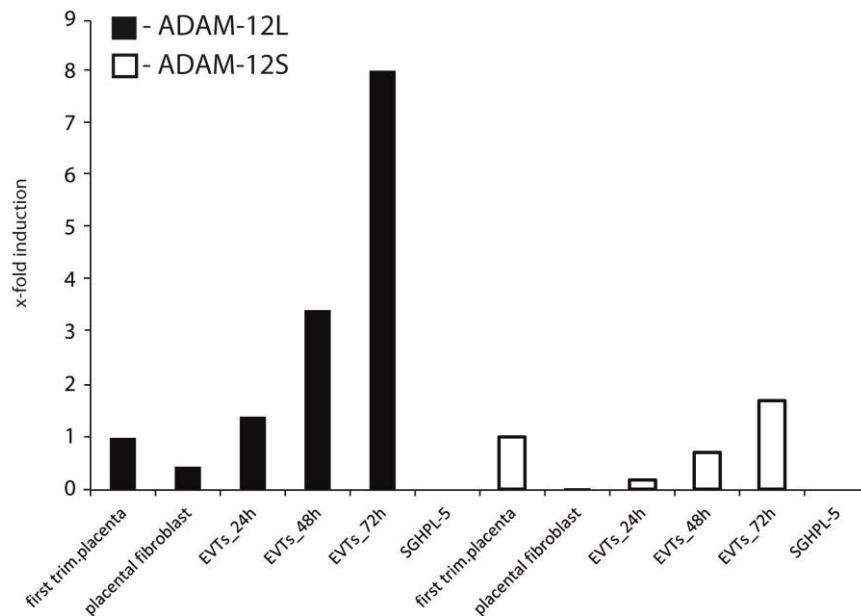


Figure 11. qRT-PCR of ADAM-12L and ADAM-12 in the first trimester placenta tissue

The placental fibroblasts, differentiating EVT and trophoblast cell line SGHPL-5. EVTs were seeded in 24 wells plate, coated with fibronectin and harvested after 24h, 48h and 72h. The cells and total tissue were lysed and RNA was extracted by direct lysis in the culture dishes using PegGOLDTriFast Reagent. Subsequently, total mRNA was rewrite to cDNA and real-time PCR was performed using TagMan gene expression assays as described earlier [128].

Furthermore, detailed analyses of the expression of ADAM-12 were also performed by immunofluorescence (IF) staining of the paraffin-embedded, first trimester placenta tissue (**Fig.12**). The IF analyses show that ADAM-12 is highly expressed in invasive, differentiated EVTs and very weakly in proliferative, cell column CTBs. I observed that the expression of ADAM-12 increases drastically during EVTs differentiation in vivo (**Fig.11**) All EVTs strongly express integrin alpha 5 [36], as well in line with existing literature, weaker expression of integrin alpha 5 was observed in the cell column CTBs. Ki67 staining, indicating the cells in proliferative stage indicate that only cell column CTBs and cytotrophoblasts proliferate actively, but in contrast ADAM-12 expression in these cells remain on very low level. Furthermore, serial sections prepared from paraffin-embedded first trimester decidua tissue were stained, to identify differentiated, invasive EVTs. IF analyses indicate very high expression level of ADAM-12 in EVTs invading the decidua (**Fig.13**).The IF of the term trimester placenta confirmed that ADAM-12 is also expressed in the term placenta (**Fig. 13B**).

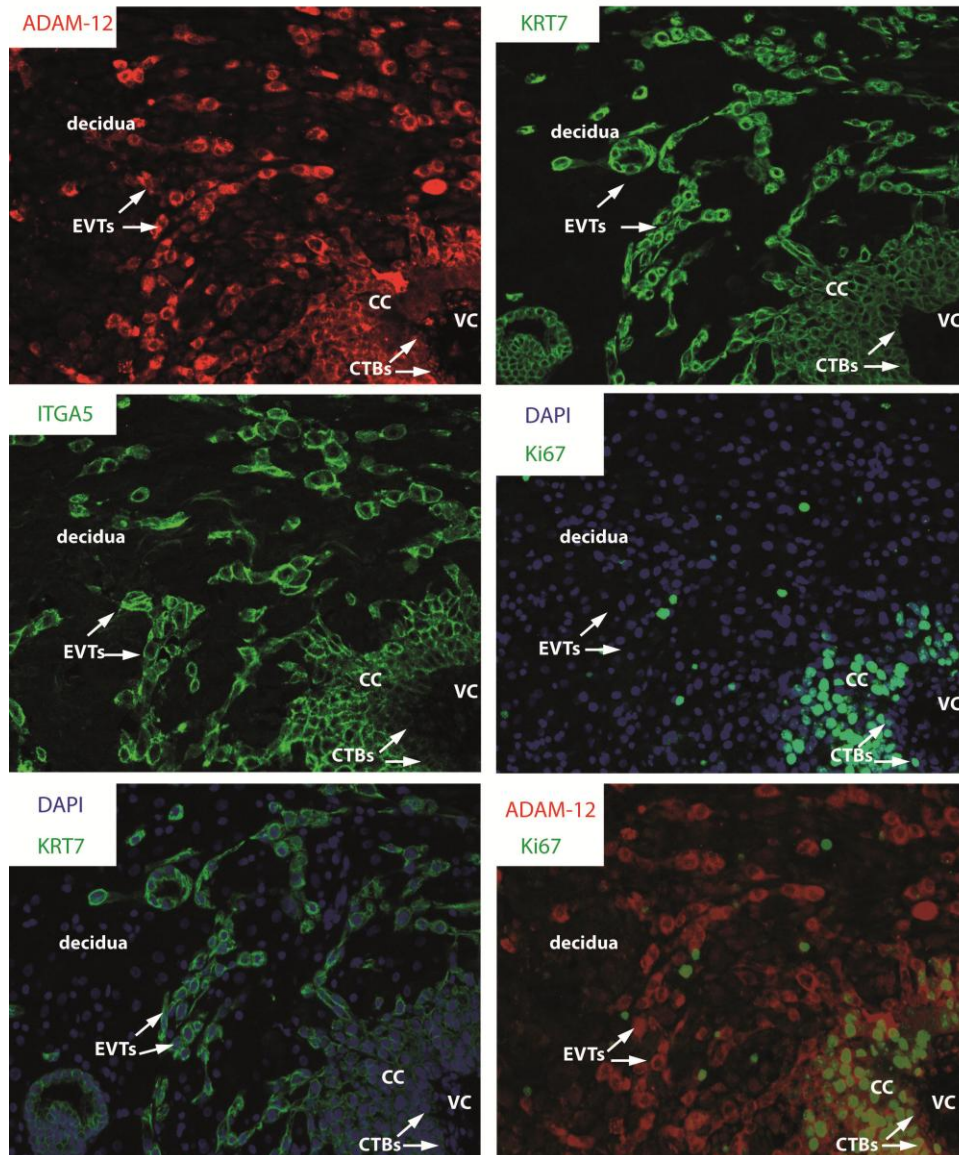


Figure 12. Expression of ADAM-12 in the first trimester placenta (12th week of gestation)

IF staining of paraffin-embedded placental tissue was performed as described previously [69] using primary antibodies against ADAM-12, ITGA5, Ki67, KRT7 and nuclei were counterstained with DAPI, the IF stainings were digitally photographed under the fluorescence microscope. CC, cell column; CTBs, cytotrophoblasts; EVT, extravillous trophoblasts; KRT7, cytokeratin-7; VC, villous core.

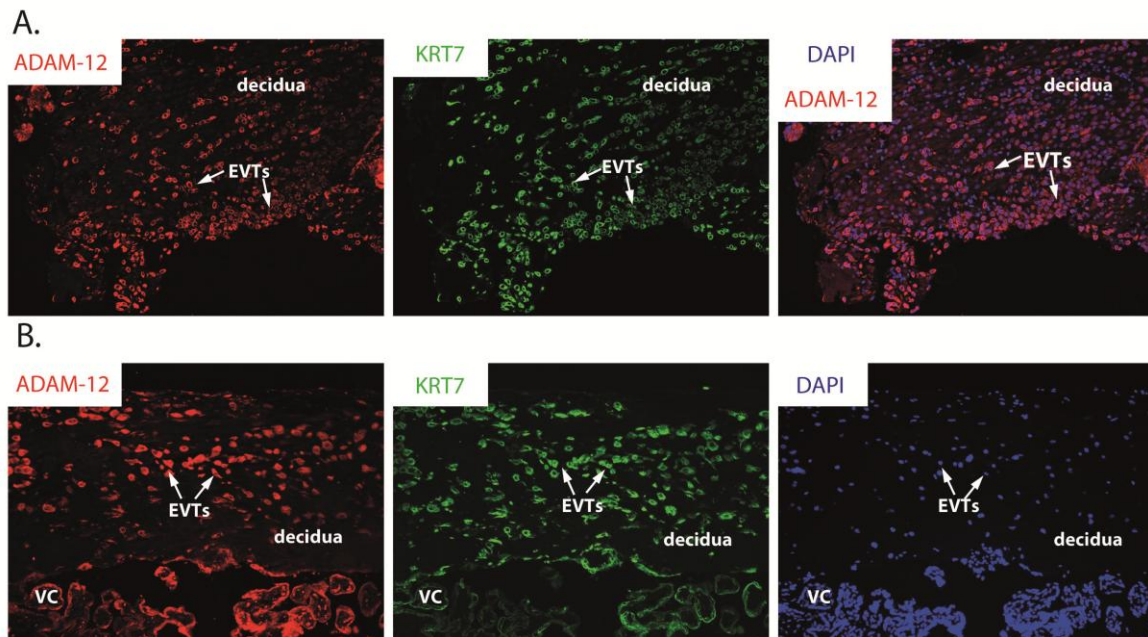


Figure 13. Expression of ADAM-12, in first trimester decidua and term trimester placenta

A, First trimester placenta (12th week of gestation). B, Term trimester placenta (38th week of gestation). IF staining of paraffin-embedded tissue was performed as described [69]. The primary antibodies against ADAM-12, KRT7 were used; nuclei were visualized with DAPI. The IF stainings were digitally photographed under fluorescent microscope. EVT, extravillous trophoblasts; VC, villous core.

Additionally, the expression of ADAM-12L and –S was analyzed in outgrowing placental explants. Dissected placental explants pieces were cultivated for 72h on matrigel, prior to fixation. Serial sections of first trimester placental explants were stained with antibodies against ADAM-12L/S and KRT 7. IF analysis of the outgrowing explants on matrigel indicated that in the proliferative, CC CTBs ADAM-12 is weakly expressed, however its expression is strongly induced in differentiated, distal EVTs, as shown (**Fig.14**).

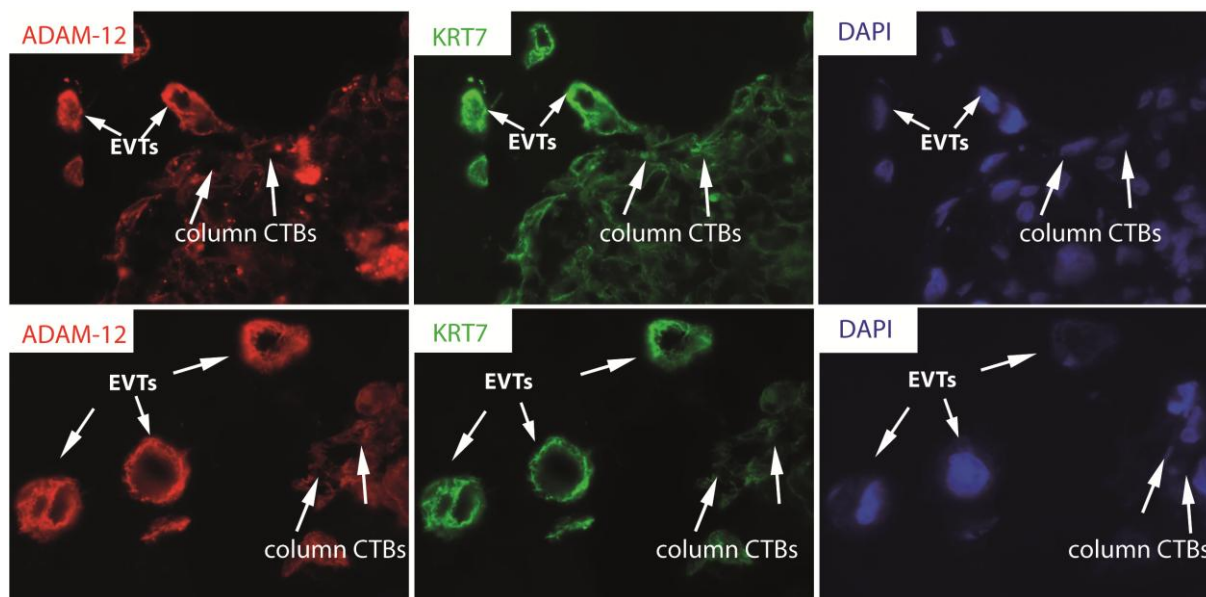


Figure 14. IF of first trimester explants cultivated for 72h on matrigel

Placental explants were cultivated for 72h on matrigel, then fixed in 2% paraformaldehyde and incubated with 30% sucrose solution for 15 min, next frozen in the liquid nitrogen and serial sections were cut. IF staining was performed as earlier described [69]. Primary antibodies against ADAM-12, KRT7 were used. Nuclei were visualized with DAPI. The IF staining was digitally photographed under fluorescent microscope.

7.1.2 Induction of ADAM-12 during differentiation of EVTs in vitro

Similar to EVTs in vivo isolated trophoblasts in vitro, when cultivated on ECM undergo differentiation. qRT-PCR and IF staining indicated that expression of ADAM-12 in vivo increases during EVTs differentiation as shown (**Fig.11**). To further investigate the upregulation of ADAM-12 in differentiating EVTs in vitro we performed WB analyses (Fig. 12) qRT-PCR and WB analyses revealed that ADAM-12L and -S increase considerably during EVTs differentiation in vitro on fibronectin (**Fig.15**). According to qRT-PCR, expression of ADAM-12L after 48h increased 7,5 fold and after another 24h 20 fold in comparison to the control. ADAM-12S increased 2 fold after 48h and after 72h was 10 fold higher compared to control. In contrast the expression of ADAM-12 in SGHPL-5 remains undetectable as indicated by WB analysis and qRT-PCR. As a control for differentiation of EVTs in vitro, well-known described marker for EVTs differentiation was analyzed [77]. WB of ITGA5 showed constant increase of the protein during differentiation in vitro (**Fig.15**).

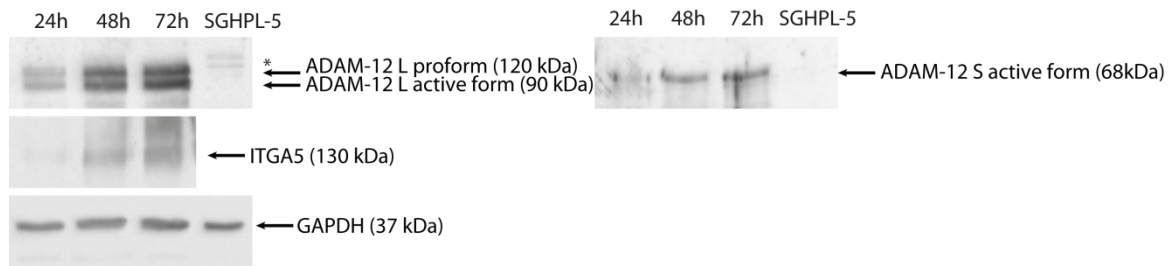


Figure 15. Induction of ADAM-12 during primary EVTs differentiation

EVTs differentiation in vitro on fibronectin. Western blot were performed as described [69], using commercially available antibody against ADAM-12L(1:500), ITGA5 (1:2000). The rb116 antibody (ADAM-12S) (1:500) was kindly provided by our collaborators (Fröhlich C., University of Copenhagen). Expression of ADAM-12L was analyzed in cell lysates of primary EVTs and SGHPL-5 cells and ADAM-12S in cell supernatants of primary EVTs and SGHPL-5, respectively. GAPDH was shown as a loading control. Arrows indicate ADAM-12L proform (120kDa), ADAM-12L active form (90kDa), ADAM-12S active form (68kDa), ITGA5 (130kDa) and GAPDH (37kDa). * - indicates unspecific band. Representative pictures of three independent experiments are shown.

7.1.3 siRNA-mediated ADAM-12L/S knock-down in primary EVTs and first trimester placental explants

To examine the function of ADAM-12 in invasion of human trophoblasts loss of function studies were performed. Knock-down of ADAM-12L/S was generated by using small interfering RNA (siRNA) oligonucleotides in primary EVTs and first trimester placental explants. The obtained knock-down resulted in decrease of approximately 80% mRNA according to qRT-PCR analysis (**Fig.16B**). The knock-down of ADAM-12 in the primary cells was evaluated also with Western Blot analysis showing similar decrease in protein expression of ADAM-12(**Fig.16A**).

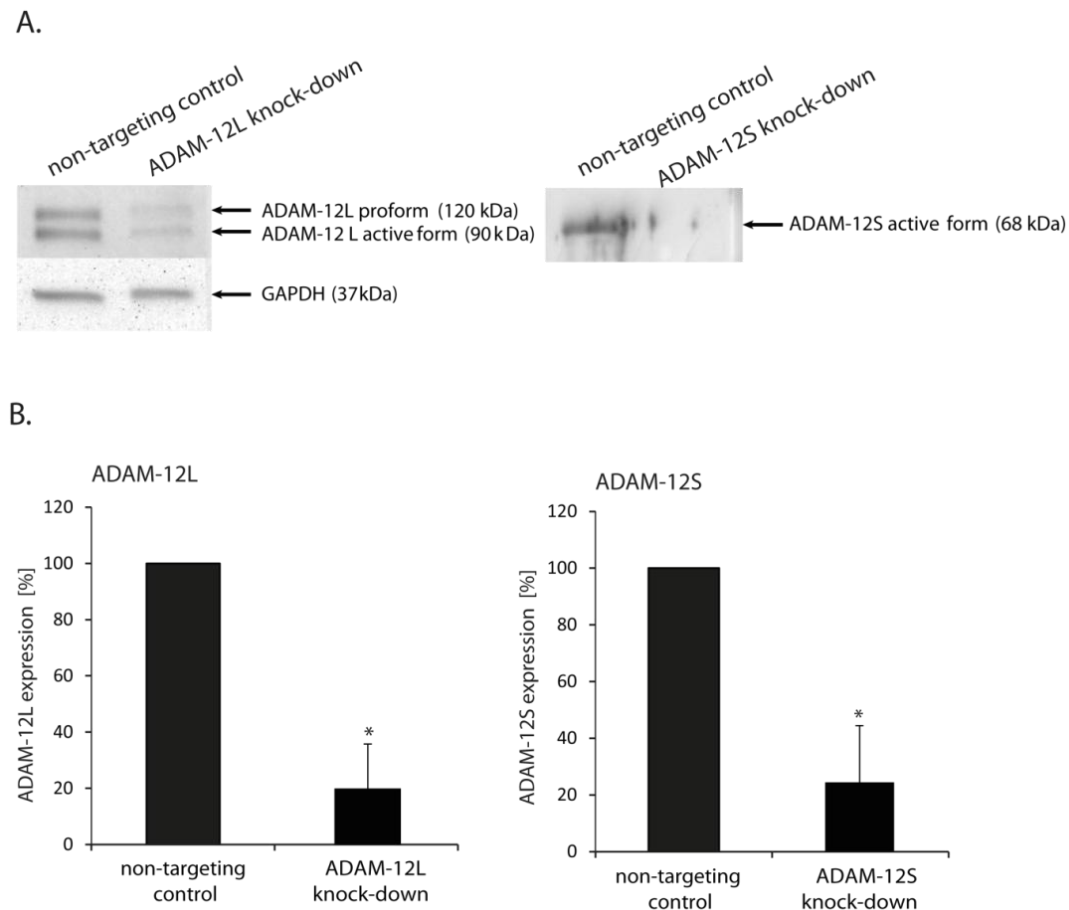


Figure 16. siRNA mediated knock-down of ADAM-12 in primary EVTs

A. Western blot analyses showing the degree of the gene silencing using siRNA specific to ADAM-12L/S in primary EVTs. Arrows indicate ADAM-12L proform (120kDa), ADAM-12L active form (90kDa), ADAM-12S active form (68kDa), and GAPDH (37kDa). GAPDH was used as a loading control. B. qRT-PCR of the ADAM-12L/S knock-down efficiency in primary EVTs, 48h after transfection with siRNA oligos. Representative WB of three independent experiments was shown. The non-targeting control was set to 100%. Bar graphs show mean values $\pm$ SD of 3 independent experiments. *, $P < 0.05$ compared with controls, respectively.

The qRT-PCR analysis from three independent experiment indicated that ADAM-12L level was decreased to 18.5% in ADAM-12 knock-down cells in comparison to non-targeting control and ADAM-12S was decreased to 20% in comparison to non-targeting control set to 100%.

7.1.4 Knock-down of ADAM-12 impairs trophoblasts motility

To analyze the functional role of ADAM-12L and ADAM-12S in trophoblast invasion, motility and adhesion assays were performed (Fig.17).

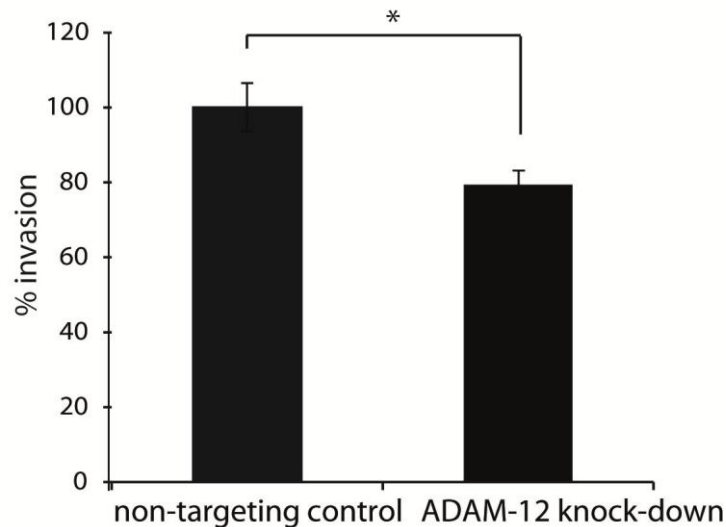


Figure 17. Invasion assay of the gene silenced primary EVT_s on fibronectin coated transwell invasion inserts

Invasion assay on fibronectin of ADAM-12 silenced primary EVT_s. Primary EVT_s were treated with siRNA oligos after isolation and seeded onto transwell invasion inserts, coated with 25 μ l of fibronectin (1mg/ml). Invasion inserts were placed in 24 wells plate containing 500 μ l culture medium, containing 10% serum. 1×10^5 primary EVT_s were seeded in each invasion chamber in 200 μ l medium containing 0,1 % serum and 0,1 μ M of siRNA oligos. After 48h cells were fixed and stained as described earlier in [69] The non-targeting control was set at 100%. Bar graphs show mean values $\pm$ SD of three independent experiments. *, $P < 0.05$ compared with controls, respectively.

Primary EVT_s were used for transfection immediately after isolation and seeded on fibronectin coated transwell invasion chambers. The result of three independent experiments performed from different ADAM-12 gene silenced cell pools revealed 20% decrease in invasion of ADAM-12 knock-down cells in comparison with non-targeting control (**Fig.17**). The reason of such small decrease in invasion after ADAM-12 deletion could be that ADAM-12 is one of many proteolytic enzymes controlling the invasion process. The other unaffected MMPs might compensate the lack of ADAM-12 and facilitate the invasion process. Besides MMPs there are many other factors, such as growth factors and integrins which were still present in the cells and may compensate the absence of ADAM-12.

Moreover, migration of primary EVT_s was analyzed with utilizing first trimester primary explants. The model system seems to be more adequate to perform the studies with ADAM-12 because it allows observation of the invasive behavior of EVT_s and measurement of the migration distance of EVT_s after gene silencing. The structure of the villi and anchoring site remain intact. It is possible to observe formation of CC and

subsequently formation of cell sprouts and invasion of single cells away from the CC.

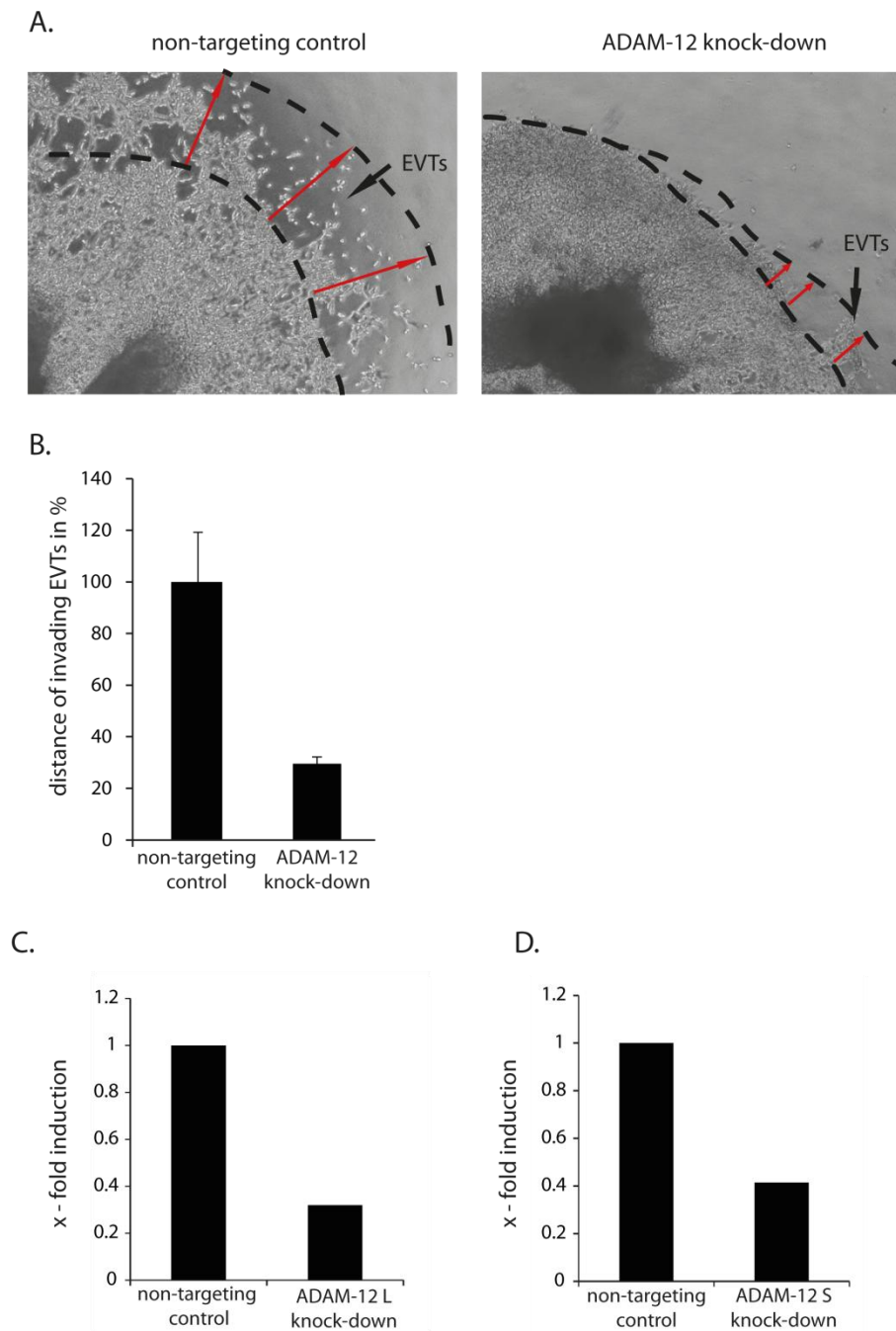


Figure 18. siRNA mediated ADAM-12 silencing in first trimester placental explants

A, The extend of EVTs migration upon ADAM-12 silencing in placental first trimester explants cultivated on collagen for 48h. Placental explants were seeded on collagen drops earlier moisturized with culture medium, left for 4h in the incubator to attach. Next, 400 μ l of medium, containing 10% serum with 0,1 μ M siRNA oligos was added. C, D, Evaluation of the knock-down efficiency of the placental first trimester explants. After 72h of transfection, explants were treated with RNAiLeter for 15 min; next explants were removed with the forceps. EVTs remained in the collagen were lysed with pegGOLD TriFast reagent, homogenized with Precellys homogenizer, 20sek, 5000 RPM. Next, RNA was isolated according to standard protocol [129] and qRT-PCR was performed as described [129]. Non-targeting control was set to 1.

The evaluation of the knock-down efficiency in placental outgrowth was 70% in ADAM-12L isoform and 60% in ADAM-12S, respectively (**Fig.18B**). As shown in (**Fig. 18A**), the explants treated with siRNA oligos for 48h show significant difference when compared with control explants, transfected with non-targeting control. The distance of invading EVTs, detaching from the CC, after treatment with siRNA oligos were 70% shorter in comparison to the migration distance of EVTs from non-targeting control (**Fig. 18B**). This indicated to us that ADAM-12 knock-down limits trophoblasts motility.

In addition, proliferative capacity of primary trophoblasts in the presence of siRNA targeting ADAM-12 or non-targeting siRNA was evaluated. The proliferation was measured with using BrdU labeling kit (data not shown) and anti Ki67 antibody in separate experiments. Ki67 antigen is cell cycle related nuclear protein, expressed by proliferating cells in all phases of the active cell cycle (G1, S, G2 and M phase). It is absent in resting (G0) cells. The analysis revealed no significant difference between proliferation of control cells and ADAM-12 knock-down cells (**Fig.19**).

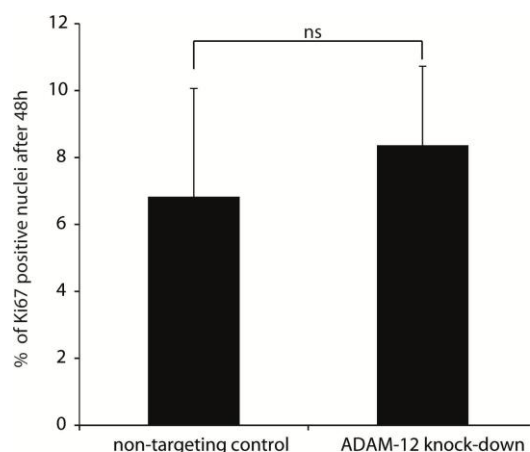


Figure 19. Proliferation measured with anti Ki67 antibody in EVTs, 48h after transfection with siRNA oligonucleotides

1.25×10^5 primary EVTs was transfected with ADAM-12 targeting siRNA oligos for 48h. The cells were fixed with 4% paraformaldehyde and stained according to IF protocol described earlier [69]. The non-targeting control was set to 100%. Bar graphs show mean values $\pm$ SD of three independent experiments. ns, not significant at $P \geq 0.05$, compared to control.

7.1.5 Gene silencing of ADAM-12 does not influence the cell adhesion

It is known that ADAM-12 interacts via disintegrin and cysteine-rich domains with different integrins and syndecans and therefore regulates cells adhesion and motility [99,

101, 130]. Therefore, we performed adhesion assay utilizing ADAM-12 knock-down cells and compared to non-targeting siRNA-treated trophoblasts. Adhesion of primary EVT_s was measured after 30 min and after 3h. EVT_s were fixed with 4% paraformaldehyde and stained with an antibody recruiting KRT7 positive cells. The analysis revealed no significant difference between control cells and ADAM-12 knock-down cells both after 30 min and after 3h of incubation.

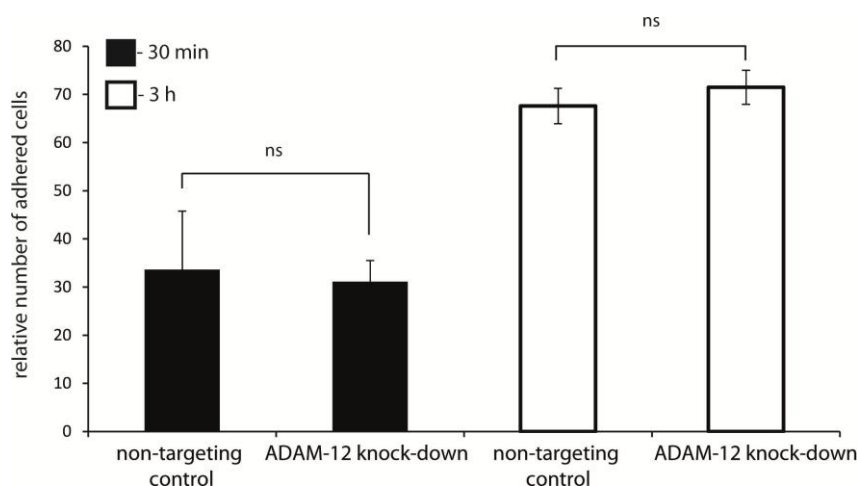


Figure 20. Adhesion assay of primary isolated EVT_s on fibronectin

Primary EVT_s, 48 after transfection with siRNA oligos targeting ADAM-12 and non-targeting control, were treated with trypsin and seeded on plastic dishes coated with fibronectin (0,02 mg/ml). Cells were fixed after 30 min and after 3h and stained using an antibody against KRT7. KRT7 positive trophoblasts were counted after indicated periods of cultivation. Non-targeting control was set to 100%. Bar graphs show mean values $\pm$ SD from two independent experiments ns, not significant at $P \geq 0,05$, compared to control.

7.1.6 Proteolytic function of ADAM-12S

Soluble isoform ADAM-12 S was shown to possess cleavage properties. It was described that ADAM-12 S degrades IGFBP-3 and -5, via its cysteine rich domain [97, 131, 132]. To analyze cleavage properties of ADAM-12S in vitro, an IGFBP-3 cleavage assay was performed using serum free supernatants, collected from ADAM-12 knock-down cells and control cells respectively. Cleavage assay of IGFBP-3 by ADAM-12S was evaluated by WB analysis using an antibody against IGFBP-3. As shown in **Fig.21A**, ADAM-12S degraded rhuIGFBP-3, resulting in several cleavage products. In the supernatants of the knock-down EVT the cleavage products were decreased approximately 40% and level of undigested rhuIGFBP-3 was higher when compared to control supernatant (**Fig.21B**).

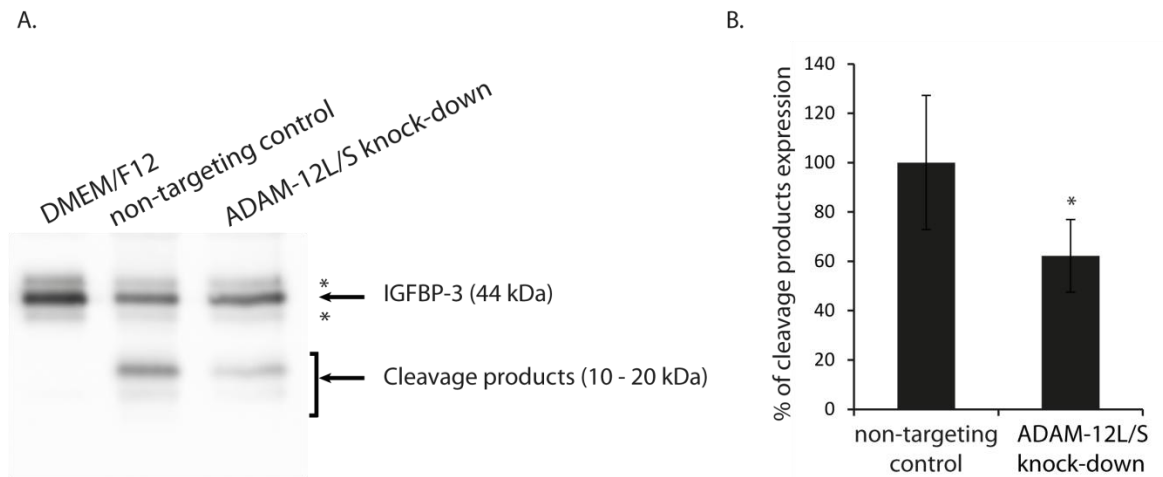


Figure 21. Cleavage of rhuIGFBP3 by secreted ADAM-12S

0.5×10^6 primary EVT_s was transfected with siRNA oligos targeting ADAM-12. After 24h medium was changed for serum free. The supernatants were collected 48h after medium change, concentrated and incubated with recombinant human IGFBP-3 (rhIGFBP-3) for 16h at 37 °C. The reaction was stopped with 1xSLAB buffer. The cleavage of IGFBP3 by ADAM-12S was evaluated by WB analysis. A, Representative WB analysis of the cleavage assay. As primary antibody IGFBP-3 antibody 1:500 (R&D Systems). Protein loading was controlled by Ponceau S staining. B, Densitometrical quantification indicating expression of IGFBP-3 cleavage products in supernatant from ADAM-12 knock-down EVT_s and non-targeting control EVT_s. Bar graphs show mean values $\pm$ SD from three independent experiments, *, $P < 0.05$ compared with control. Quantification of blots were done using ImageJ program. The non-targeting control was set to 100%

Additionally the effect of IGFBP-3 on EVT_s invasion was tested. Two different concentrations of rhuIGFBP-3 (0,5 μ g/ml and 2,5 μ g/ml) were added to the upper and lower chamber and left for 48h. Afterwards invaded trophoblasts were stained with cytokeratin7 and DAPI; cytokeratin 7 positive cells were photographed and counted. The addition of 2,5 μ g/ml of IGFBP-3 to the invading cells resulted in a significant decrease of invasiveness (about 30%) in comparison to unstimulated, control cells (**Fig. 22**).

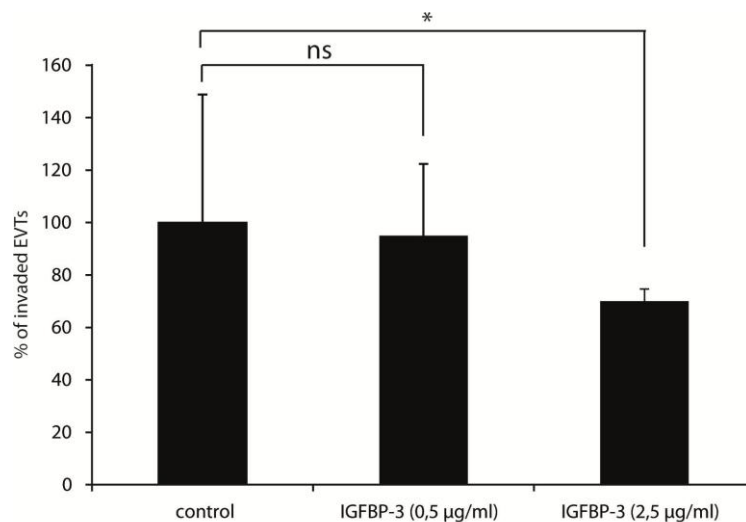


Figure 22. Invasion of primary EVT's after administration of rhuIGFBP-3

rhuIGFBP-3 decreases invasion of primary EVT's. Invasion assay was performed on fibronectin coated transwell inserts, for 48h. Two different concentrations of human recombinant IGFBP-3 were added to the lower and upper chamber of invasion assay. Afterwards, the assay was terminated, cells were fixed, stained and counted as described earlier [69]. The control was set to 100%. Bar graphs show mean values $\pm$ SD, ns, not significant at $P \geq 0,05$, compared to control, *, $P < 0.05$ compared with control, respectively.

7.1.7 Overexpression of both splice variants of ADAM-12 enhances motility of the trophoblastic cell line SGHPL-5

Finally, the invasive capacity of SGHPL-5 cells overexpressing either ADAM-12L or ADAM-12S was analyzed. We used ADAM-12L and ADAM-12S constructs kindly provided by our collaborators (K.Fröhlich, University of Copenhagen). Both ADAM-12L and -S constructs have catalytic mutation E351Q. In addition, ADAM-12L construct deltacyt is lacking the cytoplasmatic tail, in order to facilitate efficient translocation of the protein from the membrane to the cell surface. 3 independent experiments have shown that overexpression of ADAM-12 results in increase of the cell motility 60% in case of ADAM-12L and 40% in case of ADAM-12S respectively.

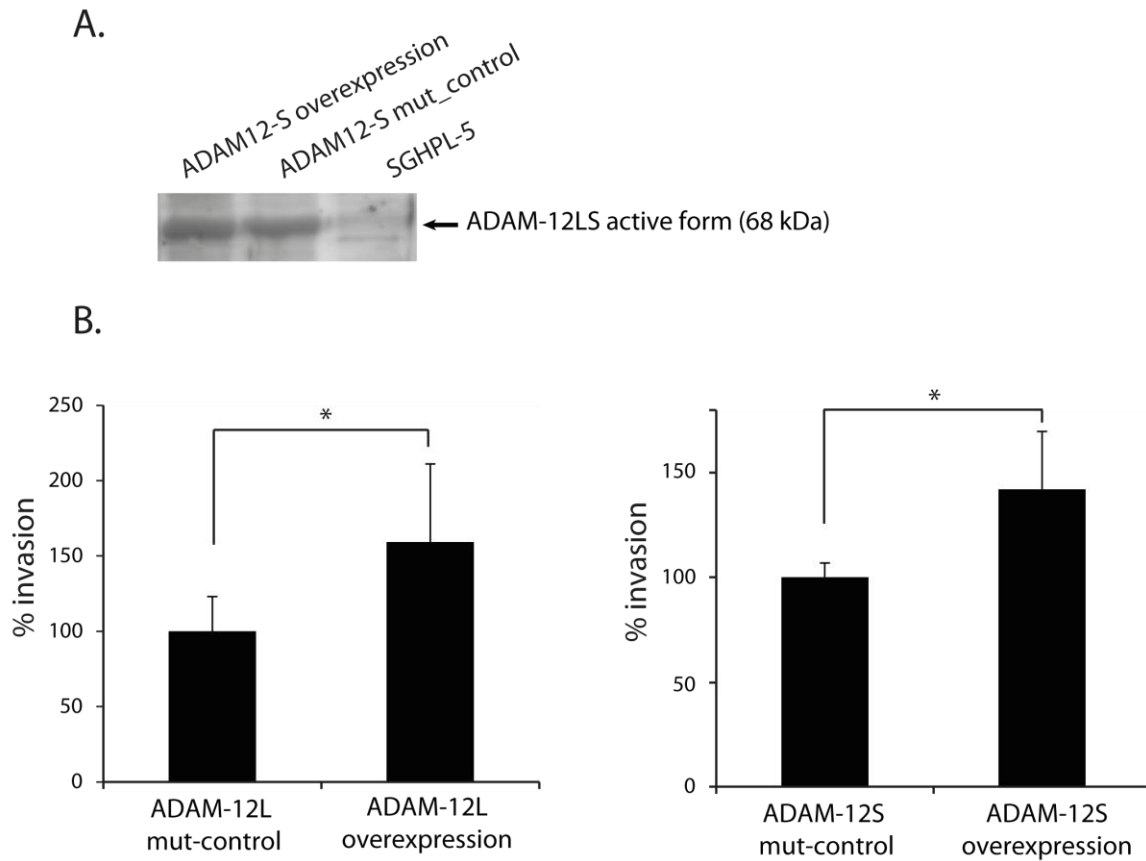


Figure 23. Transient overexpression of ADAM-12 in SGHPL-5 cell line

Overexpression of ADAM-12 affects SGHPL-5 cells invasion. A, Western Blot indicates overexpression of ADAM-12S in SGHPL-5 cells. The SGHPL-5 cells were transiently transfected with constructs containing ADAM-12 kindly provided by our collaborators (Fröhlich C., University of Copenhagen). Supernatants were collected 48h after transfection and concentrated, equal amounts of supernatant were used for WB analysis. Representative WB of ADAM-12S overexpression was shown. Transfection rate in ADAM-12L overexpression was evaluated by analyzing green fluorescent protein (GFP) positive cells (~20%). B, Invasion assay on matrigel coated transwell inserts of ADAM-12L and -S overexpressing SGHPL-5 cells and control cells. Briefly, 48h after nucleofection, cells were trypsinized counted and 2.5×10^4 cells were seeded onto invasion chambers coated with matrigel. After 24h cells were fixed with methanol and stained with DAPI to visualize the nuclei. The ADAM-12L/S mutant control was set to 100%. Bar graphs show mean values $\pm$ SD, from three independent experiments, *, $P < 0.05$ compared with controls, respectively.

8. DISSCUSSION

There are numerous growth factors, transcription factors, pregnancy hormones and proteolytic enzymes controlling the development of early placenta and therefore controlling the EVT's invasion of the maternal decidua.

Early placental development starts after attachment of the blastocyst to the uterus wall. The formation of the placental villi, attachment of anchoring villi and invasion of the extravillous trophoblasts is a fundamental step to successful pregnancy. Factors controlling invasion of EVT's come either from the maternal environment and are so-called paracrine factors or they are produced by the EVT's itself and therefore of autocrine origin. In addition, among the molecular factors controlling trophoblasts differentiation, proliferation and motility are the numerous transcription factors.

Abnormalities in invasion process and improper remodeling of the spiral arteries may lead to life threatening gestational diseases like, PE, IUGR, molar pregnancies and placenta percreta or may be the cause of the recurrent abortions. However, the molecular mechanisms regulating the pathogenesis of these disorders remain still unclear.

AP-2 α is a member of the AP-2 transcription factor family. AP-2 α plays role in the biological and pathological processes. In human AP-2 α was shown to regulate cell processes such as: proliferation and differentiation, apoptosis and differentiation during embryogenesis [133]. Moreover, two of AP-2 isoforms, AP-2 α and AP-2 γ were detected in the human placenta [115]. In addition, earlier was shown that AP-2 α controls genes that regulate trophoblast differentiation [115]. It was found that level of AP-2 α increases during differentiation of trophoblasts and controls syncytiotrophoblasts specific genes like; PL, CG and corticotrophin - releasing - hormone (CRH) [115]. Interestingly in AP-2 α knock-out mice studies have indicated, no placental phenotype but important role of AP-2 α factor in neural crest development and defects in cranio-facial structures [134]. Previously it was also found that AP-2 α is downregulated in some aggressive carcinoma like melanoma and prostate [67, 68, 135, 136]. In human melanoma AP-2 α regulates c-KIT and adhesion molecule MCAM/MUC18. C-KIT controls invasion of tumor cells and decreases during tumor development, this correlates with decrease of AP-2 α in this type of cancer [137]. However, AP-2 α was also indicated to be overexpressed in many breast cancer cell lines. Moreover, AP-2 binding sites are present in ERBB-2 and

estrogen receptor promoters, both genes are typically upregulated in most of breast cancer cases [138].

During my studies I found that AP-2 α transcription factor is expressed in vCTBs and invasive EVTs. Furthermore, I have shown that AP-2 α plays an important role in the invasion of first trimester trophoblasts by transcriptional regulation of target genes important for the invasion process. After AP-2 α gene silencing in SGHPL-5 trophoblast cell line and in primary trophoblasts I performed functional studies in order to study a role of AP-2 α in trophoblasts motility. The results of invasion assay from both cell types showed that AP-2 α does not significantly affect invasion of trophoblasts. At this step, I decided to stimulate the invading cells with EGF, which is a well-known trigger of trophoblasts invasion [124]. When cells were stimulated with EGF the invasion was significantly induced in control cells but decreased in the AP-2 α knock-down cells when compared to EGF untreated AP-2 α knock-down and control cells. Furthermore, AP-2 α was shown to play role in controlling cell proliferation, differentiation and apoptosis [139]. Studies with AP-2 α knock-out mice indicated an AP-2 α target gene, Krüppel-box transcription factor KLF-4 implicated in regulation of fibroblasts proliferation. KLF-4 was found to be expressed in AP-2 α null fibroblasts and alter their proliferation. Interestingly, I found, either by measuring cumulative cell number (SGHPL-5) cells or by BrdU labeling (EVTs) that depletion of AP-2 α does not affect trophoblasts proliferation. Another goal in the project was to identify AP-2 α target genes which may be responsible for the pro-invasive effect of AP-2 α . Analyses of AP-2 α knock-down showed EGF dependent expression of the following genes: MMP-2, CG and uPA. In addition regulation of critical invasion related metalloprotease MMP-2 was regulated by AP-2 α with and without EGF stimulation confirming studies of another group [78]. In opposite after AP-2 α depletion in SGHPL-5 cells the expression of PAI-1 and PAI-2 were increased, moreover PAI-1 expression was increased in control cells upon EGF stimulation. It was earlier shown that AP-2 α is important transcriptional regulator of CG β [116, 140]. CG is a fundamental hormone for pregnancy development and establishment, it was already earlier shown that it increases the invasion of the human trophoblasts [72]. Obtained data have shown that indeed CG β decreases in SGHPL-5 cells and primary EVTs after AP-2 α depletion. Moreover, I found that depletion of AP-2 α decreases level of CG by performing luciferase assay with CG β 5 promoter construct. This analysis showed that the activity of the CG β 5 promoter was decreased when AP-2 α was silenced.

All these data together are consistent with the fact that decrease in AP-2 α expression in primary EVT₁s directly influence CG expression. The overexpression of AP-2 α in stable cell line SGHPL-5 was performed in order to analyze AP-2 α upregulation. Overexpression studies confirmed the results obtained after loss of function studies. We could see opposite effect of invasion studies in comparison with invasion results after AP-2 α depletion. The invasiveness of trophoblast cells was slightly but significantly increased after transfection with the AP-2 α containing construct.

Another group of critical factors controlling trophoblasts invasiveness are proteases. Many of the proteolytic enzymes were shown to play a fundamental role during tumorigenesis including EMT-mediated process and tumor angiogenesis. The most frequently investigated proteases in the cancer field but also in the field of reproduction are MMPs. MMP-2 and MMP-9 were shown to be important and broadly investigated during trophoblasts invasion [73]. A member of ADAMs family belongs together with MMPs and ADAMT₁s to metzincins family of zinc-based proteinases [141]. ADAM-12 is classified as an active metalloprotease due to possessing metalloprotease, cysteine-rich and EGF-like domains in its structure. ADAM-12 was indicated to be upregulated in many cancer types and its expression level correlates with tumor aggressiveness [123, 131]. It was found among different human tissues samples including; lung, kidney liver and placenta that ADAM-12 was detected only in the placental tissue [142]. The data which I have been generated during my research suggest that ADAM-12 plays an important role in trophoblasts invasion. Descriptive analyses including IF staining, WB and qRT-PCR of the first trimester placenta, first trimester decidua, term trimester placenta and SGHPL-5 cell line clearly indicate that expression of ADAM-12 is restricted to invasive, differentiated EVT₁s, both in the first trimester and term trimester placenta. In detail, expression of ADAM-12 in vivo in the placental tissue and in vitro in primary isolated EVT₁s drastically increases during their invasive differentiation pathway as shown (Fig.15, Page 65) As observed at the IF staining the most ADAM-12 positive cells reside in the first and term trimester decidua as shown (Fig.13, Page 63). EVT₁s which reside in the decidua are concerned to be differentiated and highly invasive EVT₁s, strongly expressing ADAM-12. This may suggest that ADAM-12 play fundamental role in EVT₁s invasion. The presence of highly ADAM-12 positive EVT₁s was also detected by IF staining in the term trimester placenta (Fig 13B, Page 63); this may suggest that high level of ADAM-12 is important not only for the development of early pregnancy but also

for the maintenance of the pregnancy until its termination. Moreover, the qRT-PCR indicated expression of both spliced variants ADAM-12L and -S in the first trimester tissue and in isolated differentiating primary EVTs. qRT-PCR suggests that mRNA of both ADAM-12 variants is present in first trimester placenta, but absent in placental fibroblasts. Interestingly SGHPL-5, trophoblast cell line appeared to be negative for ADAM-12 (Fig.11, Page 61). Analysis of placental explants grown on matrigel has shown that the cell columns formed by proliferative CTBs is negative for ADAM-12. However, we noticed a cytoplasmatic, dotted ADAM-12 signal in distal, growth arrested CC trophoblasts. This could indicate freshly synthesized protein remaining in vacuoles for secretion. It might be that during differentiation pathway of EVTs, matured active metalloprotease ADAM-12 is transported to extracellular space and finally visible as membranous expression. ADAM-12S was shown to be detectable in serum of women with healthy pregnancies, decreased level of ADAM-12S may correlate with; Down syndrome pregnancies [143], ectopic pregnancy [121] and fetal trisomy 18 [144, 145]. In PE the level of secreted ADAM-12S to mother plasma depends on gestational age. It was shown that in first trimester samples the levels of ADAM-12 remain unchanged as compared to the control samples [146]. On the other hand when samples of serum from second trimester pregnancies were analyzed it revealed a significant decrease of ADAM-12S in maternal serum in comparison to the healthy controls [122]. The reason could be that during second trimester of the pregnancy there is greater involvement of the trophoblasts in pathogenesis of PE, corresponding with appearance of clinical syndromes. Therefore, a use of ADAM-12S as a biochemical marker for PE detection makes sense after first trimester of pregnancy [122]. However, studies from another group clearly indicate that ADAM-12S level in maternal serum is decreased already during first trimester of pregnancy in women who later develop PE [125]. Apparently, lower level of secreted ADAM-12 may suggest that during PE lower number of EVTs is produced, when compared to normal pregnancies, and therefore less ADAM-12S is detected in the serum.

The invasion assay on fibronectin coated transwell inserts with ADAM-12 knock-down primary EVTs, suggests that ADAM-12 indeed regulates invasion of human trophoblasts. Previously has been shown that ADAM-12 induces invasion of cancer cells. Keeping in mind the partial analogy between EMT and early placenta development, I was curious if ADAM-12 regulate invasion of placental trophoblasts. One of the most important steps

in the project was generation of the transient ADAM-12 knock-down in primary EVT_s (Fig 16, Page 66). The aim of knock-down generation in primary EVT_s was testing ADAM-12 role in functional assays after transfection of primary EVT_s. After ADAM-12 depletion in isolated primary EVT_s the invasiveness on fibronectin decreased approximately 20% when compared to control. Interestingly, proliferation of the cells was not significantly affected. ADAM-12 knock-down in primary EVT_s revealed no difference in Ki67 antibody positive staining when compared with control cells (Fig. 19, Page 69).

Rather subtle decrease in EVT_s invasion on fibronectin after ADAM-12 depletion may be caused by choice of inconvenient method for investigating invasion after ADAM-12 knock-down. Furthermore lack of ADAM-12 in the EVT_s might be compensated by other factors, such as growth factors and MMPs facilitating invasion of trophoblasts. Therefore in addition, I tested another model system to study invasion after ADAM-12 knock-down, namely primary placental explants. Evaluation of the knock-down in placental explants revealed approximately 70% decrease of the ADAM-12, when compared to control explants (Fig.18, page 68). Depletion of ADAM-12 in the first trimester placental explants cultivated on collagen indicate a stronger decrease in EVT_s motility when compared to invasion assay on transwell inserts with primary EVT_s. ADAM-12 knock-down in placental explants have a deficiency in developing migratory, detaching EVT_s, moreover the migratory distance of existing EVT_s from the CC was decreased approximately 60%, when compared to the explants treated with non-targeting control (Fig 18B, Page 68). Therefore, we suggest that first trimester placental explant model seems to be a better model system to investigate the role of ADAM-12 role in EVT_s motility

ADAM-12 is an active metalloprotease and therefore mediates proteolytic cleavage of certain factors. Numerous studies indicate ADAM-12 proteolytic activity, ectodomain shedding and therefore activation of membrane bound factors to soluble ones e.g. degradation of IGFBP-3 and -5 by secreted ADAM-12S [97, 132]. Due to IGFBP-3 degradation, IGF-II is released which is well-known factor regulating trophoblasts motility and play important role in fetus growth [82, 122]. One of our goals was to study ADAM-S mediated cleavage of IGFBP-3. The assay was performed with the serum free supernatants collected after ADAM-12 depletion in primary EVT_s. The WB analyses revealed significant decrease in the amount of the cleavage products in the supernatants

collected from ADAM-12 knockdown EVT_s (Fig.21A and B, Page 71). Additionally, a higher amount of rehIGFBP added to both supernatants remained undigested in supernatant collected from ADAM-12 deficient EVT_s when compared to the control (data not shown). Finally, an invasion assay on matrigel coated invasion inserts using ADAM-12 overexpressing SGHPL-5 cells, confirmed that ADAM-12 controls motility of trophoblasts. Overexpression of ADAM-12L and ADAM-12S caused 60% and 40% increase in invasiveness, respectively (Fig.23, Page 73). This may suggest that upregulation of ADAM-12 lead to increase in trophoblasts motility.

Taken together, both investigated regulatory factors; AP-2 α and ADAM-12 seem to play an important role in the regulation of first trimester trophoblasts motility. AP-2 α by its transcriptional activity regulates EGF dependent invasion specific target genes controlling cell motility. ADAM-12 as an active protease acts as a molecular scissors and activates growth factors like EGF and IGFI/II which then switch on signaling pathways responsible for control of cell motility. My studies have shown that AP-2 α and ADAM-12 are important for early placental development, particularly regulating trophoblasts motility, moreover abnormalities in their expression level may lead to pregnancy complications.

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10. CURRICULUM VITAE



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Publication list:

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