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Local regulation of implantation at the human fetal-maternal interface

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ABSTRACT Embryo implantation and formation of a functional placenta are complex processes that require a plethora of regulatory molecules. In recent years, many of these mediators have been identified, often from studies in experimental animals. Furthermore, their expression patterns at the embryo-maternal interface in women have been characterized and provide clues to their potential actions. What has been missing in most cases is any experimental demonstration of their function. Proteases, cytokines and chemokines are among the molecules identified at the embryo-maternal interface. Functional studies of the protease, proprotein convertase (PC)6, the gp130 cytokines, leukemia inhibitory factor (LIF) and interleukin (IL)11 and the chemokines, CX3CL1 and CCL14 demonstrate potential actions within the uterine cavity. These actions include: enhancing blastocyst development, modifying adhesive properties of the blastocyst and the uterine epithelial surface, and providing chemotactic guidance to the blastocyst. As implantation proceeds, PC6 and IL-11 also act to drive decidualization. The products (proteases, chemokines and cytokines) produced by these decidual cells provide a unique environment. This is important for both directing and restraining trophoblast invasion and for leukocyte trafficking into the decidua until the placenta is fully established.

KEY WORDS: *PC6, LIF, IL-11, chemokine, trophoblast, endometrium*

Introduction

Implantation in humans is a continuum, starting with apposition and attachment of the blastocyst to the apical surface of the endometrial luminal epithelium and continuing throughout the first trimester of pregnancy until the extravillous trophoblast taps into and remodels the maternal vasculature. A plethora of regulatory molecules have been demonstrated to play functional roles in many of the processes, including preparation of the endometrial stroma (decidualization) and epithelium for implantation and control of trophoblast adhesion, invasion and directional trafficking. These regulatory molecules include cytokines, chemokines and proteases, many of which are expressed in different cell types and with slightly different functions as implantation progresses. Although initial functional studies demonstrating critical functions at implantation have been performed in mice or in other mammalian species with different forms of placentation, subsequent studies of expression patterns in women, in whom *in vivo* functional studies are not possible, suggest that the same molecules have been adapted at various stages of implantation. This review will examine the evidence for roles for members of these three important classes of regulatory molecules at different stages during implantation in women (Fig. 1).

Proteases that regulate bioactivities of regulatory molecules: proprotein convertases

Biologically active proteins and peptides are often generated through post-translational modifications following gene transcription and protein synthesis. One such important modifica-

Abbreviations used in this paper: ECM, extracellular matrix; EVT, extravillous trophoblast; HB-EGF, heparin bound epidermal growth factor; HESC, human endometrial stromal cell; HSPG, heparan sulfate proteoglycans; IGFBP, insulin-like growth factor binding protein; IL-11, interleukin 11; IL-11Rα, interleukin 11 receptor α; JAK/STAT, janus kinase/signal transducer and activator of transcription; LIF, leukemia inhibitory factor; LIF-R, leukemia inhibitory factor receptor; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; NK cells, natural killer cells; PC, proprotein convertase; SOCS, suppressor of cytokine signaling; TIMP, tissue inhibitor of metalloproteinases.

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tion is limited proteolysis which converts precursor proteins into their active forms. The most critical enzymes responsible for proteolytic activation of precursor proteins are seven closely related serine proteases (furin, PC1/3, PC2, PC4, PACE4, PC5/6, PC7/PC8/LPC) known as proprotein convertases (PCs) (Bergeron *et al.*, 2000; Fugere and Day, 2005; Seidah and Chretien, 1999; Seidah *et al.*, 2008). PCs post-translationally process and hence regulate the temporal and spatial activation of a large number of pro-proteins, including growth factors, peptide hormones, neuropeptides, ECM proteins, adhesion molecules, proteolytic enzymes, and integral membrane proteins through a controlled proteolytic cleavage at basic residues (usually arginines) within the general motif of (K/R)-(X)n-(K/R)↓ (where n= 0, 2, 4 or 6, X is any amino acid, and ↓ indicates the site of cleavage) (Seidah and Chretien, 1999). PCs are thus regarded as "master switch" molecules and recognized as promising targets for therapeutic approaches.

Gene discovery studies, especially through the use of microarray and proteomics technologies, have identified a number of genes and their protein products that are important for implantation (Carson *et al.*, 2002; Giudice, 2003; Giudice and Irwin, 1999; Kao *et al.*, 2002; Riesewijk *et al.*, 2003; Sharkey and Smith, 2003). These include cell surface proteins (eg. selectins), ECM components (eg. proteoglycans), tissue remodeling proteases (eg. metalloproteinases), cell adhesion molecules (eg. integrins), vasoactive factors (eg. prostaglandins), growth factors (eg. epidermal growth factor), cytokines (eg. leukemia inhibitory factor) and peptide hormones (eg. calcitonin). Many of these proteins are synthesized initially as inactive precursor proteins and require post-translational activation.

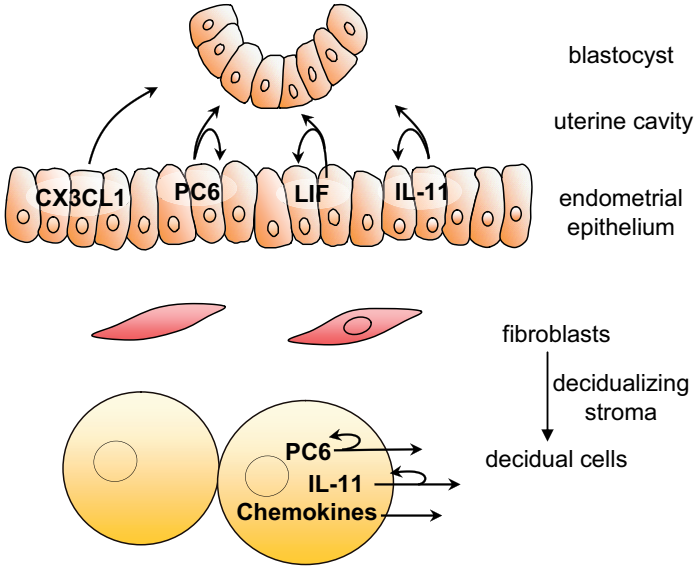

Fig. 1. Molecular interactions at implantation. *Interactions between the blastocyst/trophoblast, uterine epithelium and decidualized stromal cells at implantation are in part mediated by the cytokines, interleukin 11 and LIF, and chemokines including CX3CL1 which are released into the uterine cavity: their receptors are present on the blastocyst. The cytokines also act in an autocrine manner on the epithelium. PC6, an enzyme that processes molecules to their biologically active forms, is likewise produced in the receptive epithelium. Such mediators are also produced by decidualizing stromal cells: PC6 and IL-11 are critical for decidualization, while the secreted chemokines play important roles in both leukocyte and trophoblast trafficking through the decidual compartment.*

One of the PCs, PC6, was identified as being dramatically up-regulated in the mouse uterus at implantation, with the up-regulation being implantation site specific, transient and coinciding with the time of embryo attachment and implantation (Nie *et al.*, 2003). When PC6 protein synthesis was knocked down in the uterus using antisense morpholino oligos (designed to target the initiation site for PC6 protein translation), prior to the expected time of PC6 up-regulation during early pregnancy, implantation was completely inhibited (Nie *et al.*, 2005b). Thus endometrial PC6 is critical for implantation in the mouse.

Gp130 cytokines: LIF and IL-11

The gp130 cytokines (which include leukemia inhibitory factor (LIF) and interleukin (IL)-11) share an accessory signal transducing subunit gp130 while having separate specific low affinity α receptor (R) subunits. Binding of each cytokine to its cognate Rα triggers dimerization with gp130, forming a high affinity receptor and leading to activation of the janus kinase/signal transducer and activator of transcription (JAK/STAT) signal transduction pathway (Heinrich *et al.*, 1998). Signal transduction is attenuated via the suppressors of cytokine signaling (SOCS) family of cytoplasmic proteins that complete a negative feedback loop (Stewart *et al.*, 1992).

LIF and IL-11 have both been implicated as critical players in the implantation process. LIF was the first cytokine to be shown to be essential for implantation in mice (Stewart *et al.*, 1992). LIF is upregulated in the uterine epithelium just prior to implantation and mice null for the LIF gene fail to become pregnant. However, LIF-null embryos transplanted into wild-type mothers implant and develop normally, proving that it is endometrial LIF that is needed for implantation (Bhatt *et al.*, 1991). Mice lacking the receptor for IL-11 also have a fertility defect, which, unlike that in the LIF deficient mice, occurs in the post-implantation response to the implanting blastocyst and results from defective decidualization of the endometrial stroma (Bilinski *et al.*, 1998; Robb *et al.*, 1998).

Chemokines: CX3CL1 and CCL14

Chemokines are small chemotactic cytokines, best known as key regulators of leukocyte recruitment and activation. There are more than 50 chemokines and a lesser number of specific receptors: thus there is promiscuous ligand-receptor binding, considerable redundancy and overlap of functions. Although first identified for their role in leukocyte trafficking, a number of chemokines and their receptors have recently been identified at the fetal-maternal interface (reviewed in Hannan and Salamonsen, 2007) suggesting roles in implantation. These include CX3CL1 (also known as fractalkine) and CCL14 (also known as HCC-1). CX3CL1, exists as both a membrane-anchored adhesion molecule that can capture/coordinate leukocyte migration in an integrin- and selectin-independent manner, and as a soluble chemotactic peptide that cleaves from the cell surface. Unusually, the binding of CX3CL1 to its receptor CX3CR1, is highly specific. CCL14 exists in a range of proteolytically processed forms which have different agonist activities for its receptors, CCR1 and CCR5. Both chemokines are produced maximally by the human endometrium at the time of embryo implantation and during early

pregnancy (Hannan *et al.*, 2004; Jones *et al.*, 2004) while their receptors are present on trophoblast (Hannan *et al.*, 2006) providing an opportunity for functions in directional trophoblast migration as well as in leukocyte migration to the implantation site.

Roles of endometrial factors during implantation

Factors produced by endometrial epithelium during the 'window of receptivity'

In women, the endometrium is receptive to implantation only during a very short period of time in the mid-secretory phase of each menstrual cycle; this is known as the 'window of receptivity'. Under the influence of progesterone, the epithelial cells become highly secretory: indeed analysis of uterine fluid and data from immunohistochemical studies indicate that regulatory molecules including LIF (Laird *et al.*, 1997), CX3CL1 and CCL14 (Hannan *et al.*, 2004; Jones *et al.*, 2004) are released into the uterine lumen where they can affect the blastocyst even prior to attachment. Other changes to the luminal epithelium include alterations to cell surface glycoconjugates such as mucins (Aplin *et al.*, 2001; Gipson *et al.*, 2008), and to adhesion molecules on both apical and lateral membranes (Aplin, 1997; Murphy, 2004). In women, PC6, LIF, IL-11, CX3CL1 and CCL14 are all maximally expressed in the luminal and glandular epithelium during the mid-secretory phase of the menstrual cycle (see below). Interestingly, this expression pattern for PC6 and IL-11 is different from that seen in mice, in which these molecules are detected only in decidual cells as decidualization of the stroma proceeds, most likely reflecting the much higher importance of the epithelium during implantation in women: in mice the epithelium is lost immediately following attachment. The potential importance of epithelial production of such factors is emphasized by the reduced intensity of epithelial immunostaining for LIF and IL-11 in certain women with infertility (Dimitriadis *et al.*, 2007a; Dimitriadis *et al.*, 2006a).

In the rhesus monkey, PC6 is expressed in the glandular epithelium during a conception cycle, but the expression is dramatically increased at the expected time of implantation (Nie *et al.*, 2005b). In the human, PC6 expression in the epithelium is also increased significantly in the mid-secretory phase in anticipation of implantation (Nie *et al.*, 2005b). We have also investigated the expression of the other PC family members in the human endometrium (Freyer *et al.*, 2007). RT-PCR detected endometrial expression of mRNA for furin, PACE4, PC4 and PC7, but a negligible level for PC1/3 and PC2. None, however, showed clear up-regulation at the expected time of implantation. Furin, PACE4 and PC7 were immunolocalized to the epithelium with no apparent up-regulation in the mid-secretory phase.

In endometrium of women of proven fertility, LIF mRNA is expressed during days 18 to 28 of the menstrual cycle (Arici *et al.*, 1995; Charnock-Jones *et al.*, 1994; Dimitriadis *et al.*, 2000; Kojima *et al.*, 1994; Sharkey *et al.*, 1995; Vogiagis *et al.*, 1996). Both LIF mRNA and protein are localized in uterine glandular and luminal epithelium (Sharkey *et al.*, 1995; Vogiagis *et al.*, 1996), with immunoreactive protein being maximal in both luminal and glandular epithelium between day LH+6 and +9 coinciding with pinopode formation (Aghajanova *et al.*, 2003). Immunoreactive LIF has also been observed in stroma (Aghajanova *et al.*, 2003; Baird *et al.*, 1996; Danielsson *et al.*, 1997; Vogiagis *et al.*, 1996). LIF-R immunostaining is also maximal in both luminal and glandular epithelium between day LH+6 and +9 (Aghajanova *et al.*, 2003).

It appears that progesterone is a major regulator of LIF expression in primates. Not only does endometrial expression coincide with progesterone domination of the tissue, but treatment of women with the progesterone receptor antagonist, mifepristone (RU486) immediately after ovulation, reduces LIF immunoreactivity at the expected time of implantation (Danielsson *et al.*, 1997). However, locally produced factors, including heparin bound epidermal growth factor (HB-EGF) (Arici *et al.*, 1995; Lessey, 2002) prokineticin 1 (Evans *et al.*, 2008) and human chorionic gonadotrophin (Licht *et al.*, 2007; Perrier d'Hauterive *et al.*, 2004) have been shown to regulate LIF secretion by cultured endometrial epithelial cells in culture and its production *in vivo.* Interestingly, p53 which is widely known for its function as a tumor suppressor, regulates transcription of the mouse endometrial *Lif* gene, probably by interaction with the estrogen receptor (Hu *et al.*, 2007); whether this is the case also in women remains to be determined.

The biological actions of endometrial LIF are not yet fully understood although evidence suggests that LIF is important for human fertility. The glandular expression indicates likely secretion into the uterine lumen and indeed LIF protein is maximal in uterine flushings in the mid-late secretory phase of the menstrual cycle at the time of expected implantation in fertile women. This is reduced in flushings from women with unexplained fertility (Laird *et al.*, 1997; Ledee-Bataille *et al.*, 2002). Should LIF be released also basally from uterine epithelium, paracrine effects on the underlying stroma and leukocytes would be possible. To support this data, endometrial explants from infertile women secrete less LIF compared to biopsies from fertile women (Delage *et al.*, 1995); however, the effects of culture on cytokine production from explants were not taken into account. Another study revealed that LIF mRNA levels did not differ between fertile and infertile women (Sherwin *et al.*, 2002) although higher levels of soluble gp130 were found, suggesting inhibition of LIF action. LIF immunostaining is also reduced in endometrium of women who were infertile compared to fertile women (Tsai *et al.*, 2000) and in a cohort of women with infertility and endometriosis (Dimitriadis *et al.*, 2006a).

IL-11 is also expressed in glandular and luminal epithelium in mid-secretory phase endometrium suggesting a role in uterine receptivity (Cork *et al.*, 2001; Dimitriadis *et al.*, 2000) while prokineticin 1, which provides a potent stimulus for IL-11 production is likewise produced in endometrial epithelium (Evans *et al.*, 2008). Importantly, IL-11Rα and gp130 are also expressed in both luminal and glandular epithelium in the mid secretory phase (Cork *et al.*, 2002; Cullinan *et al.*, 1996). However, it appears that there is no cyclical variation in IL-11Rα expression and thus the expression pattern of ligand may be critical for IL-11 function in the endometrium. IL-11 is decreased in peri-implantation endometrium of excessive ovarian responders during IVF treatment suggesting that IL-11 is involved in the establishment in pregnancy, since high responders lead to failed IVF pregnancies (Makkar *et al.*, 2006). Furthermore, both IL-11 and IL-11Rα are reduced in the glands of some women with infertility and endometriosis (Dimitriadis *et al.*, 2006a), suggesting that disturbance of the production of IL-11 and its local action may result in infertility. It remains to be evaluated whether epithelial-derived IL-11 is secreted apically

into the uterine lumen or basally into the stroma.

An abundance of literature exists regarding chemokine expression in the endometrium, mostly focused on individual chemokines. However, the most abundant chemokines in the non-pregnant endometrium were recently identified using a non-biased gene array screen and tissue from all stages of the menstrual cycle. Among the chemokines subsequently validated by immunohistochemistry (summarized in Hannan and Salamonsen, 2007), and found to be elevated particularly in the epithelium during the mid-secretory phase of the cycle, were CCL8, CX3CL1, CCL4 and CCL14 (Hannan *et al.*, 2004; Jones *et al.*, 2004). Importantly, these are all chemoattractants for macrophages and all but CCL14 can also recruit natural killer cells: both these leukocyte subsets start to rise in number in the endometrium in the mid-secretory phase. However, the epithelial location of expression suggests alternative functions for these chemokines at implantation. Roles for chemokine action later in pregnancy have been proposed: these include cytotrophoblast targeting to maternal spiral arterioles and novel immune mechanisms for fetal allo-transplantation (Red-Horse *et al.*, 2001).

Factors of importance for decidualization

Decidualization can be defined as the process whereby endometrial stromal cells differentiate into a quite different cell type, the decidual cell, which along with specific leukocytes (uterine natural killer (NK) cells and macrophages) and altered blood vessels comprise the decidua of pregnancy. In the human, this process is initiated in the mid-secretory phase of each menstrual cycle, and when progesterone levels are maintained in early pregnancy, it continues throughout the pregnancy to maintain the decidua. IL-11 and PC6 mRNA and protein are strongly expressed in the decidualizing / decidualized stromal cells which start to appear in the normal menstrual cycle during the mid-late secretory phase: immunostaining for these factors is further enhanced in first trimester decidua. Both factors have been shown to be essential for decidualization.

Actions of interleukin 11

Decidualization in mice does not progress in the absence of IL-11 action (Robb *et al.*, 1998) while in women, IL-11 advances *in vitro* progesterone-induced decidualization of endometrial stromal cells (Dimitriadis *et al.*, 2002). Upregulation of IL-11 mRNA has been detected by gene array during progesterone or cAMP induced *in vitro* decidualization of endometrial stromal cells (Popovici *et al.*, 2000; Tierney *et al.*, 2003). Since both IL-11 and IL-11Rα immunolocalize in decidualized stromal cells of mid-late secretory phase endometrium in the human, it is likely that the action of IL-11 during decidualization is paracrine or autocrine (Cork *et al.*, 2002; Dimitriadis *et al.*, 2002). IL-11 secretion and mRNA expression by human endometrial stromal cells are stimulated by locally produced factors, relaxin and prostaglandin E_2, acting at least in part via cAMP during human endometrial stromal cell decidualization (Dimitriadis *et al.*, 2005). Importantly, IL-11 and progesterone mediated pathways converge during human endometrial stromal cell (HESC) decidualization (Dimitriadis *et al.*, 2006b). Progesterone stimulates both STAT3 protein (which is then phosphorylated upon activation of the IL-11 receptor complex) and SOCS3 protein (which attenuates IL-11 action and provides a feed-back loop) during decidualization. IL-11 initiates and progresses decidualization of HESC via pSTAT3 and SOCS3 (Dimitriadis *et al.*, 2006b) leading to highly regulated expression of other endometrial proteins associated with decidualization. In particular, IL-11 inhibits IGFBP-5 production and stimulates latent but not active IL-1β during decidualization of HESC (White *et al.*, 2005; White *et al.*, 2007). Importantly, IL-11 secretion is reduced during decidualization from HESC isolated from infertile women compared to fertile women (Karpovich *et al.*, 2005).

Actions of proprotein convertase 6

Likewise, endometrial PC6 is critical for decidualization and hence implantation in the mouse: when PC6 protein synthesis was knocked down using antisense morpholino oligos (designed to target the initiation site for PC6 protein translation), prior to the expected time of PC6 up-regulation during early pregnancy, implantation was completely inhibited (Nie *et al.*, 2005b). Endometrial PC6 (mRNA and protein) expression is also tightly associated with stromal cell decidualization both in the rhesus monkey and human (Nie *et al.*, 2005b). In the human, stromal PC6 expression is detected only in the decidual cells when decidualization occurs spontaneously prior to implantation during the menstrual cycle, and the decidual PC6 expression intensifies with the establishment of implantation. In the monkey, no stromal PC6 expression was detected during the menstrual cycle as no decidualization occurs spontaneously, but PC6 is highly expressed in decidual cells at implantation sites during early pregnancy (Nie *et al.*, 2005a).

Investigation of the expression of the other PC family members in the stromal cells across the menstrual cycle has not shown particular association of their expression with decidualization. Unlike PC6 which is localized specifically to decidual cells, furin, PACE4 and PC7 were localized to both non-decidualised and decidualised stromal cells. The lack of association of PCs other than PC6 with decidualization was further confirmed in an *in vitro* decidualization model using primary endometrial stromal cells isolated from human endometrial tissues (Freyer *et al.*, 2007; Okada *et al.*, 2005). Subsequently, the *in vitro* model was used to determine PC6's importance for decidualization (Nie *et al.*, 2005b). Knocking down of PC6 in stromal cells during the course of

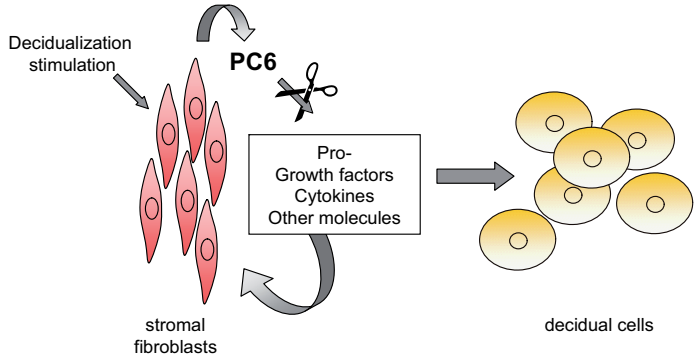

Fig. 2. PC6 is pivotal in human stromal cell decidualization. *PC6 expression is first seen in stromal cells as they start to decidualize and is critical for this process. Its proteolytic activity cleaves pro-forms of certain growth factors and cytokines and other molecules (particularly caldesmon and tropomyosin) to produce biologically active forms that are essential as decidualization proceeds.*

decidualization significantly inhibited the process of decidualization (Okada *et al.*, 2005), providing evidence that PC6 is critical for decidualization of in the human (Fig. 2).

An important remaining question is what substrates are cleaved by PC6 during decidualization? Two dimensional differential in gel electrophoresis of decidualized stromal cell lysates that had or had not been treated with active PC6 *in vitro*, recently identified caldesmon, tropomyosin-2 and tropomyosin-4 as being cleaved by PC6. In confirmation, caldesmon co-localizes with PC6 in decidual cells in the human endometrium *in vivo* (Nie *et al.*, 2008). Since caldesmon and tropomyosin are structural proteins previously found to be involved in actin filament reorganization, it appears that at least one action of PC6 is as a mediator of the structural remodeling of stromal cells during decidualization.

Decidual chemokines

Many chemokines are very strongly expressed in decidualized stromal cells (but not in non-decidualized stromal cells) (Hannan *et al.*, 2004; Jones *et al.*, 2004) and these appear to be among the molecules regulated by the 'molecular switch' which dramatically changes the phenotype of stromal cells as they decidualize. Importantly, expression of some of these (CXCL1, CXCL2, CCL8) can be further increased in decidualized stromal cells in culture following exposure to culture medium from trophoblast cells (Hess *et al.*, 2007).

Regulation of trophoblast function by factors of endometrial epithelial origin

Uterine fluid: influence on blastocyst development

The content of uterine fluid is likely to be of considerable importance in providing an optimal environment for pre-implantation blastocyst development, apposition and attachment; whether reduced levels of any individual factor play a key role in failure of blastocyst attachment and invasion remains to be established. While the high number and complexity of proteins present in uterine fluid is apparent when such fluid is analysed by two dimensional gel electrophoresis, the identity of most of these proteins is currently unknown; the most abundant are serum proteins (Parmar *et al.*, 2008). Whether these serum proteins are important for the blastocyst is not yet known.

A number of cytokines have been detected in uterine fluid by individual assay: these include LIF (Laird *et al.*, 1997; Mikolajczyk *et al.*, 2003), activin A (Florio *et al.*, 2003) and IL-18 (Ledee-Bataille, 2004) while recent analysis using Luminex technology has confirmed the presence of an abundance of cytokines, but with considerable variability between individuals (Boomsma *et al.*, 2008). There is also suggestive evidence that many other regulatory molecules are secreted from endometrial epithelial cells into the uterine lumen at the time of implantation. For example, certain chemokines including CX3CL1 and CCL8 are immunodetected first in the glands in the early secretory phase where they appear to be localized basally within the cells. As the cycle progresses into the mid-secretory phase, the protein becomes localized to the apical surface of these cells and can be detected also within the lumen of the glands (Fig. 3). Whether IL-11 or PC6 are secreted into the uterine lumen is not yet known: it is possible that the secreted form of PC6 could act on soluble substrates within the uterine cavity to regulate their bioactivity.

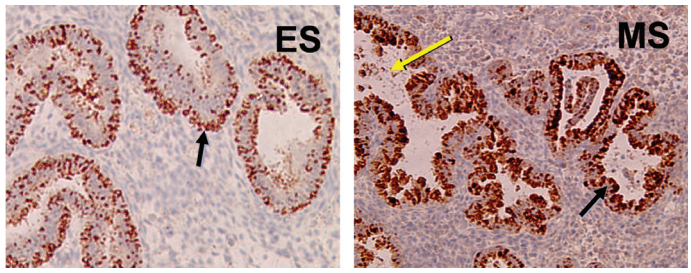

Fig. 3. Immunostaining for CCL7 in secretory phase endometrium. *The pattern of staining (brown) suggests secretion into the uterine lumen. Immunoreactive CCL7 is highly expressed during the early secretory phase (ES) of the cycle, basally in glandular and luminal epithelial cells (black arrow). By the mid-secretory phase (MS), the protein is identified towards the apical surface of these cells (black arrow) and also within the lumen of the glands (yellow arrow). (Reproduced with permission from Jones* et al.*, 2004).*

Stage specific expression of a number of cytokine receptors occurs on pre-implantation embryos (Sharkey *et al.*, 1995). Interestingly, while mRNA encoding receptors for c-fms and c-kit (receptors for colony-stimulating factor-1 and stem-cell factor respectively) appears not to be stage-specific, mRNA for the LIF receptor (LIF-R), IL-6 receptor and gp130, do not appear until the blastocyst stage, suggesting differing roles for the specific ligands. In confirmation, blastocysts produced by *in vitro* fertilization and cultured to the peri-implantation stage likewise express LIF-R transcripts (Charnock-Jones *et al.*, 1994). *In vitro*, LIF promotes human blastocyst formation rates of human embryos cultured in serum-free medium (Dunglison *et al.*, 1996); whether LIF exerts similar or additional effects on human pre-implantation blastocysts *in vivo* is not yet known.

Chemokine receptors (specifically CCR2B and CCR5) are also present on the pre-implantation blastocyst (Dominguez *et al.*, 2003): it may be that other receptors are also present. However identification of receptors and actions of their ligands on human blastocysts is limited by the very restricted access to these worldwide and most studies have therefore been carried out on trophoblast cell lines, that generally reflect somewhat later stages of trophoblast development (Hannan *et al.,* 2009). Chemokines present in uterine fluid are likely to set up concentration gradients to assist blastocyst apposition, while their actions on trophoblast adhesion that have been demonstrated in cell lines (Hannan and Salamonsen, 2008), may represent actions on pre-implantation blastocysts.

Uterine epithelium: interaction with trophoblast during adhesion and attachment

Changes to the luminal epithelium are necessary for successful implantation. In particular, these include changes to the apical surface to facilitate attachment and alterations at the lateral surface to facilitate penetration of trophoblast between the epithelial cells.

PC6 is known to process a number of adhesion related molecules including integrins (Lissitzky *et al.*, 2000; Stawowy *et al.*, 2004) and neural adhesion protein-L1 (Kalus *et al.*, 2003). It was recently demonstrated that PC6 interacts with heparan sulfate proteoglycans (HSPGs), thereby recruiting itself to the cell surface where it can activate a number of HSPG-bound substrates

both at the cell surface or in the extracellular space (Mayer *et al.*, 2008; Seidah *et al.*, 2008). Experiments are needed to identify which proteins PC6 actually activates in the endometrial epithelium, but it is tempting to speculate that PC6 likely regulates adhesion molecules and other cell surface proteins during the establishment of endometrial receptivity, to facilitate epithelial-trophoblast interaction for embryo attachment and implantation.

Functional roles for both LIF and IL-11 on the uterine epithelium during the peri-implantation period have recently been demonstrated. IL-11 stimulates (but LIF has no effect on) the adhesion of human trophoblast to human endometrial epithelial cells, possibly by its demonstrated stimulation of epithelial integrin $\alpha 2$ mRNA and protein (Marwood *et al.*, 2009). By contrast, both IL-11 and LIF stimulate the adhesion of primary human endometrial epithelial cells to fibronectin via phosphorylation of STAT3, actions that are abolished by specific inhibitors (Dimitriadis *et al.*, 2007b). The contraceptive potential of blocking LIF action in the uterus has also been demonstrated. Administration of a long-acting LIF antagonist blocked LIF action in the uterine luminal epithelium and prevented implantation in mice (White *et al.*, 2007).

Uterine decidual cells: interaction with trophoblast during invasion

Once the trophectoderm has breached the uterine epithelium and formed a syncytium, certain extravillous trophoblast (EVT) cells break through the syncytium and migrate and invade through the maternal decidua to eventually establish a blood supply for the fetus. In humans, trophoblast invasion is unusually aggressive; interstitial EVT invade up to the first third of the uterine myometrium and home to the vicinity of the spiral arteries which they invade (becoming endovascular EVT) and remodel. Extensive communication between trophoblast subtypes and endometrial cells is required for the entire invasive process. Furthermore, the decidua plays an important role in restraining trophoblast invasion which can be lethal if it occurs outside of the uterus.

During pregnancy, LIF and LIF-R have been detected in the decidua and chorionic villi of first-trimester and term placenta in humans (Kojima *et al.*, 1994; Kojima *et al.*, 1995; Sawai *et al.*, 1995; Sawai *et al.*, 1997; Sharkey *et al.*, 1999). LIF-R mRNA and immunoreactivity localizes in both villous and extravillous trophoblast throughout pregnancy, and in endothelial cells of the fetal villi (Sharkey *et al.*, 1999). Strong expression of mRNA encoding LIF has also been detected in decidual leukocytes, which are abundant at the implantation site, suggesting that LIF may mediate interactions between maternal decidual leukocytes and invading trophoblast cells (Sharkey *et al.*, 1999).

Recent data supports a role for LIF in adhesion and differentiation of trophoblast cells to the invasive phenotype. Both of these are involved in trophoblast invasion, a process that if not finely regulated can result in abnormal placentation. Indeed, an abnormally persistent expression of LIF receptor on EVT cells in placental bed biopsies has been demonstrated in patients with early onset pre-eclampsia combined with intrauterine growth retardation (Reister *et al.*, 2006). In functional studies, LIF increased EVT cell adhesion to fibronectin, vitronectin and laminin (Fig. 4A) (Tapia *et al.*, 2008) and decreased integrin β 4 mRNA levels compared to controls. Although matrix metalloproteinase (MMP)2 and MMP9 secretion from EVT were not affected, LIF increased secretion of tissue inhibitor of metalloproteinases (TIMP)-1, thus altering the MMP/TIMP balance. These data therefore suggest a local mechanism for decidual cell restraint of invasion.

Both IL-11 and IL-11 receptor α (Rα) mRNA and protein are present in decidual cells during early pregnancy (Chen *et al.*, 2002; Cork *et al.*, 2002; Dimitriadis *et al.*, 2003; Dimitriadis *et al.*, 2002; Karpovich *et al.*, 2003). Furthermore, invasive trophoblast cells are a source of IL-11 and IL-11Rα during early pregnancy in primates, suggesting an involvement in placentation (Chen *et al.*, 2002; Dimitriadis *et al.*, 2003). In subsequent detailed studies of the receptor, immunoreactive IL-11Rα was localized to the cell column and subpopulations of interstitial and endovascular EVT cells *in vivo* (Paiva *et al.*, 2007).

The EVT-hybridoma cell line, AMI88, likewise expresses IL-11Rα but does not produce IL-11, thus providing a model to study effects of IL-11 on EVT. Both chemotactic migration and invasion are features of cellular movement through a tissue. IL-11 acted as a chemotactic agent in stimulating migration of the EVT-hybridoma cells (Fig. 4B) but was without effect on their proliferation. In primary EVT, blocking endogenous IL-11 inhibited EVT migration by 30-40%, confirming the effect on migration is relevant *in vivo* (Paiva *et al.*, 2007). Conversely, IL-11 significantly inhibited invasion of EVT cells by 40-60% and this IL-11-mediated inhibition occurred via STAT-3 and not MAPK signaling pathways. The

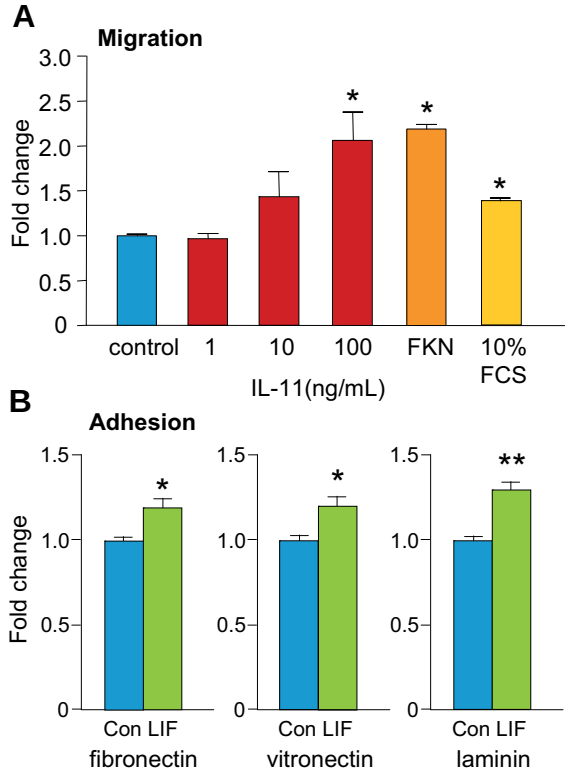

Fig. 4. Endometrial epithelial LIF and IL-11 have paracrine actions on trophoblast. (A) *IL-11 stimulates migration of extravillous trophoblast cells in a dose dependent manner. CX3CL1 (Fractalkine, FKN) and fetal calf serum (FCS) which likewise stimulate migration, were used as positive controls.* **(B)** *LIF stimulates adhesion of extravillous trophoblast to the matrix components, fibronectin, vitronectin and laminin. (Reproduced with permission from Paiva* et al.*, 2007; Tapia* et al.*, 2008).*

mechanism by which IL-11 inhibits EVT invasion remains unclear since IL-11 had no effect on common protease systems (i.e. MMP2 or -9, TIMPs 1-3, the urokinase plasminogen activator system) and cell adhesive properties that are known to play important roles during EVT invasion (Paiva *et al.*, 2008). This anti-invasive action of IL-11 is in contrast to its pro-invasive role during tumorigenesis of human cancers, reinforcing the tissue / cell specificity of such actions and the important role of decidua in restraining trophoblast invasion.

Chemokines have recently emerged as key players in directing migration of trophoblast using mechanisms akin to those used in leukocyte trafficking (Dominguez *et al.*, 2002; Hannan and Salamonsen, 2007). Indeed, there are considerable similarities between the events of implantation, where trophoblast must attach and invade, and those of leukocyte extravasation from the lumen of the blood vessel, through the endothelial cell basal lamina and into tissue. Once the trophoblast has penetrated the epithelial layer it lies within the unique chemokine-rich environment. A wide range of chemokines are among the molecules that appear once decidualization of stromal cells has occurred, and their production by decidual cells continues throughout the first trimester. Several studies have demonstrated that EVT within first trimester decidua express several chemokine receptors, including CX3CR1, CCR1 and CCR3 (Hannan *et al.*, 2006) and indeed, CCR1 appears to be switched on just as the EVT takes on invasive properties (Sato *et al.*, 2003).

Trophoblast (EVT cell) migration has been demonstrated in response to CX3CL1, CCL14 and CCL4 and in particular, conditioned medium from both epithelial and decidualized but not non-decidualized stromal cells likewise stimulated trophoblast migration (Hannan *et al.*, 2006). This was partly blocked by neutralization of CX3CL1 and CCL4, demonstrating that at least these two chemokines are likely to have such effects *in vivo*. This is in accord with other published work showing that CXCL12, CXCL16 and CCL12, that are also present in first trimester decidua, likewise stimulated migration (Red-Horse *et al.*, 2001). Such cross talk from decidual cells to trophoblast at the maternal fetal interface also appears to occur in reverse (Hess *et al.*, 2007).

Chemokine regulation of leukocyte trafficking is via regulation of adhesion molecules: similar mechanisms have now been demonstrated to apply also to trophoblast migration. Alterations in adhesion and extracellular matrix (ECM) molecules in response to chemokine stimulation of EVT were examined by pathway specific array analysis and findings validated by real-time RT-PCR, Significant changes in the mRNA transcripts of α-catenin *(CTNNA1),* extracellular matrix protein-1 (*ECM1*), osteopontin (*SPP1*), integrin α6 (*ITGA6*), MMP12 (*MMP12*) and integrin β5 (*ITGB5*) followed treatment by CXCL2 and/or CCL14. Immunohistochemistry confirmed the presence of integrin α6, SPP1 and ECM1 protein in first trimester human implantation sites (Hannan and Salamonsen, 2008). This inter-related temporal and spatial expression of chemokines, their receptors, adhesion and ECM at the maternal-fetal interface emphasizes an important role in the controlled directional migration of trophoblast through the maternal decidua.

Conclusions

It is of considerable interest that the same factors appear to be repetitively used, albeit by different cell types and with different functional outcomes, throughout the early stages of pregnancy in the human and also that factors which are expressed only in limited cell types during establishment of pregnancy in mice, are more widely expressed and over a longer relative time span in humans. Apparently the same regulatory molecules are adapted for different functions in different physiological situations. It is to be hoped that understanding the actions of these molecules throughout the establishment of pregnancy, will inform choices for targets for therapeutic interventions to reduce the impact of infertility and of pregnancy-related problems such as early miscarriage or inadequate placentation and the resultant pre-eclampsia.

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