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Review

Basic aspects of implantation

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Abstract

Implantation, a critical step for establishing pregnancy, requires molecular and cellular events resulting in healthy uterine growth and differentiation, blastocyst adhesion, invasion and placental formation. Successful implantation requires a receptive endometrium, a normal and functional embryo at the blastocyst stage and a synchronized dialogue between maternal and embryonic tissues. In addition to the main role of sex steroids, the complexity of embryo implantation and placentation is exemplified by the number of cytokines and growth factors with demonstrated roles in these processes. Disturbances of the normal expression and action of these cytokines result in absolute or partial failure of implantation and abnormal placental formation in mice and humans. Members of the gp130 cytokine family, interleukin (IL)-11 and leukaemia inhibitory factor, the transforming growth factor-β superfamily, colony-stimulating factors, and the IL-1 and IL-15 systems are all crucial for successful implantation. In addition, chemokines are important both in recruiting specific cohorts of leukocytes to the implantation site, and in trophoblast trafficking and differentiation. This review provides discussion on embryonic and uterine factors that are involved in the process of implantation in autocrine, paracrine and/or juxtacrine manners at hormonal, cellular, and molecular levels.

Keywords: *blastocyst, endometrium, implantation, pregnancy, uterine biology*

Introduction

Development of the embryo to the blastocyst stage, its implantation into the endometrium, and the formation of a functional placenta are essential steps in the establishment of pregnancy. Like many developmental processes, a complicated series of genetic, molecular and cellular interactions are involved, all of which must be executed within an optimal time frame. In mammals, the fertilized egg undergoes many cell divisions to form a blastocyst, which is able to attach to the uterine epithelium. The implantation process starts when a free-floating blastocyst communicates with the endometrium. Next, adhesive interaction between the trophoblasts and endometrial surface epithelium, followed by local invasion, ensues (Dey *et al.*, 2004; Red-Horse *et al.*, 2004; Makrigiannakis and Minas, 2007). Therefore, implantation requires the synchronous development of a blastocyst competent to implant and an endometrium able to respond to the signals from the blastocyst (Nardo *et al.*, 2006).

During implantation, many endocrine, paracrine and autocrine interactions occur among maternal–maternal, embryonic–embryonic and maternal–embryonic cells that mediate a complex dialogue between endometrium and the conceptus. These developmental events are mainly carried out by sex steroids, human chorionic gonadotrophin (HCG), growth factors, cytokines, adhesion molecules, the extracellular matrix proteins and prostaglandins (Dey *et al.*, 2004).

The incidence of early pregnancy loss, during or immediately after implantation, is high, estimated at 25–40%. Of the pregnancies that are lost, 75% represent a failure of implantation. Failed implantation is also a major limiting factor in assisted reproduction (Norwitz *et al.*, 2001). This review focuses on recent developments in implantation biology, and discusses basic aspects of molecular, cellular and morphological mechanisms involved in the implantation.

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Early embryonic development

Following successful fertilization, in most mammals the zygote starts a 4–5-day journey from the ampulla portion of the Fallopian tube towards the uterus, developing to the morula stage by a striking increase in cell number. Most animals devote their early development to rapid and synchronous cell cycles. Whereas the overall amount of cytoplasm in the embryo remains constant, the number of nuclei and the amount of DNA increase exponentially. During this period, mRNA and proteins provided to the egg by the mother drive development. In contrast, the embryonic genome is transcriptionally activated only at later cell cycles. The gene expression patterns required for continued development are established by zygotic genome activation (Bettegowda and Smith, 2007). The morula undergoes reorganization processes at the cellular level, and becomes a compacting embryo. This is the first stage during which the most obvious morphological differentiation can be observed. The appearance of the inner cavity within the mass of cells is a sign of initiation of the formation of the blastocyst (Yamanaka *et al.*, 2006), and results in appearance of two distinct types of cells: the outer cell mass, also called the trophectoderm (TE), and the inner cell mass (ICM). TE gives rise to trophoblast and extra-embryonic structures such as placenta, while ICM gives rise to the embryo (Kodaman and Taylor, 2004; Lunghi, 2007). Investigations have identified several genes that are crucial for lineage segregations, which include *Oct4*, *Sox2*, *Nanog*, *Cdx2* and Eomesodermin (*Eomes*) (Johnson and McConnell, 2004). Their cell-specific expression and functions are summarized in **Figure 1** and **Table 1**.

Recently, Takaoka and co-workers identified the presence of an antagonist of Nodal, *Lefty-1*, a gene determining the left–right axis in mouse embryos at day 3.5 and later in a specific position in ICM. Surprisingly, *Lefty* expression is restricted to a tiny subset of cells in the ICM of the early blastocyst. In the late blastocyst,

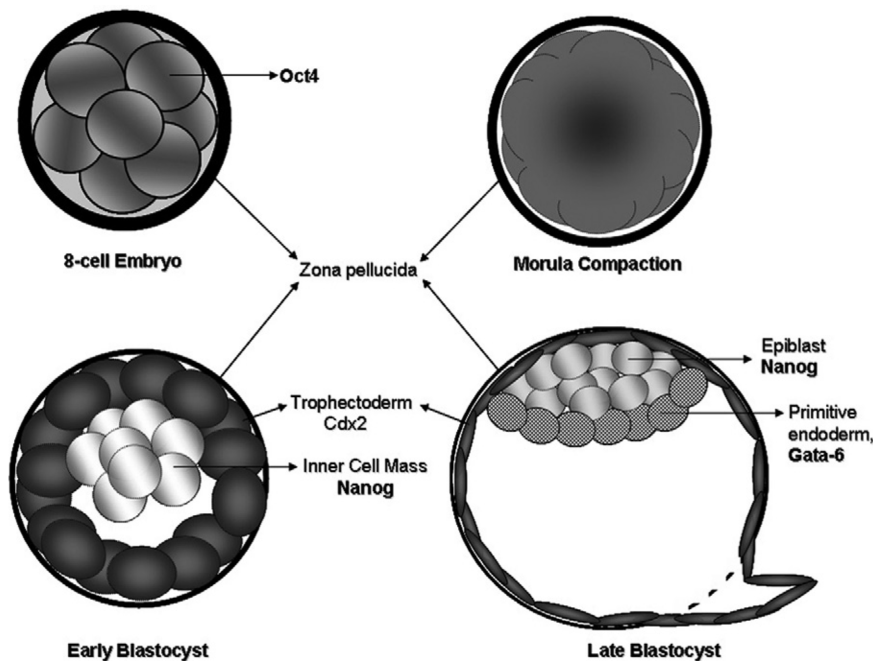

Figure 1. Lineage differentiation during 8-cell to blastocyst transition and gene expression patterns during blastocyte formation. *Oct4* is expressed throughout the embryo before the late morula, and crucial for the formation of inner cell mass. The expression of *Nanog* is induced in the inside cells of late morula. *Cdx2* is expressed in trophectoderm. *Gata6* is expressed in the primitive endoderm of the late blastocyst.

Table 1. Function of transcription factors known to be important for the fate of trophectoderm (TE) and the inner cell mass (ICM).

Gene	*Function*	*Reference*
Oct4	Expressing in nuclei of all cells of the early embryo, but its expression becomes restricted to the ICM upon blastocyst formation, preventing TE formation.	Kirchhof *et al.*, 2000
Sox2	A similar expression profile to *Oct4*, preventing trophoblast specification.	Avilion *et al.*, 2003
Nanog	Sustaining the self-renewal capacity of embryonal stem cells and inhibiting their differentiation ICM formation, visceral-parietal endoderm formation.	Mitsui *et al.*, 2003
Cdx2	Segregating ICM and TE lineages by ensuring the suppression of *Oct4* and *Nanog*.	Niwa *et al.*, 2005
Eomes	Required for TE proliferation and differentiation at the blastocyst stage.	Simmons and Cross, 2006
Lefty-1	Determining the left–right axis in mouse embryos.	Takaoka *et al.*, 2006

Lefty expression is localized to a small region of the primitive endoderm (PE) (Takaoka *et al.,* 2006). These observations support the notion that the ICM is initially a mosaic of epiblast (EPI) and PE cells, with a suggestion that an embryonic axis prepattern may exist as early as the blastocyst stage (Tabin, 2006). While there are many unanswered questions in terms of the relationships between the asymmetries of the blastocyst and the later axes of the embryo, there is accumulating evidence that early asymmetries in the relationships between the different lineages of the blastocyst will play roles in establishing later embryonic patterning. During the blastocyst stage, TE starts to hatch from the glycoprotein coat (the zona pellucida), and acquires the ability to attach the endometrium. At the same time, the uterine environment is able to support blastocyst growth, attachment, and the subsequent events of implantation (Tabibzadeh *et al.,* 1999). Therefore, a successful implantation depends on two important factors: blastocyst competency and endometrial receptivity, which are responsible for the embryo–maternal interaction necessary for the attachment and invasion of the blastocyst into the endometrium.

Recent studies on mice have also shown that new genes are involved in differentiation of trophoblast cell subtypes. *Hand1*, *Stra13* and *Imfa* are shown to be expressed specifically in trophoblastic giant cells, the main invasive cell type mediating implantation as well as the main endocrine cells of the placenta, producing several hormones that regulate the maternal endocrine and immune systems and promote maternal blood flow to the implantation site. It has been shown that decidual *Prlpa* expression decreases in the uterus adjacent to *Hand1* and *Ets2*-deficient embryos, suggesting that normal trophoblast giant cells in the placenta are required for the conceptus-dependent effects on *Prlpa* expression in the mesometrial decidua. Moreover, these cells are also shown to be positive for placental lactogen 1 and 2, type I interferon-like molecules that have been shown to have ability to induce *G1p2* expression in endometrial stromal cells (Simmons and Cross, 2005). However, these genes remain to be investigated in human embryos and decidual tissues to see if the human homologues, or similar genes, are expressed and function as in mouse embryos and decidua.

Morphological steps of implantation

The process of implantation is classified into three phases: apposition, attachment (adhesion) and penetration (invasion). Apposition is identified as the unstable adhesion of blastocyst to the endometrial surface. During this stage, the trophoblast becomes closely apposed to the luminal epithelium (Tabibzadeh and Babaknia, 1995). This is followed by a stable adhesion or attachment phase. Local paracrine signalling between the embryo and endometrium is believed to occur to trigger a stronger attachment. The first sign of attachment reaction occurs on the evening of day 4 in mice and on day 20–21 in humans, and coincides with a localized increase in the stromal vascular permeability at the site of blastocyst attachment (Sharkey and Smith, 2003). Penetration involves invasion of the embryo through the luminal epithelium and its basal lamina into the stroma, to establish a vascular relationship with the mother. Trophectoderm cells from the blastocyst migrate between the epithelial cells, displacing them and penetrating as far as the basement membrane (Nikas, 2000). The trophoblast then invades through the basement membrane, coming into contact with the underlying stroma. In response to this invasion, the endometrial stromal cells undergo decidualization. The timely completion of attachment and decidualization are essential for the viability of the pregnancy. In most pregnancies, HCG secreted by the embryo is detectable in maternal serum within 8–10 days of ovulation (Enders, 2000; Sharkey and Smith, 2003).

Human endometrium undergoes a complex series of organized proliferative and secretory changes in each menstrual cycle (Strowitzki *et al*., 2006). When implantation does not occur, there is a deliberate and timely destruction of the fully developed endometrium, which leads to menstruation, only for the same cycle of events to be repeated once again to prepare for the next generation blastocyst (Kayisli *et al.,* 2004; Strowitzki *et al*., 2006).

Blastocyst competency

The process of embryo implantation is far from being fully understood. In the past decade, many factors influencing the implantation process have been identified, and many researchers have focused on embryonic and maternal factors such as calcitonin, growth factors, integrins, cytokines and ovarian hormones. For mammalian development to continue beyond the first week, the embryo must establish intimate contact with uterine tissues and form a placenta that will provide an interface between the growing fetus and the maternal circulation (Aplin, 2006).

Blastocyst implantation in the uterine endometrium is a highly coordinated event that is dependent on the intrinsic embryonic programme operating in conjunction with extrinsic signals from the receptive uterus. Uterine-derived signals, e.g calcitonin, heparin-binding epidermal growth factor-like growth factor (HB-EGF), insulin-like growth factor binding protein-1 (IGFBP-1) and lysophosphatidic acid (LPA) maintain the pace of blastocyst development and mobilize embryonic receptors used for subsequent signalling. The blastocyst is primed to advance in development only after it receives the necessary external signals, most probably from the decidua (Armant *et al.,* 2000).

Mammals have evolved to sequentially deliver extrinsic signals to the preimplantation embryo by taking advantage of its location within the reproductive tract, temporal changes in the endometrium, and accessibility of embryonic cells to maternal compartments. However, preimplantation embryos are initially surrounded by an extracellular matrix, the zona pellucida, which limits paracrine signalling; only soluble molecules present in the fluids of the oviduct and the uterus can penetrate the zona and reach the embryo. Once freed from the zona pellucida, the blastocyst can interact directly with endometrial cells (Herrler and Beier, 2000). Growth factors tethered to cell surfaces become accessible for juxtacrine signalling, widening the scope of interactions. For example, epithelial cells adjacent to the hatched blastocyst produce the transmembrane form of HB-EGF, which activates receptors on the surface of trophoblast cells (Raab *et al.,* 1996). HB-EGF accelerates the rate of blastocyst differentiation *in vitro*, in part by stimulating $\alpha^5\beta^1$ integrin trafficking to the apical surface of trophoblast cells (Wang *et al.,* 2000). Later, the epithelial cells adjacent

to the embryo are lost and extracellular matrix components of the underlying basement membrane become accessible to the trophoblast. An important second messenger-integrating signal that reaches the embryo from both growth factors and extracellular matrix is Ca^{2+}, which plays a similar role in oocyte activation by initiating protein trafficking. The fine-tuning of trophoblast differentiation by extrinsic signals, including *Cdx2*, *Err2*, and *Eomes*, orchestrates the molecular interactions required for implantation in a precise and metabolically efficient sequence.

Hormonal and molecular determinants of implantation

Endometrial receptivity can be defined as the capacity of the uterine mucosa to facilitate successful embryonic implantation. Embryonic implantation is the process by which the embryo attaches to the endometrium, first invading the epithelium and then the endothelium of spiral arteries, forming the placenta. The timing of implantation has now been firmly established, occurring around 7–10 days after ovulation, corresponding to days 21–24 of an idealized 28-day menstrual cycle (Lessey *et al.,* 2006). Endometrial receptivity is manifested by molecular and genetic evidence, including cytokines [interleukin (IL)-6, IL-11], growth factors (EGF, TGF-β, FGF), transcription factors (*Hox*), and ovarian hormones (Achache and Revel, 2006).

Role of ovarian hormones in implantation

Ovarian steroids, progesterone and oestrogen are the major factors for blastocyst implantation. Progesterone is essential for implantation and pregnancy maintenance in all mammals, whereas the requirement for oestrogen is species specific (Dey *et al.,* 2004). In humans, proliferative-phase oestrogen plays a role in preparing the endometrium for progesterone action (Huet-Hudson and Dey, 1990). The uterine effects of oestrogen and progesterone are primarily executed by nuclear oestrogen and progesterone (ER and PR) receptors (Carpenter and Korach, 2006). The recent discovery of ER (ERα and ERβ) and PR (PR-A and PR-B) isoforms and studies of the effect of their selective deletion provide evidence for their isoform-specific functions in uterine biology and implantation. $ER\alpha^{-/-}$ mice uteri are hypoplastic and unable to support implantation (**Table 2**, Hewitt and Korach, 2000), whereas $ER\beta^{-/-}$ mice uteri retain biological function that allows normal implantation (Dey *et al.,* 2004). Interestingly, progesterone is sufficient for decidualization in $ER\alpha^{-/-}$ mice in response to artificial stimuli, which indicates that ERα might be essential for blastocyst attachment, but dispensable for subsequent decidualization (Curtis *et al.,* 1999; Paria *et al.,* 1999). In PR-A knockout mice, in which the expression of the PR-A isoform is selectively ablated (PRAKO), the PR-B isoform functions in a tissue-specific manner to mediate a subset of the reproductive functions of PR. Ablation of PR-A does not affect responses of the mammary gland or thymus to progesterone, but instead results in severe abnormalities in ovarian and uterine function, leading to female infertility (**Table 2**). These tissue-selective activities of PR-B are due to this isoform's ability to regulate a subset of progesterone-responsive target genes in reproductive tissues rather than to differences in its spatiotemporal expression relative to the PR-A isoform (Lydon *et al.,* 1995; Conneely and Jericevic, 2002). Even though many of the physiological roles of oestrogen and progesterone have been outlined, the molecular networks that mediate their actions are largely unknown. Further studies are required to elucidate the oestrogen- and progesterone-dependent molecular pathways leading to successful implantation.

Leukaemia inhibitory factor

Leukaemia inhibitory factor (LIF) is a secreted glycoprotein first described as a factor that induces macrophage differentiation of the myeloid leukaemia cell line M1. LIF, a member of the IL-6 type cytokine family, has a variety of functions, including stimulation of cell proliferation, differentiation and survival,

Table 2. Reproductive function in animals with targeted disruption of genes important in implantation.

Gene	*Reproductive function*	*Reference*
ERα	Infertile because of complete ovarian inefficiency, immature uteri, unresponsive to oestrogen	Hewitt and Korach, 2000
ERβ	Subfertile because of reduced ovarian efficiency, enhanced response to oestrogen	Harris, 2007
PRKO	Defective uterine implantation, lack of decidualization of uterine stromal cells in response to progesterone	Conneely *et al.*, 2002
IL-6	Reduced fertility, viable implantation sites decreased 48%	Robertson, 2000
LIF	Failure of implantation, unable to induced decidualization	Stewart *et al.*, 1992
LIFR	Intrauterine lethality	Ware *et al.*, 1995
IL-15	Fertile, but display impaired decidual integrity, unmodified spiral arteries and lack of uNK at the implantation sites	Ashkar *et al.*, 2003
IL-11	Fertility defect that, unlike that in the LIF-deficient mice, occurs in the post-implantation response to the implanting blastocyst	Laird *et al.*, 2006
HoxA10	Oviductal transformation of the proximal one-third of the uterus, defective implantation and embryonic reabsorption in the early post-implantation period	Benson *et al.*, 1996
HoxA11	No uterine glands; partial homeotic transformation of uterus to oviduct	Hsieh-Li *et al.*, 1995

all functions that are essential for blastocyst development and implantation (Laird *et al.,* 2006). LIF acts on cells by binding to heterodimeric LIF receptor, which consists of two transmembrane proteins, LIF receptor (LIFR) and gp130. LIF receptor activates several signalling pathways in diverse cells types, including Jak/STAT, MAPK, and PI3-kinase pathways (Duval *et al.,* 2000). In the endometrium of fertile women, LIF mRNA is expressed on days 18–28 of the menstrual cycle (Arici *et al.,* 1995; Sharkey *et al.,* 1995; Dimitriadis *et al.,* 2000). Moreover, both LIF mRNA and protein are localized mostly in uterine glandular and luminal epithelium in humans (Sharkey *et al.,* 1995; Vogiagis *et al.,* 1996), whereas immunoreactive LIF has also been observed in stroma (Baird *et al.*, 1996; Vogiagis *et al.,* 1996; Aghajanova *et al.,* 2003). LIFR mRNA is restricted to luminal and glandular epithelium in the mid-secretory phase in humans (Cullinan *et al.,* 1996; Aghajanova *et al.,* 2003). Strong expression of LIF mRNA has also been detected in human decidual leukocytes, which are abundant at the implantation site, suggesting that LIF may mediate interactions between maternal decidual leukocytes and invading cytotrophoblasts (Sharkey *et al.*, 1999). Progesterone is a likely regulator of LIF expression. However, locally produced factors, including HB-EGF and transforming growth factor-β1 (TGF-β1), have been shown to regulate LIF secretion in endometrial cells *in vitro* (Arici *et al.,* 1995; Lessey *et al.*, 2002) and may also be relevant *in vivo*. LIF protein is maximal in human uterine flushing in the mid–late secretory phase of the menstrual cycle at the time of expected implantation, suggesting a role in uterine receptivity (Laird *et al.,* 1997; Ledee-Bataille *et al.,* 2002). Furthermore, LIF appears to stimulate the expression of progesterone-regulated genes in the luminal epithelium in mice (Sherwin *et al.* 2004). LIF may also act on the embryo, as the blastocyst at the preimplantation stage expresses LIFR transcripts (Charnock-Jones *et al.,* 1994). A role for LIF in trophoblast cell growth and differentiation has also been shown (Kojima *et al.,* 1995; Sawai *et al.,* 1995; Ren *et al.,* 1997).

The uterine milieu in LIF-mutant mice fails to induce implantation irrespective of the blastocyst genotype, since $LIF^{(-/-)}$ blastocysts can implant after transfer to wild-type pseudopregnant recipients (**Table 2**; Stewart *et al.*, 1992; Escary *et al.*, 1993). These reciprocal embryo transfer experiments suggest that maternal LIF is essential for blastocyst implantation. However, a role for this cytokine in embryonic functions cannot be ignored, because LIFR and gp130 are expressed at the blastocyst stage, and administration of exogenous LIF improves embryo viability and hatching in several species (Dunglison *et al.,* 1996; Nichols *et al.,* 1996). Taken together, these data suggest that both the preimplantation embryo and the uterus are sites of LIF action. However, embryos lacking either LIFR or gp130 develop to the blastocyst stage and implant normally, but die during the perinatal period (Ware *et al.,* 1995). Thus, these results show that LIF may signal to both embryonic and uterine tissues during implantation (Laird *et al.,* 1997). Gp130 deficiency in mice leads to embryonic lethality (Yoshida *et al.,* 1996). Gp130 mRNA localizes predominantly to glandular and luminal epithelium in human endometrium (Cullinan *et al.,* 1996), and has also been demonstrated in human embryos from 3-cell stage onward (Sharkey *et al.,* 1995). Soluble gp130 is secreted from endometrial tissues obtained from women between days 20 and 26 of the menstrual cycle at a 20-fold higher concentration compared with secretion from endometrial tissue obtained during the proliferative phase. Importantly, secretion of soluble gp130 from endometrial tissues obtained between days LH +6 and LH +13 is reduced in infertile women compared with fertile women (Sherwin *et al.,* 2002). The presence of such soluble receptors in the endometrium has important implications in cytokine action.

Interleukin-6

IL-6 is a multifunctional cytokine that regulates various aspects of the immune response, acute phase reaction, and haematopoiesis. IL-6 has some functional redundancy with IL-11 and LIF. IL-6-deficient mice have reduced fertility and a decrease in viable implantation sites (**Table 2**; Robertson, 2000). In human endometrium, IL-6 is weakly expressed during the proliferative phase, but strong immunoreactivity is present during the mid-secretory phase, predominantly in the glandular and luminal epithelial cells (Tabibzadeh *et al.,* 1995; Vandermolen, 1996). Furthermore, the IL-6 receptor (IL-6R) is predominantly localized in glandular epithelium and to a lesser extent in the stroma throughout the menstrual cycle (Tabibzadeh *et al.,* 1995). Therefore, a role in human implantation could also be postulated for this cytokine, as for LIF and IL-11. On the other hand, when levels of IL-6 secretion were measured from endometrial biopsies obtained between days LH +6 and LH +13 from infertile women compared with fertile women, no difference was found (Sherwin *et al.,* 2002).

Interleukin-11

IL-11 was initially described as a growth factor acting at multiple stages during haematopoiesis, synergizing with other factors (Du and Williams, 1994). IL-11 has important anti-inflammatory activities (Sands *et al.,* 1999) as well as pleiotrophic actions in multiple cell types (Du and Williams, 1994). Mice lacking the receptor for IL-11 have a fertility defect, which, unlike that in the LIF-deficient mice, occurs in the post-implantation response to the implanting blastocyst (**Table 2**, Laird *et al.,* 2006). There is increasing evidence that IL-11 has an important function in human implantation. IL-11 and its receptor (IL-11Rα) have recently been demonstrated in the human endometrium. While all the major cell types in the endometrium expressed IL-11 with cyclical variation, the most dominant immunoreactive IL-11 was seen in the decidualized stromal cells late in the menstrual cycle (Dimitriadis *et al.,* 2000; Cork *et al.,* 2001; von Rango *et al.,* 2004). 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. Several studies have identified both IL-11 and IL-11Rα mRNA and protein in decidual cells from the late secretory phase and early pregnancy endometrium (Chen *et al.,* 2002; Cork *et al.,* 2002; Dimitriadis *et al.,* 2002). Furthermore, invasive trophoblast cells are a source of IL-11 and IL-11Rα during early pregnancy in primates, suggesting their involvement in placentation (Chen *et al.,* 2002) . IL-11 is involved in in-vivo and in-vitro decidualization (Dimitriadis *et al.,* 2002). Up-regulation of IL-11 mRNA was detected by gene array analysis during progesterone or cAMP-induced *in vitro* decidualization of endometrial stromal cells (Popovici *et al.,* 2000; Tierney *et al.,* 2003). Moreover, IL-11 and IL-11Rα are immunolocalized to decidualized stromal cells of mid–late secretory phase endometrium, demonstrating a paracrine and autocrine source of action (Cork *et al.,* 2002; Dimitriadis *et al.,*

2002). Interestingly, recent evidence in mice shows that IL-11 signalling is required for decidual-specific maturation of natural killer (NK) cells in mice (Ain *et al.,* 2004). This evidence indicates that IL-11 may be important in the establishment of viable pregnancies. To date, knowledge of IL-11 functions in implantation is only at a preliminary stage. Future studies should validate and further investigate the importance of this cytokine in human implantation.

Adhesion molecules

Integrins are a family of cell adhesion molecules intensively studied in the endometrium. Heterodimers of α and β integrins serve as receptors for extracellular matrix ligands such as collagen, laminin, and fibronectin, as well as transducing signals from soluble ligands such as osteopontin (Aplin, 2006). In humans, integrins $\alpha^2\beta^1$, $\alpha^3\beta^1$, $\alpha^6\beta^1$, $\alpha^9\beta^1$, $\alpha v\beta^1$, $\alpha^v\beta^3$, $\alpha^v\beta^5$, and $\alpha^v\beta^6$ have been described on the uterine luminal epithelium. The glandular epithelium expresses the same proteins, with the exception of $\alpha^v\beta^5$, and $\alpha^v\beta^6$ and the addition of $\alpha^1\beta^1$ and $\alpha^4\beta^1$ (Hoozemans *et al.,* 2004; Minas *et al.,* 2005). Some integrins such as α^4 and β^3 exhibit cycle-dependent changes in gene expression in both the stroma and epithelium throughout the cycle (Lessey and Castelbaum, 2002). In normal fertile women, the α4 subunit is up-regulated in the glandular epithelium at the time of ovulation and it disappears around cycle day 24. In contrast, β^3 expression in both glandular and luminal epithelium is up-regulated on day 19 or 20 (Gonzalez *et al.,* 1999; Acosta *et al.*, 2000*)*. Since β^3 up-regulation and α4 down-regulation coincides with the window of receptivity, this has led to the hypothesis that these integrins could be markers of the functionally receptive endometrium. In addition to the endometrium, the cytotrophoblasts also exhibit temporal and spatial regulation of integrin expression. At the pre-implantation phase, the human blastocyst expresses a number of integrin subunits, including α^v, α^3, β^1, β^3, β^4, and β^5. During this stage, integrins may be important for blastocyst cleavage and proper development, as well as initial adhesion to the uterine surface epithelium (Aplin, 1997). In addition to integrins, many ligands and their receptors are expressed in the uterine luminal epithelium and blastocyst cell surface such as selectins, heparin sulphate proteoglycans (HSPG), Muc-1, and cadherins as reviewed in detail by Kimber and Spanswick (2000).

Growth factors

The expression of various growth factors and their receptors in the uterus in a temporal and cell-specific manner during the preimplantation period suggests that some of these factors are important for implantation, for example members of the epidermal growth factor (EGF) family, TGFβ, fibroblast growth factors (FGF), insulin-like growth factors (IGF), and platelet-derived growth factors (PDGF) (Tazuke and Giudice, 1996). The EGF family of growth factors includes EGF itself, TGFα, HB-EGF, amphiregulin, beta-cellulin, epiregulin, and neuregulins (Dey *et al.,* 2004). HB-EGF is the earliest molecular marker found in the mice uterus, exclusively at the sites of active blastocysts, appearing several hours before the attachment reaction. This induction is followed by the expression of beta-cellulin, epiregulin, neuregulin-1, and Cox-2 around the time of the attachment reaction (Das *et al.,* 1995). Although strong expression of amphiregulin in the luminal epithelium is found only around the implanting blastocysts, and this expression is absent by day 5 of pregnancy, knockout mice for EGF/TGFα/amphiregulin do not exhibit implantation defects, because HB-EGF, betacellulin, epiregulin, neuregulin, and amphiregulin all show overlapping uterine expression patterns around the implanting blastocyst at the time of attachment in mice (Das *et al.,* 1995, 1997a,b). The EGF-like growth factors interact with the receptor subtypes of the *erbB* gene family, which is comprised of four receptor tyrosine kinases: ErbB1 (EGF-R), ErbB2, ErbB3, and ErbB4. They share common structural features, but differ in their ligand specificity and kinase activity (Olayioye *et al.,* 2000). Spatiotemporal expression patterns of EGF gene family members and ErbB in the uterus during the peri-implantation period suggest compartmentalized functions of EGF-like growth factors in implantation. ErbB1 (EGF-R), ErbB2, and ErbB4, the receptor subtypes for the EGF family of growth factors, are expressed in the mouse blastocyst. HB-EGF appears to play a role in implantation and embryonic development. Its expression is maximal during the late secretory phase, when the endometrium becomes receptive for implantation (Rathjen *et al.*, 1990) and cells expressing the transmembrane form of HB-EGF adhere to human blastocysts displaying cell surface ErbB4 (Chobotova *et al.,* 2002).

Matrix metalloproteinases (MMP)

Tissue remodelling and angiogenesis are hallmark events during implantation and decidualization. The changing endocrine state of the female during the reproductive cycle and pregnancy results in extensive remodelling of the uterine tissue (Curry and Osteen, 2003). For example, in human endometrium, various basement membrane components, such as type IV collagen, laminin, fibronectin, and proteoglycans, undergo changes throughout the menstrual cycle and pregnancy (Aplin *et al.,* 1988). Matrix metalloproteinases (MMP) and tissue inhibitors of MMP (TIMP) are thought to be key mediators for matrix degradation during implantation and decidualization (Das *et al.,* 1997a,b; Vu and Werb, 2000). Evidence for in-vivo role for MMP-9 in implantation comes from studies of the Ets-2 and MMP-9-null mice, as well as of TIMP-1 overexpressing mice and pharmacogenetic studies using chemical inhibitors. Ets-2 is a member of the Ets family of transcription factors that regulate the transcription of diverse genes, particularly MMP. The Ets-2-deficient mice die early in embryogenesis, because of defective development of the placenta (Yamamoto *et al.,* 1998). The embryos die by embryonic day (E)8.5. At E7.5, there is a smaller amount of attached trophoblastic tissue. At E6.5, the ectoplacental cone is small, apparently because of failure of trophoblast migration. A membrane, immunostained for laminin, covers the ectoplacental cone and extends into and over the trophoblasts. There is also poor connection with the maternal circulation. Interestingly, in trophoblasts, expression of MMP-9, which is a transcriptional target of Ets-2, is significantly decreased in the Ets-2-null mice. Thus, the placental phenotype in Ets-2 deficiency may be due to insufficient MMP-9 activity in the trophoblast. In support of this model, TIMP-1 overexpressing, metalloproteinase inhibitor-treated mice (Alexander *et al.,* 1996) and MMP-9-null mice on some genetic backgrounds (Rinkenberger *et al.,* 1997) display similar placental phenotype, even though these do not lead to lethality. There is evidence that a balance between a select set of MMP and TIMP is important for implantation. Mechanisms regulating the MMP and TIMP genes during the

periimplantation period are not clear, although growth factors and cytokines including the EGF and TGFβ family members and LIF have been shown to modulate MMP and TIMP (Vu and Werb, 2000).

Homeobox genes

Hox genes are transcription factors that belong to a multigene family and are evolutionarily highly conserved and act as regulators of embryonic morphogenesis and differentiation. They are developmentally regulated and share a common highly conserved sequence element called the homeobox that encodes a 61-amino acid helix–turn–helix DNA binding domain. Although animal species differ widely in appearance, they all use *Hox* genes to establish their body plans (Krumlauf, 1994). There are two *Hox* genes that are thought essential for implantation in mice because homozygous mutants of either of these genes are infertile as a result of unreceptive endometrium. The genes *HoxA10* and *HoxA11* are expressed in the endometrial glands and stroma of the uterus throughout the menstrual cycle in humans (Benson *et al.*, 1996). The expression of both of these genes rises dramatically at the time of implantation during the mid-luteal phase, and thereafter remains elevated throughout the remainder of the cycle as well as in the decidua of pregnancy in humans. This pattern of expression in the adult suggests that *Hox* genes play a role in human implantation, as seen in mice (Benson *et al.*, 1996; Lim *et al.*, 1999). The mechanisms by which the *Hox* genes interfere with implantation is unknown, but mice homozygous for *HoxA10* deficiency show oviductal transformation of the proximal one-third of the uterus, defective implantation and embryonic reabsorption in the early post-implantation period, suggesting that *HoxA10* may be important during morphogenesis for proper patterning of the reproductive tract and in adult endometrium for adequate implantation events (Benson *et al.*, 1996). Uterine stromal cells in *HoxA10*-deficient female mice show reduced proliferation in response to progesterone, leading to decidualization defects (Benson *et al.*, 1996; Lim *et al.*, 1999). Furthermore, because several progesterone-responsive genes are dysregulated in the uterine stroma of *HoxA10* mutant mice, *HoxA10* may convey progesterone responsiveness in the uterine stroma by regulating gene expression. Similarly, the uterus in *HoxA11*-deficient mice is hypoplastic and devoid of uterine glands due to developmental defects (Hsieh-Li *et al.*, 1995; Daftary and Taylor, 2001). Thus, a gene therapy approach that involves the manipulation of *Hox* gene expression may have a role in the enhancement of endometrial receptivity and implantation.

JAK/STAT intracellular signalling

The importance of the JAK/STAT signal transduction pathway in embryo implantation has been demonstrated by the embryonic lethality of STAT3-deficient mice. Interestingly, STAT3-deficient embryos implant but die rapidly due to placental defects (Takeda *et al.*, 1997).

LIF and IL-11 induce activation of the STAT family of signal transducers via the JAK/STAT pathway. In the mouse uterus, LIF acts primarily through the activation of STAT3 (Cheng *et al.*, 2001). Similarly, in human endometrial stromal cells, IL-11 acts via activated STAT3 (Salamonsen, 2003; Underhill-Day *et al.*, 2003). It remains to be determined when phosphorylated-STAT3 can be detected in human endometrium, as this will indicate when the signal-transduction pathway is activated. Interestingly, STAT3 protein production is stimulated by progesterone (Dimitriadis *et al.*, 2003) and is activated by IL-11 in human endometrial stromal cells *in vitro* (Dimitriadis *et al.*, 2003; Underhill-Day *et al.*, 2003). Furthermore, a role for STAT3 activity in trophoblast invasiveness has also been proposed (Corvinus *et al.*, 2003).

Immune acceptance of pregnancy

The immune system is a complex entity designed to eliminate foreign intruding antigens and is influenced by and, in turn, influences the function of the reproductive system. During implantation and embryogenesis, the maternal immune system is not indifferent to the presence of paternal alloantigens in the fetus. Implantation is known to be followed by a local immune response characterized by the presence of a large population of T cells, many of which express surface markers characteristic of activated cells. These T cells specifically recognized paternal alloantigens (Zhou and Mellor, 1998). The immunological action against the embryo can be described as maternal restraint. This maternal restraint might be a cause of implantation failure or failure of adequate placentation (Chao *et al.*, 1999; Herrler *et al.*, 2003). Some sort of immunomodulation might be necessary to prevent the maternal immune system rejecting the embryonic transplant. The nature of this immunomodulation is only partially known. IL-1, IL-15, human leukocyte associated antigen-DR-α (HLA-DR-α), and the FasFas ligand (FasFasL) system are known to be important for immune acceptance of pregnancy (Hoozemans *et al.*, 2004; Crncic *et al.*, 2005).

FasFas ligand system

Fas ligand (FasL or CD95L) is a protein belonging to the TNF family that induces apoptosis of T cells expressing the membrane receptor Fas (CD95). FasL is expressed in murine placentas, especially in labyrinthine trophoblast and giant cells (Hunt *et al.*, 1997). Placentas from *gld* mice that lack FasL have extensive leukocyte infiltrates and necrosis in the decidual placental interface. FasL is also expressed on the surface of the placenta in human pregnancy, localizing to cytotrophoblast and syncytiotrophoblast of first trimester and term placentas (Runic *et al.*, 1996; Bamberger *et al.*, 1997). Immunohistochemistry has shown augmented FasL concentrations in the invasive, interstitial trophoblast cells of first trimester pregnancies among CD45C leukocytes in the maternal decidua (Hammer *et al.*, 1999; Kauma *et al.*, 1999). Fas-mediated apoptosis of CD45 leukocytes co-cultured to trophoblast cells is partially abrogated when monoclonal antibody to FasL is added to cultures of human trophoblasts (Kauma *et al.*, 1999). Taken together, these findings suggest a potential role for Fas-dependent induction of apoptosis in lymphocytes in human materno–fetal interface, which may play a role in the immune acceptance of pregnancy (Aagaard-Tillery *et al.*, 2006).

Natural killer cells

The most distinctive feature of the uterine mucosa during the reproductive cycle is the presence of a large population of natural killer (NK) cells. NK cells are present in abundance in the human uterus at the time of implantation, and are in close contact with invading placental trophoblast cells. Human

uterine NK (uNK) cells achieve peak numbers during the first trimester, when they represent 70% of all lymphocytes, and are not found at term. uNK cells are different to mature circulating NK cells, yet phenotypically resemble the smaller unique blood NK cell subset, which is $CD56^{+bright}/CD16^{-}/CD3^{-}$ and has low direct cytotoxicity. Since IL-2 is not produced in the placenta, proliferation of human uNK cells is through production of IL-15 by placental macrophages and the IL-15 receptor on $CD56^{+bright}$ uNK cells (Miller, 2001). The stimulus for the presence of so many NK cells in the uterus is not completely understood. Recent research has focused on the identification of chemokines important for leukocyte recruitment to the human endometrium at the time of embryo implantation and menstruation (Wold and Arici, 2005).

Significant progress has been made in understanding the role of NK cells by evaluating mice that lack NK cells. These mice are able to bear offspring but with 64% fetal loss (Guimond *et al.*, 1999). In addition, they have no granulated metrial gland cells, decreased placental size, decidual oedema, and abnormalities involving the large maternal arterioles supplying the placenta (Redline, 2000). The causal relationship between this phenotype and the role of NK cells was established by showing that T cell-deficient mice do not exhibit these abnormalities and transplantation of stem cells from SCID mice (which have normal NK cells but no T cells) into the NK cell-deficient mice reverses the phenotype (Guimond *et al.,* 1998). These studies show that $CD56^{+bright}$ NK cells occupying the mesometrial side of the pregnant uterus contribute to fetal implantation of the embryo or the formation of the placenta in mice.

The number of uNK cells changes during the course of the menstrual cycle. They are few in the proliferative preovulatory phase of the cycle, gradually increase during the mid-luteal phase and reach a peak in the late secretory phase in humans. If pregnancy does not occur, progesterone concentrations fall and approximately 2 days before menstruation, the NK cells undergo nuclear changes resembling apoptotic cell death. However, if pregnancy occurs, the uNK cells persist in the pregnant endometrium, the decidua. There is a particularly dense infiltrate of NK cells in the decidua basalis, where the implanting trophoblast cells infiltrate into maternal tissues (Loke and King, 2000; Tabiasco *et al.,* 2006). The presence of NK cells in the decidua is a feature only of early pregnancy, as they become less conspicuous after approximately 20 weeks of gestation and are absent in term decidua in humans (Kazzaz, 1972; Sindram-Trujillo *et al.,* 2003; Wold and Arici, 2005).

Interleukin 15

IL-15 is a 14–15 kDa member of the four α-helix bundle cytokine family, which includes IL-2 and promotes activation of neutrophils, macrophages and T cells, but importantly, is a core chemokine that controls lymphocyte function and maintenance (Kang and Der, 2004). IL-15 is essential for NK cell development in bone marrow and stimulates the proliferation, cytokine production and cytotoxicity of activated blood NK cells. IL-15 is reported to be essential for type 2 cytokine production by the uNK cells (Eriksson *et al.* 2004). Unlike its effects on blood NK cells, it does not transform the uNK cells into potent cytolytic cells (Verma *et al.,* 2000). This is critically important for a cell that is present at the maternal–fetal interface where cytolytic activity would destroy trophoblast. It is therefore reasonable to assume that in the human uterus, IL-15 may play a role in promoting uNK cell survival and expansion. IL-15 mRNA and protein have been demonstrated in non-pregnant human endometrium, decidua and placenta (Verma *et al.,* 2000) with the protein being immunolocalized perivascularly in secretory-phase stromal cells, in glandular epithelial cells during the proliferative phase and in decidua in the first trimester of pregnancy (Kitaya *et al.,* 2003). IL-15 knockout mice are fertile, but display impaired decidual integrity, unmodified spiral arteries and lack of uNK at the implantation sites (Ashkar *et al.,* 2003).

IL-15 mRNA expression and protein secretion increase during in-vitro decidualization of endometrial stromal cells in culture, although there are some discrepancies in the literature, probably because of the different culture conditions used. It is clear that cells decidualized using either cAMP or progesterone show enhanced IL-15 mRNA expression and protein secretion (Kitaya *et al.,* 2003) and that this is further enhanced in the presence of interferon-γ, although the latter cytokine alone cannot stimulate IL-15 production. The probable source of interferon-γ in the endometrium is the uNK cells (Dunn *et al.*, 2002), and thus the likelihood exists of enhancement of IL-15 production from decidualizing cells by adjacent uterine NK cells. IL-1β appears to play an opposing role as it acts as a negative regulator of IL-15 mRNA and protein during in-vitro decidualization.

Conclusion

A better understanding of the implantation process, which involves two synchronous and complementary events [capacitation of endometrium (endometrial receptivity) and proper embryonic differentiation], will improve not only the pregnancy rate of assisted reproductive techniques but also will assist the understanding of several other pathophysiological processes such as tumour development, angiogenesis and tumour metastasis. Endometrial receptivity is mainly controlled by sex steroids, directly by their specific receptors, and/or indirectly by cytoplasmic or nuclear interactions. Endometrial receptivity may also be regulated by growth factors, e.g. HB-EGF, amphiregulin, and adhesion molecules, e.g. integrins, E-cadherin, laminin, fibronectin, which are necessary for embryo attachment. Many cytokines are expressed during the implantation window but only a few have been shown to be required for embryonic implantation such as LIF, IL-6, and IL-11. A significant advance has been made in recent years towards understanding the molecular mechanisms governing trophoblast and ICM differentiation.

Appearance of early blastocyst formation is the first morphological sign of proper differentiation. *Oct-4*, *Nanog*, *Sox2* and *Gata6* are involved in ICM and its subsequent differentiation to epiblast and primitive endoderm. On the other side, *Cdx*-2, *Eomes*, *Stra13*, *Hand1*, and *Tpbpa* are involved in trophectoderm development and subsequent differentiation to trophoblastic giant cells, spongiotrophoblasts. These genes are detected through the generation of knockout mice and further studies are required to confirm their relevance in human embryos.

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