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.

KEY WORDS: 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 syncytiotrophoblast which covers the floating 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).

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, extracellular regulated kinase; EVT, extravillous trophoblast; FAK, focal adhesion kinase.

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 extracel-
lular 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 polyplody, and differentiation into placental
bed giant cells are some of the remarkable functions of this
particular invasive trophoblast cell type (Genbacev et al., 1993;
Handschu 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 mater-
nal 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 inter-
stitial invasion were detected in the placental bed of women
suffering from preeclampsia or severe intrauterine growth restric-
tion (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 plasmino-
gen activator (Bischof et al., 2000; Lala and Chakraborty, 2003;
Varanou et al., 2006). However, decidual cells produce tissue
inhibitors of metalloproteinase (TIMPs) and plasminogen activa-
tor 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 predomi-
nant laminin-5 receptors. In contrast, trophoblasts of the cell
columns induce surface expression of α5β1 integrin which is
thought 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 criti-

![Image](312x202 to 568x442)

**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 syncytiun (S).
blasts 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 tumorogenesis 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 Matrilg, 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 mechanisms 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 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 fibroblastosid 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 CTB 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 α5β1 and α1β1 (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 syncytiot and EVT or whether each differentiated trophoblast cell type has its own progenitor cell (Baczyn 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.

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 vitro* 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 placentation 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 (PIGF), CSF-1, IGF-I or IGF-II are abundantly secreted from diverse cell types of the fetal-maternal interface 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/iCTBs also produce a variety of inhibitory proteins such as TGFβ family members, interferon-γ, endostatin, kispeptin-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, CCL4, CCL21 (Hanna et al., 2006), CXCL16 (Huang et al., 2006) were shown to increase trophoblast migration or invasion. Similarly, the interleukins IL-1, IL-6, IL-8 or IL-11 secreted from CTB/iCTB and 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 IGFBP5s (Giudice and Irwin, 1999; Lala and Chakraborty, 2003), soluble TNF receptors (Knöfler et al., 1998), sFlt-1 (Clark et al., 1998), a vEGF/PIGF 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 fulfill 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, TGFβ 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 (Handschooh 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)-

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 (arrow) as well as inhibitory effects on expression / secretion are depicted.
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; 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 syncytiotum 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 signals in trophoblast invasion

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 toPIP3. ElevatedPIP3 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 suggest 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., 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 GPCRs 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, paxillin 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 protein 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 (Illic et al., 2001; MacPhee et al., 2001). Down-regulation of FAK expression in villous explant cultures or CTB reduced in vitro migration and invasion (Illic 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 Rho-GDP 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 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 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.

Rule of Wnt/TCF in trophoblast differentiation and invasion

Throughout the years evidence accumulated that Wnt signalling could also be involved in placentation 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 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 EVTs.

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 counterparts 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 trophoderm differentiation in vitro but not in any of the different trophoblast cell types of first trimester placentae (Knöfler et al., 2002; Peifer 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 (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 (Handschu 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 cascade 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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References


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