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Critical growth factors and signalling pathways controlling human trophoblast invasion

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Abstract

Invasion of placental trophoblasts into uterine tissue and vessels is an essential process of human pregnancy and fetal development. Due to their remarkable plasticity invasive trophoblasts fulfil numerous functions, i.e. anchorage of the placenta, secretion of hormones, modulation of decidual angiogenesis/lymphangiogenesis and remodelling of maternal spiral arteries. The latter is required to increase blood flow to the placenta, thereby ensuring appropriate transfer of nutrients and oxygen to the developing fetus. Since failures in vascular changes of the placental bed are associated with pregnancy diseases such as preeclampsia or intrauterine growth restriction, basic research in this particular field focuses on molecular mechanisms controlling trophoblast invasion under physiological and pathological conditions. Throughout the years, an increasing number of growth factors, cytokines and angiogenic molecules controlling trophoblast motility have been identified. These factors are secreted from numerous cells such as trophoblast, maternal epithelial and stromal cells, as well as uterine NK cells and macrophages, suggesting that a complex network of cell types, mediators and signalling pathways regulates trophoblast invasiveness. Whereas essential features of the invasive trophoblast such as expression of critical proteases and adhesion molecules have been well characterised, the interplay between different cell types and growth factors and the cross-talk between distinct signalling cascades remain largely elusive. Similarly, key-regulatory transcription factors committing and differentiating invasive trophoblasts are mostly unknown. This review will summarise our current understanding of growth factors and signal transduction pathways regulating human trophoblast invasion/migration, as well as give insights into novel mechanisms involved in the particular differentiation process.

Keywords

human placenta; trophoblast invasion; growth factor; signal transduction

Introduction

General aspects of trophoblast differentiation

Placenta morphogenesis and formation of specialised trophoblast cell types are initiated after implantation and have to be completed during the first weeks of gestation to guarantee successful progression of pregnancy. Trophoblast progenitor cells residing at the basement membrane of placental villi give rise to distinct epithelial cell types. Fusion of cytotrophoblasts (CTBs) generates the multinucleated syncytium which covers the floating

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villi and is mainly responsible for protein transport and hormone production. In addition, different invasive CTB cell types develop which migrate from villous structures and hence are commonly termed extravillous trophoblasts (EVT).

Invasive differentiation program of the anchoring villus—In anchoring villi attaching to the uterine epithelium, mononuclear CTBs form cell columns through proliferation. Interaction of L-selectin with carbohydrate ligands might play a role in maintaining integrity of these columns throughout pregnancy (Prakobphol *et al.*, 2006). However, at their distal anchoring sites CTBs detach from the columns and invade the maternal decidual stroma (Fig. 1). Cell cycle exit and differentiation into these interstitial CTBs (iCTBs) is thought to be influenced by extracellular matrix contact and endometrial components. In addition, oxygen concentrations are thought to be critically involved since hypoxia promotes trophoblast proliferation whereas normoxia inhibits proliferation and induces migration (Genbacev *et al.*, 1997). However, spontaneous local invasion of trophoblasts *in vitro* as well as in ectopic pregnancies supports the hypothesis of a strongly activating, intrinsic differentiation program (Fisher *et al.*, 1989; Wells, 2007). Production of pregnancy hormones, such as human chorionic gonadotrophin (hCG) and placental lactogen (hPL), acquisition of polyploidy, and differentiation into placental bed giant cells are some of the remarkable functions of this particular invasive trophoblast cell type (Genbacev *et al.*, 1993; Handschuh *et al.*, 2007a; Pijnenborg *et al.*, 1980; Zybina *et al.*, 2002).

Besides iCTB another population of migratory trophoblasts develop which are thought to play an important role in vascular remodelling of maternal spiral arteries. Apoptosis of vascular smooth muscles around these vessel and displacement of maternal endothelial cells by endovascular cytotrophoblasts (eCTB) are key features of the particular invasive differentiation process (Harris and Aplin, 2007; Pijnenborg *et al.*, 1983). The eCTBs acquire endothelial-like functions expressing typical vascular adhesion molecules (Zhou *et al.*, 1997). These modification steps may finally result in enlargement of the vessel diameter which is thought to initiate and sustain blood flow into the intervillous space.

Failures in transformation of spiral arteries and shallow interstitial invasion were detected in the placental bed of women suffering from preeclampsia or severe intrauterine growth restriction (IUGR) indicating that the EVT differentiation program could be disturbed in these pregnancies (Khong *et al.*, 1986; McFadyen *et al.*, 1986; Pijnenborg *et al.*, 1991). Poor perfusion of placental tissue is thought to result in the release of cytokines and products of oxidative stress into the maternal circulation which may cause endothelial dysfunction (Hung *et al.*, 2002; Many *et al.*, 2000).

Mechanisms controlling trophoblast invasion—Since in uncomplicated pregnancies interstitial/endovascular invasion do not occur beyond the decidua and the first third of the underlying myometrium, the extent of trophoblast invasiveness is thought to be precisely controlled by trophoblast-derived as well as maternal factors in a time- and distance-dependent manner (Bischof *et al.*, 2000; Lala and Hamilton, 1996). In general, iCTBs are equipped with different protease systems allowing them to degrade extracellular matrix (ECM) proteins to promote cell migration, whereas decidua was shown to express a variety of inhibitory proteins thereby restricting invasiveness. Migratory trophoblasts express different family members of matrix metalloproteinase (MMPs), cathepsins and urokinase plasminogen activator (Bischof *et al.*, 2000; Lala and Chakraborty, 2003; Varanou *et al.*, 2006). However, decidual cells produce tissue inhibitors of metalloproteinases (TIMPs) and plasminogen activator inhibitor (PAI) (Lala and Graham, 1990; Schatz and Lockwood, 1993). The importance of the MMP/TIMP and the uPA/PAI system in trophoblast invasion is further emphasized by the fact TIMPs/PAI and MMPs are expressed by iCTB and decidua, respectively (Feinberg *et al.*, 1989; Bischof, 2000; Lala and Hamilton, 1996).

Besides degradation of ECM components trophoblast are known to switch expression of adhesion molecules along the invasive differentiation pathway *in vivo* as well as *in vitro* (Damsky *et al.*, 1994; Vicovac *et al.*, 1995). CTB residing at the villous basement membrane express α6β4 integrin, one of the predominant laminin-5 receptors. In contrast, trophoblasts of the cell columns induce surface expression of α5β1 integrin which is though to play a role in the stabilisation of cell columns by binding to fibronectin. Distally located iCTBs, however, suppress α6β4 integrin and upregulate α1β1 integrin which is thought to promote invasiveness upon interaction with collagens and distinct laminins, such as laminin-2, which is abundantly expressed in the decidua (Church *et al.*, 1996).

The adherens junction protein E-cadherin, another marker of the polarised epithelium, is transiently downregulated during trophoblast invasion (Zhou *et al.*, 1997). Hence, the invasive differentiation of trophoblasts shares features with a process termed epithelial-mesenchymal transition (EMT) which is critically involved in development and tumorigenesis. Typically, iCTBs display nuclear expression of critical transcription factors involved in EMT such as Snail (S. Sonderegger and M. Knöfler, unpublished observation) and T-cell factor family (TCF) members, the latter being regulated by Wnt signalling (Pollheimer *et al.*, 2006). However, differently to cancer cells migratory trophoblasts do not induce critical (mesenchymal) markers of EMT such as vimentin and retain epithelial characteristics such as cytokeratin 7 expression. Despite their high invasive potential it is currently unclear why iCTB do not fully undergo EMT. With respect to that it is interesting to mention that during tumorigenesis epithelial cells switch to a metastable cell phenotype, characterised by co-expression of mesenchymal and epithelial genes, before they fully transform into a mesenchymal cell. It could well be that iCTB are kept in a similar metastable phenotype (Lee *et al.*, 2006). Maintenance of critical (epithelial) markers may ensure that cell cycle exit and differentiation occur in order to limit decidual invasion. Interestingly, invasive trophoblasts of complete hydatidiform mole (CHM) placentae, which eventually deeply infiltrate into maternal tissue, strongly express TCF molecules as well as nuclear β-catenin providing the activation domain of the Wnt-dependent transcription factor family (Pollheimer *et al.*, 2006). Appearance of nuclear β-catenin is a typical feature of EMT and cancer cells suggesting that invasive trophoblasts of CHM placentae may have further progressed towards a tumor cell-like, mesenchymal phenotype.

Model systems of trophoblast invasion

Trophoblast cell lines—Several investigators have established trophoblast cell lines derived from first trimester placentae which are commonly used to study trophoblast invasion. Similar to choriocarcinoma cells HTR-8/SVneo cells, which had been generated by transformation of HTR-8 cells with large T antigen, display an unlimited life-span in culture. The primary HTR-8 cultures were obtained after plating and outgrowth of cells from tissue pieces of first trimester villi and share features with invasive trophoblasts such as expression of cytokeratin 18 and some EVT-specific integrins (Graham *et al.*, 1993). Similarly, SGHPL-4 and SGHPL-5 cells have been produced after trypsinisation, gradient centrifugation and SV40 large T antigen transfection of minced first trimester placentae (Choy and Manyonda, 1998; Choy *et al.*, 2000). The two presumptive EVT cell lines, which express cytokeratin 7 and HLA-G, particularly when plated on Matrigel, retain a senescence mechanism and are commonly used until passage 25 (G. Whitley, personal communication). SGHPL-4 cells migrate into fibrin-embedded spiral arteries *in vitro* suggesting that they have retained cellular functions of *in vivo* EVT (Cartwright *et al.*, 2002a). HIPEC 65 represents another SV40 large T-transformed cell line derived from early chorionic villi expressing several EVT markers (Pavan *et al.*, 2003). Whereas many researchers investigated molecular mechanism of trophoblast invasion in the above mentioned cell lines, several other putative EVT cell lines such as HT-116, or SWAN 71, exist, which however

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were hardly used for *in vitro* studies (Aplin *et al.*, 2006; Logan *et al.*, 1996; Zdravkovic *et al.*, 1999). However differently to primary EVT, downregulation of cytokeratin 7 and induction of vimentin was noticed in all trophoblast cell lines. This suggests that the selection procedure has artificially converted EVT from an epithelial or presumptive metastable phenotype into a more fibroblastoid phenotype. Indeed, gene expression profiling and cluster analyses revealed that primary EVT share more similarities with villous CTB than with trophoblastic cell lines (M. Bilban and M. Knöfler, unpublished).

Trophoblast primary cultures—Besides analyses of cell lines molecular mechanisms controlling trophoblast invasion should be also investigated in appropriate primary cell models. This, however, is hampered by fact that the amount of primary trophoblasts is limited and that cultures are eventually contaminated with other placental cell types such as fibroblasts. In addition, cells rapidly cease proliferation in culture and thus are notoriously difficult to transfect by standard protocols.

Nevertheless, villous CTBs isolated by the Kliman method, i.e., trypsinization of first trimester villous material, Percoll gradient centrifugation and immunopurification have been widely used to study aspects of trophoblast invasiveness (Fisher *et al.*, 1989; Kliman *et al.*, 1986). Similarly, the villous explant culture system was established allowing to study column formation and EVT migration/invasion in a time- and distance-dependent manner (Genbacev *et al.*, 1993). After plating of first trimester villous tissues on ECM-coated dishes, differentiated EVT develop expressing cell-specific markers such as HLA-G and integrins $\alpha 5\beta 1$ and $\alpha 1\beta 1$ (Bauer *et al.*, 2004; Vicovac *et al.*, 1995).

Whereas utilisation of primary cells is desirable, one also has to be aware of the disadvantages of the trophoblast models described above. Explant cultures correctly mimic the EVT differentiation program, however, the different processes, i.e., adhesion, proliferation and migration/invasion, cannot be studied separately. Depending on the immunopurification protocol Kliman-prepared CTB represent a mixture of different trophoblast cell types with villous CTB being the most prominent one. Thus, it is not anticipated that for example effects of growth factors on protease expression and invasion would be the same as on distal, migratory EVT. Rather, utilisation of these cells may reflect the initial step of the invasive differentiation process, i.e. detachment of CTB from villous basement membranes. Therefore, procedures were developed to isolate and purify primary EVT from first trimester villous tissue (Tarrade *et al.*, 2001a).

To overcome the limitations of isolated primary cultures different attempts were also made to isolate and propagate CTB stem cells which eventually can be differentiated into the EVT lineage. Indeed, human CTB stem cells/lines have been developed from embryoid bodies of embryonic stem cells expressing critical trophoblast (stem cell)-specific markers such as Cdx2, cytokeratin 7 and HLA-G (Harun *et al.*, 2006; Gerami-Naini *et al.*, 2004). These cells also express endothelial-specific genes upon endometrial co-cultivation reflecting the vascular adhesion phenotype of eCTB *in vivo* (Harun *et al.*, 2006). Nevertheless, further functional analyses of CTB stem cells, such as mechanisms of differentiation into EVT, are still lacking. Also, there is currently no agreement within the community whether placental villi harbour a bi-potential CTB stem cells which is capable to differentiate into both syncytium and EVT or whether each differentiated trophoblast cell type has its own progenitor cell (Baczyk *et al.*, 2006; James *et al.*, 2007). Hence, analyses of EVT cell lines in combination with the verification of critical steps in a primary trophoblast model currently seems the most appropriate way to gain more insights into mechanisms of trophoblast invasion.

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It is also worth mentioning, that recent trophoblast models systems have paid attention to the fact that, differently to standard cell culture conditions, iCTB do not grow in two dimensions *in vivo* and closely interact with different cell types of the decidua. Hence, spheroid cultures of SGHPL-4 cells were established suggesting that three dimensional growth could be critical for invasiveness and expression of some MMPs (LaMarca *et al.*, 2005). Also, co-culture systems of trophoblasts with endothelial cells, explanted spiral arteries or smooth muscle cells are being developed to unravel the role of CTB in uterine vessel modification (Aldo *et al.*, 2007; Cartwright *et al.*, 2002a; Harris *et al.*, 2006).

Growth factors of the fetal-maternal interface

Numerous growth factor have been identified at the fetal-maternal interface controlling proliferative as well as invasive capacity of trophoblasts (Bischof *et al.*, 2000; Lala and Chakraborty, 2003). Different decidual cell types which are in close contact to iCTBs, i.e. decidual stromal cells, uterine NK cells and macrophages, are thought to regulate these processes in a paracrine manner. In addition, EVT express multiple ligands and hormones as well as their receptors indicating autocrine control.

During early gestation placental development occurs in the absence of maternal arterial supply. Hence, placental growth and invasion are also potentially regulated by proteins released from endometrial glands such as EGF, vEGF or LIF which are commonly regarded as critical regulators of implantation (Burton *et al.*, 2007). Throughout gestation growth factors such as EGF, vEGF, PDGF, placental growth factor (PlGF), CSF-1, IGF-I or IGF-II are abundantly secreted from diverse cell types of the fetal-maternal interface including CTBs and were shown to promote proliferation, adhesion and/or invasion (Bischof *et al.*, 2000; Ferretti *et al.*, 2007; Lala and Hamilton, 1996; Pollheimer and Knöfler, 2005b). However, decidual cells / CTBs also produce a variety of inhibitory proteins such as TGFβ family members, interferon-γ, endostatin, kisspeptin-10 or TNFα to fine-tune and limit the extent of trophoblast invasion (Bauer *et al.*, 2004; Bilban *et al.*, 2004; Lala and Graham, 1990; Lash *et al.*, 2006; Pollheimer *et al.*, 2005a).

Besides classical growth factors a plethora of cytokines and chemokines are secreted from CTBs and decidual cell types, predominantly from macrophages and fibroblasts (Hannan and Salamonsen, 2007; Jokhi *et al.*, 1997). Many of their respective receptors were identified on CTBs (Drake *et al.*, 2004). Chemokines such as CX3CL1, CCL14, CCL4 (Hannan *et al.*, 2006), CXCL16 (Huang *et al.*, 2006) or CCL21 (Red-Horse *et al.*, 2005) were shown to increase trophoblast migration or invasion. Similarly, the interleukins IL-1, IL-6, IL-8 or IL-11 secreted from CTB/decidual NK cells were shown to promote gelatinase activity and/or trophoblast invasion (Hanna *et al.*, 2006; Librach *et al.*, 1994; Meisser *et al.*, 1999; Paiva *et al.*, 2007). Interestingly, chemokines potentially regulated by the binding of ephrin ligands to their EPH receptors may not only play a role in interstitial invasion but also in the selective remodelling of uterine spiral arteries as compared to veins (Red-Horse *et al.*, 2005). Moreover, regulatory binding proteins, for example IGFBPs (Giudice and Irwin, 1999; Lala and Chakraborty, 2003), soluble TNF receptors (Knöfler *et al.*, 1998), sFlt-1 (Clark *et al.*, 1998), a vEGF/PlGF antagonist, or soluble endoglin (sEng) (Venkatesha *et al.*, 2006), a secreted TGFβ co-receptor, have been identified at the fetal-maternal interface adding further complexity to the growth factor network regulating trophoblast invasion. Physiological levels of sFlt-1 and sEng are critical for normal progression of pregnancy since elevated concentrations of these factors are thought to be involved in the pathogenesis of preeclampsia (Tjoa *et al.*, 2007).

Whereas an increasing number of growth factors controlling trophoblast invasion are being identified, it is largely unknown whether some of these play a more predominant role than others. Similarly, importance of an individual growth factor may vary throughout gestation

depending on its temporal expression pattern and the abundance of soluble inhibitors or other receptor ligands. However, its tempting to speculate that growth factors specific to pregnancy may fulfil central functions. With respect to that it is worth mentioning that hCG may play a pivotal role in trophoblast syncytialisation. Inhibition of its receptor abolishes the fusing-promoting effects of several growth factors, i.e. EGF, TGFα and LIF, suggesting that these secreted factors signal through stimulation of hCG expression/secretion (Yang *et al.*, 2003a). It could well be that hCG which is known to promote trophoblast motility (Handschuh *et al.*, 2007b; Prast *et al.*, 2008) also plays a similar key role in the invasive differentiation process. Indeed, several factors promoting trophoblast invasion, such as IGF-I, IL-1 or EGF, were shown to increase hCG expression and secretion (Fig. 2).

Signal transduction pathways promoting trophoblast invasion

Signal transduction through sequential steps of phosphorylation represents the most common control mechanism of cellular protein function. Multiple extracellular stimuli such as growth factors, hormones, cytokines, chemokines or cell-matrix contacts initiate signalling upon interaction with receptor tyrosine kinases (RTKs), G-protein-coupled receptors (GPCRs), integrins or others. This ultimately leads to the activation of critical signalling cascades such as mitogen-activated protein kinases (MAPKs), focal adhesion kinase (FAK), the phosphoinositide 3-kinase (PI3K)-Akt pathway or Janus kinase (JAK)-Signal Transducers and Activators of Transcription (STAT) controlling a wide range of biological processes including proliferation, differentiation, migration and apoptosis. Hence, it is not surprising that the different signalling pathways also play a critical role in placental development and differentiation. Here, we will predominantly discuss signalling cascades promoting trophoblast invasion /migration. We respect to anti-invasive mechanisms we would like to refer to recent reviews discussing for example TGFβ-mediated signalling and their inhibitory targets such as PAI-1 (Lala and Graham, 1990; Lala and Hamilton, 1996; Pollheimer and Knöfler, 2005b).

Mitogen-activated protein kinases (MAPKs)

Family members and general role of MAPKs—The family of MAPKs comprises a large group of enzymes which are sequentially activated by phosphorylation at specific Ser, Thr and Tyr residues. Upon ligand binding to RTKs or GPCRs enzymes of the MAPK kinase kinase family, such as Raf, are activated through Ras. This leads to the activation of MAPK kinases, for example MEKs, which subsequently phosphorylate four different families of MAPK, i.e. extracellular regulated kinases (ERKs), ERK5, c-Jun N-terminal kinases (JNKs) and different p38 MAPKs. Whereas ERKs are mainly activated through mitogenic signals, JNK and p38 MAPK are predominantly associated with stress and inflammatory response (Kyriakis and Avruch, 2001).

MAPKs in trophoblast invasion—One particular Raf protein, Raf-B, is critically involved in vascular development of the murine placenta (Galabova-Kovacs *et al.*, 2006). The role of Raf has not been investigated in human placenta; however, the fact that various growth factors trigger activation of ERKs in different EVT cell models suggests that Ras-Raf-MEK-ERK may control trophoblast motility. Indeed, IGF-II and IGFBP-1 activated ERK-1 and −2 and promoted migration of HTR-8/SVneo cells which could be blocked by a specific MEK inhibitor (Gleeson *et al.*, 2001; McKinnon *et al.*, 2001). Inhibition of MEK also diminished HGF-induced motility of SGHPL-4 cells (Cartwright *et al.*, 2002b). Similarly, the N-terminal fragment of uPA which lacks catalytic activity, stimulated migration of HTR-8/SVneo cells which was reduced upon inhibition of MEK (Liu *et al.*, 2003). Other factors stimulating HTR-8/SVneo cell migration and phosphorylation of ERKs are endothelin, EGF and prostaglandin E2 (Chakraborty *et al.*, 2003; Nicola *et al.*, 2008a;

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Qiu *et al.*, 2004a). The pregnancy hormone hCG also promoted trophoblast motility through ERK phosphorylation since MEK inhibition affected migration of SGHPL-5 cells and of EVTs in first trimester villous explant cultures (Prast *et al.*, 2008). Interestingly, these effects might be largely attributed to the hyperglycosylated form of hCG secreted from EVT since hormone released from syncytium had no effects on trophoblast motility (Handschuh *et al.*, 2007b). The role of MAPK kinase signalling in trophoblast invasion is also emphasized by the fact that active ERKs are detectable in late-gestational EVT *in situ* using immunohistochemistry of placental bed biopsies (Moon *et al.*, 2008). Presumptive roles of ERK5 and JNK in the regulation of trophoblast motility have not been investigated, whereas p38 MAPK may not be involved. Active and inactive forms of p38 MAPK were absent from EVTs *in situ* and chemical inhibition of the enzyme did not affect migration of SGHPL-4 cells (Cartwright *et al.*, 2002b; Moon *et al.*, 2008). In general, activation of ERKs through growth factors may affect numerous processes involved in cell motility such as integrin signalling, cytoskeletal dynamics or nuclear functions (Pullikuth and Catling, 2007). In trophoblasts ERKs were shown to regulate hCG- and EGF-dependent induction of MMP-2 and MMP-9, respectively, indicating that proteinases crucial for trophoblast invasion are targets of the particular signalling pathway (Prast *et al.*, 2008; Qiu *et al.*, 2004b).

Phosphoinositide 3-kinase (PI3K) – AKT signalling

General function of the PI3K-AKT pathway—PI3K-AKT signalling is involved in a variety of cellular processes including cell growth, proliferation, migration and survival (Manning and Cantley, 2007) Activation of RTKs or GPCRs results in membrane recruitment/activation of the p85 and p110 subunits of PI3K, respectively. Active PI3K phosphorylates phosphatidylinositol-4, 5-bis-phosphate (PIP2) at the 3'position of its inositol ring and thereby converts PIP2 to PIP3. Elevated PIP3 levels result in recruitment and activation of AKT at the membrane. Subsequently, signalling is achieved by sequential phosphorylation of downstream factors, for example, activation of the mammalian target of rapamycin (mTOR). The kinase mTOR controls cell cycle progression and cell size/mass through phosphorylation of proteins controlling protein translation, for example ribosomal S6 kinases and factors involved in translation initiation. AKT, however, also phosphorylates a wide range of other target proteins that control proliferation, cell growth and survival. In particular, the anti-apoptotic function of the signalling pathway is well known. Increased PIP3 levels occur in cancer due to hyperactivation of PI3K-AKT or loss of function mutations in PTEN (phosphatase and tensin homolog deleted on chromosome 10), the phosphatase converting PIP3 to PIP2.

Role of PI3K-AKT in trophoblast motility—Activation of PI3K-AKT signalling by anti-apoptotic factors such as EGF has also been described in human trophoblasts (Johnstone *et al.*, 2005). However, AKT also seems to play a critical role in development and differentiation of the placenta/trophoblast. In mice, homozygous deletion of ATK1 affects placental development due to decreased numbers of proliferative trophoblasts (Yang *et al.*, 2003b). PI3K-AKT also seems to be involved in differentiation of murine giant cells (Kamei *et al.*, 2002).

The role of AKT in cell migration is less well understood. The three different AKT isoforms may positively or negatively influence motility depending on the cell type (Manning and Cantley, 2007). However, recent evidence from several laboratories suggests that PI3K-AKT signalling positively affects human trophoblast migration. In particular, abundant growth factors of the fetal-maternal interface, i.e. EGF and IGF-II, are potent activators of PI3K-AKT and AKT-dependent migration of trophoblastic HTR-8/SVneo cells (Qiu *et al.*, 2005; Qiu *et al.*, 2004a). In addition, rapamycin, which specifically blocks mTOR, was shown to decrease phosphorylation of S6 kinase and migration of these cells (Qiu *et al.*,

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2004a). In SGHPL-5 cells activation of PI3K with specific peptides resulted in increased motility, whereas inhibition of PI3K reduced basal and HGF-induced migration (Cartwright *et al.*, 2002b). Activation of AKT through hCG was noticed in purified EVT and SGHPL-5 cells (Prast *et al.*, 2008). Inhibition of the signalling pathway reduced migration of SGHPL-5 cells and motility of EVT in villous explant cultures (Prast *et al.*, 2008). PI3K-AKT is also required for hCG- and EGF-dependent expression of MMP-2 and MMP-9 in trophoblasts (Prast *et al.*, 2008; Qiu *et al.*, 2004b), as well as of MMP-3 (J. Prast and M. Knöfler, unpublished). Since these enzymes are also targets of ERK signalling, PI3K-AKT and MAPK signalling may have synergistic effects on protease expression and CTB invasion.

Focal adhesion kinase (FAK)

General aspects of FAK signalling—FAK is a non-receptor protein tyrosine kinase (PTK) located in focal adhesions which are particular contact sites between migratory cells and the surrounding ECM. Activation of GPRCs or clustering of integrins in focal adhesions results in activation of FAK through phosphorylation at critical residues. Signalling through FAK ultimately provokes directed polymerisation/stress fiber formation of actin filaments at the leading edge of motile cells. The active kinase contains regulatory protein regions interacting with integrin-associated proteins, paxilin and talin, but also harbours contact sites binding Src-family members and other adaptor proteins with Src homology (SH) domains (Schlaepfer and Mitra, 2004). For example, activation at Tyr-397 selectively occurs at the leading edge of motile cells suggesting a critical role in cell migration. Indeed, phosphorylation of the particular amino acid promotes high-affinity binding of Src-family PTKs resulting in activation of multiple proteins kinase cascades promoting migration, i.e. MAPK, PI3K and signalling through Rho-family GTPases (Schlaepfer *et al.*, 1999).

FAK controls trophoblast migration—Integrin-mediated signalling and FAK are also crucially involved in trophoblast migration and invasion. Integrin switching takes place during invasive trophoblast differentiation *in vivo* as well as *in vitro* (Aplin *et al.*, 1999; Damsky *et al.*, 1994) and active, Tyr-397 phosphorylated FAK was noticed in invasive trophoblasts expressing MMP-2 and α5 integrin (Ilic *et al.*, 2001; MacPhee *et al.*, 2001). Down-regulation of FAK expression in villous explant cultures or CTB reduced *in vitro* migration and invasion (Ilic *et al.*, 2001; MacPhee *et al.*, 2001). Proteins such as the amino acid transporter CD98 expressed on EVT co-localize with αvβ3 integrin and promote migration through FAK (Kabir-Salmani *et al.*, 2008). Different growth factors were shown to activate FAK in trophoblasts. IGFBP-1-treated HTR-8/SVneo cells showed elevated FAK phosphorylation and migration (Gleeson *et al.*, 2001). Treatment of EVTs with IGF-I induced FAK activation as well as stress fiber formation (Kabir-Salmani *et al.*, 2002). Besides FAK, EVT express another enzyme activated through integrin-clustering in focal adhesions, i.e. integrin-linked kinase (ILK). Expression of a dominant-negative form of ILK was shown to reduce migration of HTR-8SVneo cells (Elustondo *et al.*, 2006).

RhoGTPases and Rho-associated kinase ROCK

General role of Rho proteins—RhoA, Rac1 and Cdc42 comprise the family of Rho-GTPases regulating diverse biological processes such as proliferation, adhesion and migration (Hall, 1998). Similar to Ras, the Rho-like GTPases function as molecular switches by cycling between an active GTP-bound and an inactive GDP-bound state. Growth factor binding or focal adhesion formation result in their activation thereby promoting stress fiber formation and motility. RhoGTPases are regulated by guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), and RhoGDP dissociation inhibitors. FAK can control Rho activity since FAK-src signalling regulates binding and phosphorylation of GAPs and Rho-GEFs (Schlaepfer and Mitra, 2004). Numerous downstream effectors of Rho

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were described for example the Rho-associated kinases (ROCKs) or p21-activated kinases (PAKs) controlling actin polymerisation, microtubuli and myosin motor proteins.

Rho-ROCK signalling regulates trophoblast motility—Signalling through Rho factors has also been described in trophoblasts. In mice, ROCK2 was suggested to play a role in blood flow through the labyrinth. Targeted deletion of the gene resulted in elevated PAI-1 expression, intrauterine growth restriction and fetal death (Thumkeo *et al.*, 2003). Changes in ROCK2 expression were also noticed in preeclamptic placentae (Ark *et al.*, 2005). With respect to trophoblast motility inhibition of Rho or ROCK were shown to decrease spreading and migration of EVT through fibronectin-coated filters (Shiokawa *et al.*, 2002). Different growth factors may utilize the Rho-ROCK pathway to promote trophoblast motility. For example, inhibition/siRNA-mediated silencing of either Rho, Rac1, CDC42 or ROCK reduced prostaglandin E2-dependent migration of HTR-8/SVneo cells and of EVT in villous explant cultures (Nicola *et al.*, 2008a; Nicola *et al.*, 2008b). Interestingly, depending on the receptor type different RhoGTPases were shown to be involved in IGF-II-induced migration of HTR-8SVneo cells. Signalling of IGF-II through IGFR1 required RhoA and RhoC but not Rac1 or CDC42, whereas IGFR1-independent effects of IGF-II involved ROCK but none of the Rho factors (Shields *et al.*, 2007).

Wnt signalling

Key components of canonical Wnt signalling—Proteins of the Wingless (Wnt) family are secreted regulators playing key roles in embryonic development and tumorigenesis (Gordon and Nusse, 2006). In the canonical pathway Wnt ligands bind to the heterodimeric Frizzled (FZD)/low-density lipoprotein receptor-related protein-5/6 (LRP-5/6) receptors thereby inhibiting the β-catenin destruction complex formed by proteins that include Axin, glycogen synthase kinase 3β (GSK-3β) and adenomatous polyposis coli (APC). In the absence of Wnt ligands active GSK-3β phosphorylates β-catenin thereby keeping cytosolic levels of β-catenin low. Wnt-dependent inactivation of the destruction complex/GSK-3β results in cytoplasmic accumulation and nuclear translocation of dephosphorylated β-catenin. Nuclear β-catenin then provides the activation domain of the lymphoid enhancer binding factor 1 (LEF-1)/T-cell factor (TCF) transcription factor family which induce growth- and invasion-associated genes such as cyclin D1, c-myc, MMP-7 and MT1-MMP. To control canonical Wnt signalling cells also produce soluble proteins such as different Dickkopf (DKK) members which inhibit the pathway upon interaction with LRP-5/6.

Role of Wnt/TCF in trophoblast differentiation and invasion—Throughout the years evidence accumulated that Wnt signalling could also be involved in placental development and trophoblast differentiation. Homozygous mutation of Wnt2, Wnt7b and LEF-1/TCF in mice resulted in different placental pathologies (Galceran *et al.*, 1999; Monkley *et al.*, 1996; Parr *et al.*, 2001). Implantation in mice was shown to be associated with increased expression of Wnt4 and inhibition of canonical signalling impaired the process (Mohamed *et al.*, 2005; Paria *et al.*, 2001).

In human placenta, 14 out of 19 Wnt ligands and 8 out of 10 FZD receptors, respectively, were detectable indicating that Wnt signalling could be involved in trophoblast function/differentiation (Sonderegger *et al.*, 2007). Interestingly, expression patterns of distinct Wnt ligands varied with gestational age and between different trophoblast subtypes suggesting cell-specific functions (Sonderegger *et al.*, 2007). Indeed, trophoblast invasion was associated with nuclear accumulation of TCFs in EVTs *in vitro* as well as *in vivo* (Pollheimer *et al.*, 2006). Activation of the pathway by a recombinant Wnt ligand induced

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migration and invasion of SGHPL-5 cells and CTBs which could be specifically inhibited by soluble DKK1 (Pollheimer *et al.*, 2006).

The decidua which is generally considered as an anti-invasive environment expresses Wnt3 and DKK in a menstrual-cycle dependent manner (Tulac *et al.*, 2003). In particular, DKK1 was shown to be highly expressed during the secretory phase and could be upregulated by progesterone *in vitro* (Tulac *et al.*, 2006) suggesting that decidual expression of the inhibitor may represent another mechanism limiting trophoblast invasiveness. Indeed, inhibition with DKK1 reduced basal trophoblast migration/invasion (Pollheimer *et al.*, 2006) indicating that autocrine Wnt ligands contribute to the inherent, invasive properties of EVT.

Besides canonical signalling recent data suggest that Wnt can also stimulate trophoblast migration through activation of the PI3K-AKT pathway (S. Sonderegger and M. Knöfler, unpublished). Since phosphorylation of AKT by a recombinant Wnt ligand could not be inhibited by DKK1, non-canonical Wnt receptor(s) but not FZD-LRP-5/6 must be involved. Evidence that aberrant Wnt signalling may play a role in human placental pathologies came from analyses of CHM placentae (Pollheimer *et al.*, 2006). Increased nuclear expression of β-catenin in EVT of these placentae suggested that, similar to tumour cells, mutations of critical Wnt signalling components and /or overexpression of Wnt ligands may contribute to the pre-malignant status of CHM.

Transcription factors in trophoblast invasion

General aspects

In mice, critical tissue-specific transcription factors controlling development of different trophoblast subtypes have been described (Cross *et al.*, 2003). In humans, however, it's largely unclear which factors commit and differentiate invasive trophoblasts (Loregger *et al.*, 2003). Although transcriptional regulators important for invasive giant cell differentiation of mice were elucidated, expression and distribution of their human counter-parts do not necessarily suggest equivalent roles (Meinhardt *et al.*, 2005). This might be explained by differences in placental morphologies/trophoblast subtypes as well as by the fact that trophoblast differentiation of humans can only be monitored at few time-frames of gestation. For example, the basic helix-loop-helix (bHLH) protein Hand1 promotes differentiation of precursors into invasive giant cells during early murine pregnancy (Riley *et al.*, 1998).

In humans, however, Hand1 expression can only be detected in blastocysts and upon trophectoderm differentiation *in vitro* but not in any of the different trophoblast cell types of first trimester placentae (Knöfler *et al.*, 2002; Peiffer *et al.*, 2007). Hence, it is possible that determination of human trophoblasts subtypes occurs early in development and cannot be studied in first trimester placental tissue usually obtained between 8th and 12th week of gestation. On the other hand, several homeobox genes potentially controlling commitment and differentiation were identified in human invasive trophoblasts (Quinn *et al.*, 1997; Quinn *et al.*, 1998).

Regulatory transcription factors in humans

Several transcription factors were shown to be involved in human trophoblast invasion based on *in vitro* experiments with trophoblast cell lines or isolated CTBs/EVTs. Id-2, an inhibitor of bHLH proteins, was found to be highly expressed in cell columns but decreased upon differentiation into EVT (Janatpour *et al.*, 2000). Overexpression of Id-2 in CTBs reduced *in vitro* invasiveness.

Signal transducer and activator of transcription 3 (STAT3) was identified in JEG-3 cells and first trimester trophoblasts but not in term cells suggesting a role in trophoblast invasion

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(Corvinus *et al.*, 2003). In general, phosphorylation, dimerisation and nuclear translocation of STATs are achieved upon growth factor/cytokine-dependent activation of receptor-associated Janus kinases (JAKs). Leptin was shown to increase protease expression and STAT3 activity and siRNA-mediated down-regulation of STAT3 reduced *in vitro* invasion of JEG-3 cells and CTBs (Fitzgerald *et al.*, 2005; Poehlmann *et al.*, 2005). Similarly, invasion-promoting effects of IL-11 on primary EVT are potentially triggered through STAT3 (Paiva *et al.*, 2007). Another STAT factor involved in trophoblast invasion is STAT5. Human placental growth hormone (hPGH) stimulates STAT5 binding activity through activation of Janus kinases 2 (JAK2) as well as invasion of purified, primary EVT in a JAK2-dependent manner.

Invasive trophoblasts also express one isoform of the Ikaros (Ik) transcription factors, namely Ikx. Overexpression of a dominant-negative Ikx, lacking DNA binding, was shown to inhibit migration of HTR-8/SVneo cells (Yamamoto *et al.*, 2005).

Finally, different studies indicate that peroxisome proliferator-activated receptor-gamma (PPAR-γ) plays a critical role in placental function and trophoblast invasion (Schaiff *et al.*, 2006). Activation of PPAR-γ with synthetic ligands reduced invasion of primary CTB and trophoblastic HIPEC 65 cells whereas antagonists increased invasiveness suggesting an inhibitory role of the nuclear receptor in trophoblast motility (Pavan *et al.*, 2003; Tarrade *et al.*, 2001b). Interestingly, activation of PPAR-γ was recently shown to impair hCG expression and secretion from EVT suggesting that the negative effects of the transcription on trophoblast invasion could be mediated through reduced levels of the pregnancy hormone (Handschuh *et al.*, 2007b).

Conclusion

Multiple growth factors expressed at the fetal-maternal are involved in the regulation of trophoblast migration and invasion (Fig. 3). Signalling of these factors occurs through pathways also modulating motility in other cellular systems, for example ERK, FAK or WNT signalling. Since invasiveness represents an inherent property of EVT, signal transduction pathways which are not primarily operational in controlling cell migration, such as the PI3K-AKT pathway, are also critically involved. Similar to other cell types most growth factors do not signal through a single cascades but stimulate invasiveness through a range of pathways. Whereas the number of growth factors, cytokines and chemokines affecting trophoblast motility are steadily increasing their downstream effectors such as critical transcription factors and their target genes are largely unknown. However, some of the nuclear factors, for example STATs, PPAR-γ, homeobox genes or WNT-dependent TCFs have been identified, which based on functional analyses or their expression pattern likely play key roles in trophoblast invasion. It can be speculated that these genes may control markers of the differentiated, invasive trophoblast such as integrins, EVT-specific hormones, and different protease systems. However, much remains to be learned about physiological trophoblast invasion and whether changes in activity of certain signalling cascades may contribute to the pathogenesis of pregnancy diseases with abnormal placentation or failed trophoblast differentiation.

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Abbreviations used in this paper

CHM	complete hydatidiform mole
CTB	cytotrophoblast
DKK	Dickkopf
ECM	extracellular matrix
EMT	epithelial-mesenchymal transition
EGF	epidermal growth factor
ERK	extacellular regulated kinase
EVT	extravillous trophoblast
FAK	focal adhesion kinase.

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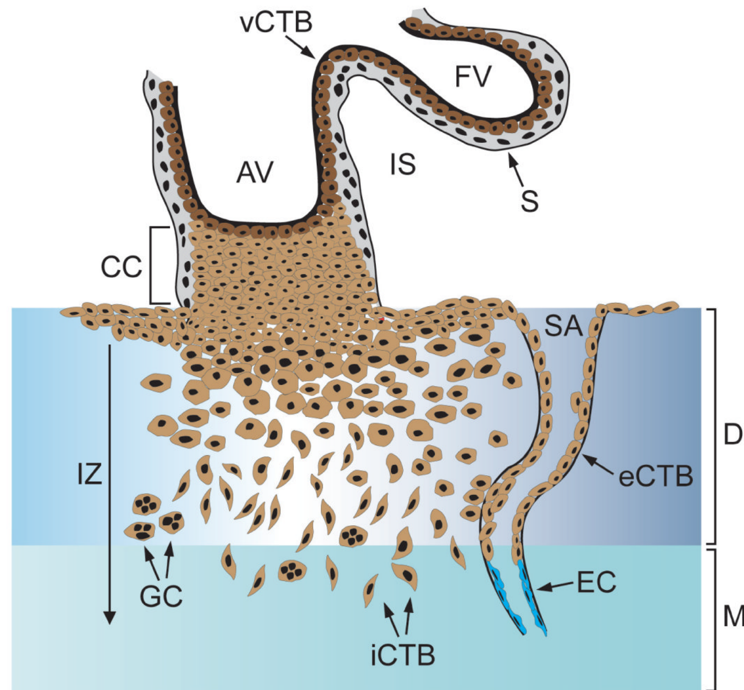

Fig. 1. Invasive differentiation of human trophoblast

After anchorage of a mesenchymal villus (formation of anchoring villi, AV) at the uterine basement membrane villous cytotrophoblast (vCTB) precursor cells give rise to proliferative cell columns (CC). At distal sites non-proliferating, extravillous trophoblasts are formed which detach from the cell columns and migrate into stromal areas of the maternal decidua (D), i.e. formation of interstitial cytotrophoblasts (iCTB). iCTB differentiate into giant cells (GC) in deeper areas of the placental bed. Endovascular trophoblasts (eCTB) migrate into spiral arteries (SA) within the decidua and inner third of the myometrium (M), replace maternal endothelial cells (EC) and acquire endothelial characteristics. In floating villi (FV) surrounded by maternal blood of the intervillous space (IS), CTB progenitors fuse to build the multinucleated syncytium (S).

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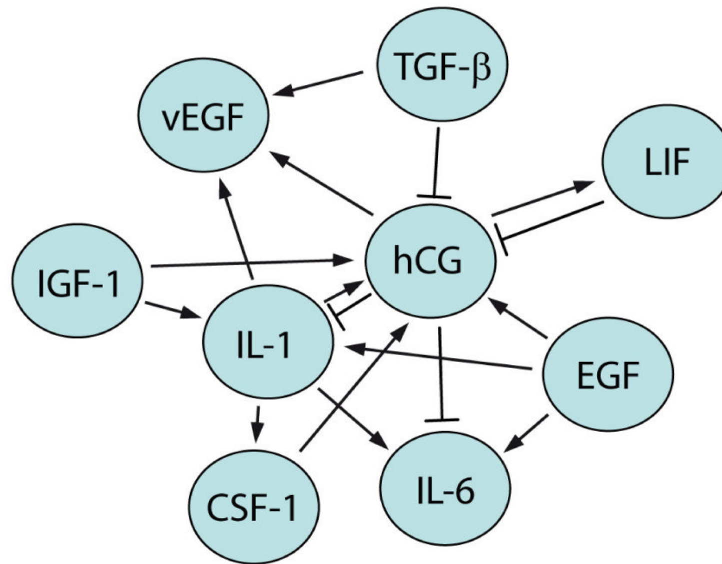

Fig. 2. Interplay between growth factors expressed at the fetal-maternal interface

Growth factor expression/secretion is stimulated by paracrine interactions of diverse maternal and placental cell types. Predominant growth factors of the fetal-maternal interface as well as their mutual stimulations published so far are summarised. With the exception of TGFβ all factors depicted were shown to positively influence trophoblast proliferation and/ or migration/invasion. Some soluble ligands such as hCG may play key roles in trophoblast motility since several growth factors trigger their secretion. On the other hand, hCG for example may also promote trophoblast migration through elevation of vEGF and LIF secretion. Hence, when studying effects of a particular growth factor on trophoblast migration direct as well as indirect effects must be considered. Stimulating (arrows) as well as inhibitory effects on expression / secretion are depicted.

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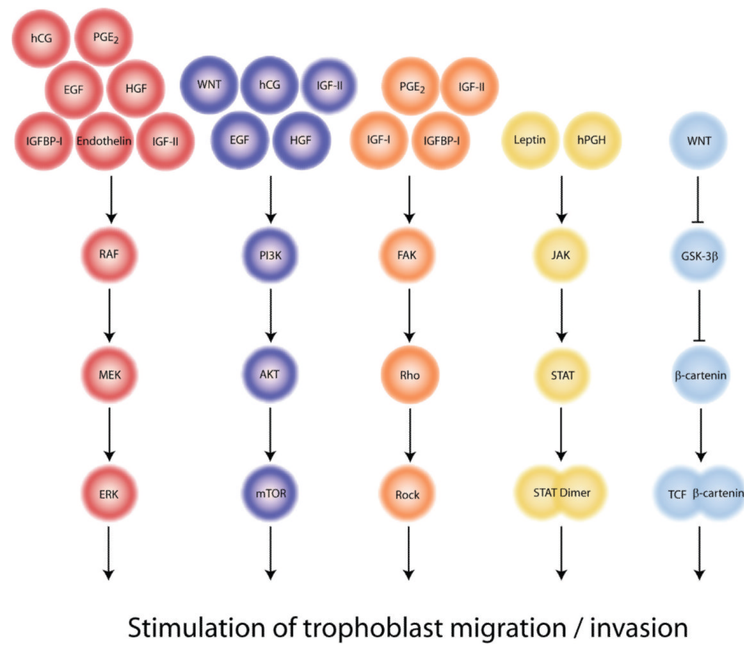

Fig. 3. Schematic presentation of signalling pathways stimulating trophoblast migration and/or invasion

Growth factors acting through these pathways are indicated. Sequential signalling steps resulting in phosphorylation (arrows) or dephosphorylation of downstream kinases or other protein targets (STAT, β-catenin) are depicted. Several critical factors such as EGF, IGFs, hCG or WNT signal through more than one pathway.