REPRODUCTION
REVIEW

Leukaemia inhibitory factor in implantation and uterine biology

Susan J Kimber

Faculty of Life Sciences, University of Manchester, Stopford Building, Oxford Road, Manchester M13 9PT, UK

Correspondence should be addressed to S J Kimber; Email: sue.kimber@manchester.ac.uk

Abstract

Leukaemia inhibitory factor (LIF) is one of the most important cytokines in the reproductive tract. Without expression of LIF in the uterus, implantation of a blastocyst cannot begin. Yet, 13 years after publication of the phenotype of the LIF knockout mouse we are only just beginning to understand how LIF functions in the uterus. This review addresses our knowledge of the role of LIF in regulating implantation through its influence on the luminal epithelium and stromal decidualization, but also its influence on reproductive tract cells such as leukocytes and glandular epithelium, during the pre-implantation phase of pregnancy.

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Introduction

Blastocyst implantation is a unique feature of mammalian reproduction and a tightly regulated process. Changes in ovarian steroids during the pre-implantation period instigate the required maturation of the endometrial epithelium and stroma. Following priming with preovulatory oestrogen, increasing ovarian progesterone produces a sensitized uterus. In rodents, the ability of the uterus to support and facilitate the implantation of the blastocyst is regulated by the downstream effects of a subsequent small transient increase in oestrogen (Finn & Martin 1970). Oestrogen induces changes which allow the uterus to support implantation of the blastocyst over a short period of the reproductive cycle, the receptive period. This spans about 18–24 h in rodents and probably several days in the human (Navot *et al.* 1991) although experimentally the amount of oestrogen can influence the duration of the receptive phase (Ma *et al.* 2003). The endometrial response involves differentiation of the endometrial epithelium and stroma to a phenotype favouring interaction with the trophectoderm. Changes in the luminal epithelium (LE), with which the trophectoderm of the activated blastocyst first interacts, are essential for the initiation of implantation (Kimber & Spanswick 2000, Aplin & Kimber 2004). The steroid-prepared stroma must also undergo a process of further differentiation, known as decidualization, triggered by an embryonic signal (or artificial mimic). Decidualization is induced in stroma around the implantation site from late on day 4 of pregnancy in mice. In women, decidual changes occur in the absence of an embryo. Decidualization involves changes in the expression of a large number of genes (Farrar & Carson 1992, Paria *et al.* 2001) and is marked by a rapid increase in vascular permeability with resulting oedema in the stroma around the implantation site (Abrahamsohn & Zorn 1993). In spite of the well-established requirement for oestrogen priming before blastocyst signals can establish a decidual response, in mice decidualization has been shown to occur in the absence of a functional oestrogen receptor-α (Curtis *et al.* 1999, Paria *et al.* 1999, Curtis-Hewitt *et al.* 2002). Following the receptive period, the uterus becomes refractory, no longer allowing implantation of any remaining blastocysts. Thus tightly regulated synchrony between embryonic development and uterine maturation is essential for successful pregnancy.

It is clear that implantation of the mammalian embryo requires co-ordinated interaction between the embryo and the uterus. Although it has long been known that ovarian steroids regulate this process, it is only in the last couple of decades that some of the local factors have been identified. Steroidal regulation of uterine function is mediated to a large extent through the action of growth factors and cytokines on their receptors (for review see Sharkey 1998, Saito 2001).

Leukaemia inhibitory factor (LIF)

One cytokine which is essential for successful implantation is Leukaemia Inhibitory Factor (LIF), a member of the interleukin (IL)-6 family. LIF is a highly glycosylated 40–50 kDa glycoprotein with a range of biological functions (Haines

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et al. 2000). It is expressed in various embryonic and adult tissues (Hilton & Gough 1991, Schafer-Somi 2003) with particularly high levels in the uterus. At the cell surface, LIF receptor-β (LIF-Rβ) binds the glycoprotein gp-130 (the common signalling receptor for IL-6 family cytokines) to form a high affinity receptor through which LIF signalling is triggered (Heinrich *et al.* 2003). Transduction of the signal can occur by activation of several pathways, the main ones being the JAK/STAT pathway, the Src homology 2-domain-containing tyrosine phoshatase (SHP-2)/Ras/extracellular signal-regulated kinase (ERK) pathway or the phosphatidylinositol-3-kinase (PI3K)/Akt pathways, the relative importance of which varies with tissue. Signalling can be inhibited by suppressors of cytokine signalling or protein inhibitor of activated STAT (PIAS) proteins (Chung *et al.* 1997, Bousquet *et al.* 1999, Duval *et al.* 2000).

LIF and implantation

In inbred female C57Bl mice, null for the LIF gene, embryos develop to the blastocyst stage but do not implant (Stewart *et al.* 1992, Cheng *et al.* 2002) and the uteri show little evidence of decidualization (Chen *et al.* 2000). We observed a similar phenotype on an outbred, MF1, background (Sherwin *et al.* 2004, Fouladi-Nashta *et al.* 2005). In spite of the presence of anti-mesometrially located blastocysts in the uterine lumen, no decidual reaction is evident, nor is there penetration of LE by trophoblast, even by day 7 of pregnancy (Fig. 1). LIF −/− embryos can implant in the uteri of wild-type female mice and delivery of LIF, either by a micro-osmotic pump or injection on day 4, restores implantation capacity to homozygous mutant females (Stewart *et al.* 1992, Chen *et al.* 2000, Sherwin *et al.* 2004). Since LIF-null embryos develop to term in heterozygous dams and can implant after transfer to wild-type

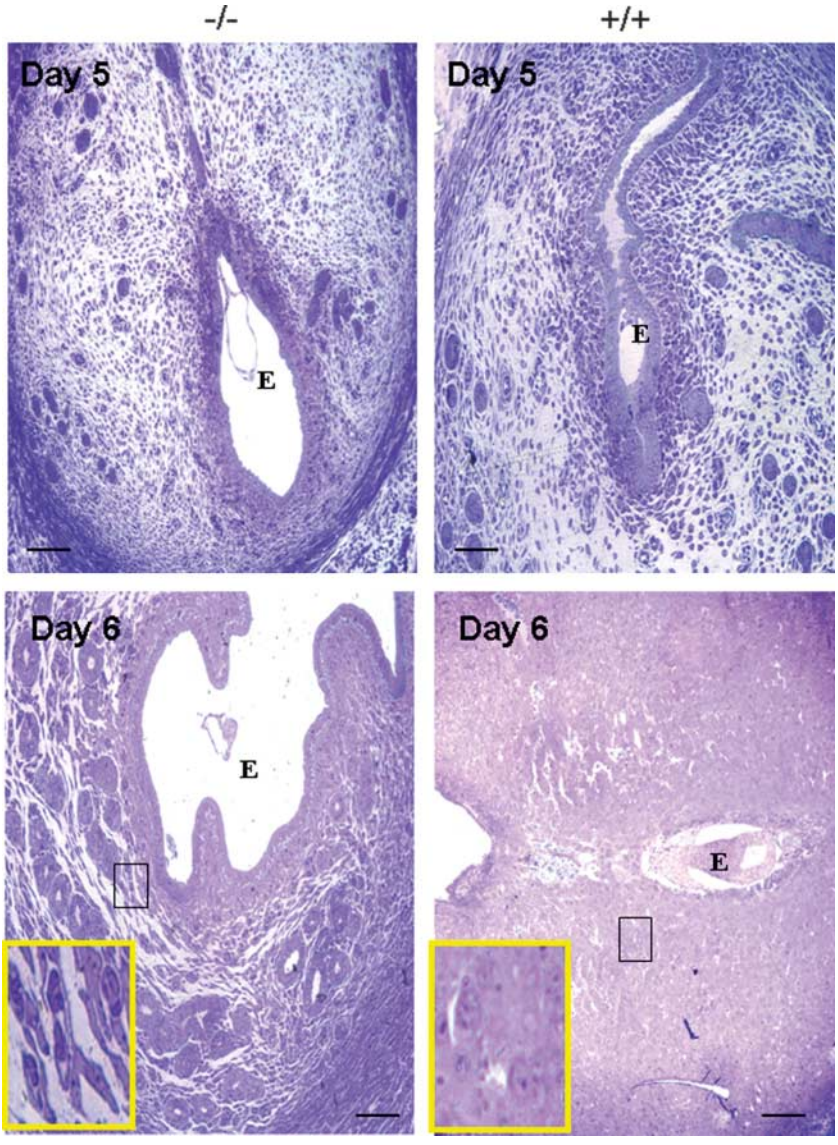

Figure 1 Histological features of the uterus in LIF-null and wild-type mice. Semi-thin resin sections from days 5 and 6 of pregnancy were stained with toluidine blue. The insets show high magnification of differentiated polygonal stromal cells in the wild-type and undifferentiated fibroblast-like stroma cells in the LIF-null mice on day 6 of pregnancy. E = embryo, scale bars = 50 μm (Reprinted from *Developmental Biology*, vol 281, Fouladi-Nashta *et al.* Characterization of the uterine phenotype during the peri-implantation period for LIF-null, MF1 strain mice, pp 1–21, 2005, with permission from Elsevier).

females of appropriate endocrine status it is clear that the major implantation defect is on the maternal side. This is supported by the absence of an implantation defect in embryos lacking components of the LIF signalling cascade. For instance, gp-130-null embryos die only during the second half of gestation with multiple abnormalities including in heart and haematopoietic progenitors (Yoshida *et al.* 1996) while LIF-Rβ knockout animals implant normally but have, for instance, placental defects and motor neuron degeneration and die at birth (Ware *et al.* 1995). Deletion of the signal transducer and activator of transcription 3 (Stat-3) is embryo lethal but only after implantation has occurred (Takeda *et al.* 1997). The lack of implantation in females carrying the LIF-null mutation suggests that the action of LIF in the uterus is essential for even early events in this process. However, the precise role of maternal LIF at the molecular level is still unclear in any species.

LIF in the human uterus

LIF probably plays a role in endometrial function in humans (Vogiagis *et al.* 1996, Hambartsoumian 1998, Lass *et al.* 2001) and domestic species (Vogiagis *et al.* 1997, Modric *et al.* 2000, Oshima *et al.* 2003, Schafer-Somi 2003). In humans, LIF mRNA and protein are expressed in the endometrial glands during the luteal phase of the menstrual cycle when implantation would occur (Charnock-Jones *et al.* 1994, Arici *et al.* 1995, Chen *et al.* 1995). LIF-Rβ and gp-130 are expressed in LE throughout the cycle in women of proven fertility (Cullinan *et al.* 1996) but LIF mRNA and protein are also expressed in decidual stroma (Kojima *et al.* 1994, Sawai *et al.* 1997, Chen *et al.* 2004) and gp-130 in decidua (Classen-Linke *et al.* 2004). gp-130 protein has been localized to the glandular epithelium in the mid- and late-secretory phase but it is unclear whether LE was examined (Classen-Linke *et al.* 2004). A soluble form of gp-130 is released by the endometrium, this is formed by proteolytic cleavage and released at highest levels in the mid- to late-luteal phases (Sherwin *et al.* 2002, Classen-Linke *et al.* 2004). It is stimulated by oestrogen together with progesterone in cultured endometrial epithelial cells. This suggests the possibility that modulating levels of a potential antagonist may regulate the activation of the membrane-bound IL-6 family receptors in the presence of ligand. The misregulation of soluble gp-130 in patients with unexplained infertility (Sherwin *et al.* 2002) is further evidence for the role of IL-6 family cytokines in normal pregnant endometrial function. Furthermore, a correlation has been suggested between LIF and LIF-R levels and LE uterodome formation, potentially indicative of receptivity (Aghajanova *et al.* 2003). Levels of LIF in uterine flushings were suggested to be lower in patients with unexplained infertility (Laird *et al.* 1997) but in another study lower levels of LIF were suggested as predictive of implantation success (Ledee-Bataille *et al.* 2002) with higher levels being indicative of inflammation. Thus the precise level of LIF may be important. It has been proposed that heterozygous mutations in the LIF gene may account for some incidences of infertility (Giess *et al.* 1999) with reduced amounts or LIF activity in the uterus leading to failure of implantation. However, out of 50 women with unexplained infertility, only one was reported with a heterozygous mutation in the LIF gene and she achieved a pregnancy after ovarian stimulation (Steck *et al.* 2004). The authors concluded that although LIF mutations may well play a role in infertility, screening for them would not be feasible because of their low prevalence. Another group (Inagaki *et al.* 2003) found no statistically significant difference between LIF levels in patients with recurrent implantation failure and control multiparous women. Nevertheless, defects in LIF expression have in some studies been associated with recurrent miscarriage and some conditions of unexplained infertility consistent with role(s) in early events in pregnancy (Hambartsoumian 1998, Lass *et al.* 2001).

Regulation of LIF expression in mice

In mice, the highest levels of LIF mRNA are found prior to implantation in glandular epithelium following the nidatory surge of oestrogen on the morning of day 4 of pregnancy (Bhatt *et al.* 1991, Chen *et al.* 2000). Highest levels of LIF protein have also been reported on day 4, mainly in the epithelium but some in stroma (Yang *et al.* 1995, Fouladi-Nashta *et al.* 2004). In ovariectomized mice, uterine LIF mRNA increases markedly within 1 h of oestrogen injection and is not affected by progesterone, strongly suggesting oestrogenic control (Bhatt *et al.* 1991, Stewart & Cullinan 1997, Chen *et al.* 2000). LIF mRNA declines to a low level by days 6–7 of pregnancy and the transient nature of the LIF signal may be important in its role in implantation. Mice null for the homeobox gene Hmx3 are infertile, lack normal decidualization and do not upregulate LIF in the glandular epithelium at the appropriate time in pregnancy (Wang *et al.* 1998). Another homeobox gene Hoxa-11 is also required for normal decidualization and completion of implantation. Again, in its absence, LIF does not show an upsurge at day 4 of pregnancy (Gendron *et al.* 1997). This suggests that these two genes may be involved in regulating the increase in LIF at implantation. LIF activity may also be regulated by soluble receptor expression.

Regulation of LIF expression in the human

Observations on cultured human endometrial cells emphasize that LIF is linked to inflammatory pathways, e.g. through IL-1 and tumour necrosis factor-α (TNF-α), which poses the question as to whether LIF is involved in inflammation or implantation (or both) in humans. IL-1β stimulates LIF secretion by endometrial epithelial cells *in vitro* (Perrier d'Hauterive *et al.* 2004). Recent evidence suggests that in these cells LIF and LIF-R, and IL-1β, its receptor and

receptor antagonist are all regulated by leptin via the leptin receptor OB-R (Gonzalez *et al.* 2003, 2004), a cytokine better known for its role in regulating satiety. Moreover, IL-1β as well as leptin upregulate LIF-R and both effects are blocked by inhibition of IL-1R type-1. Thus it appears that leptin may be a primary regulator of the LIF response at several levels at least in human endometrium and feedback loops exist between IL-1 and LIF in endometrial epithelial cells. It remains to be demonstrated whether IL-1 regulates LIF and its receptor in the mouse. In cultured endometrial epithelial cells, TNF-α stimulates LIF, along with IL-6, secretion in a nerve factor-κB-dependent manner that also requires a functional proteosome compartment (Laird *et al.* 2000). IL-1β, transforming growth factor-β (TGF-β) and TNF-α have also been shown to stimulate LIF production by first trimester human decidual cell-enriched cultures (Sawai *et al.* 1997). However, these observations may not necessarily indicate the signalling pathways involved during early stages in implantation.

A role for the human blastocyst in endometrial LIF regulation has been suggested (Perrier d'Hauterive *et al.* 2004). *In vivo* (Licht *et al.* 2001) and *in vitro* (Perrier d'Hauterive *et al.* 2004), human chorionic gonadotrophin (hCG) has been shown to stimulate LIF secretion by endometrial epithelial cells. Paradoxically, the effect *in vitro* was dramatic using follicular phase endometrial epithelium but less impressive with secretory epithelium which appears to express a higher level of hCG/luteinizing hormone receptor transcripts. Perhaps this indicates differences in the amount of receptor protein which are not reflected in transcript levels, or already near-saturated receptor stimulation in biopsied secretory phase tissue. Since it is known that LIF can stimulate hCG production by the trophoblast (Sawai *et al.* 1995, Nachtigall *et al.* 1996) these findings suggest a potential positive feedback loop. Insulin-like growth factor (IGF)-I and II and TGF-β were also found to induce a dose-dependent stimulation of LIF secretion by human endometrial epithelial cells whilst the IGFs (like hCG) also inhibited IL-6 secretion (Perrier d'Hauterive *et al.* 2004). In women, progesterone given *in vivo* was reported to inhibit subsequent *in vitro* LIF secretion by human endometrium (Hambartsoumian *et al.* 1998*b*). Interestingly, progesterone and IL-4 have both been shown to upregulate LIF in Th-2 cells (Piccinni *et al.* 1998).

Cellular targets and LIF signalling in the murine uterus

A key cellular target for LIF in the murine uterus appears to be the LE in which LIF-Rβ mRNA increases between days 3 and 4 of pregnancy, as the time of implantation approaches. However, both LIF-Rβ and gp-130 protein can be detected in LE on days 3–5 of pregnancy (Cheng *et al.* 2001). gp-130 expression in LE is stimulated by oestrogen together with progesterone (Cheng *et al.* 2001, Ni *et al.* 2002). LIF signalling in the uterus occurs mainly through the JAK/STAT pathway and not the alternative SHP-2/RAS/ERK pathway (Cheng *et al.* 2001). Stat-3 is present in LE throughout early pregnancy but is only susceptible to activation (via tyrosine phosphorylation) and nuclear translocation on day 4 at the onset of the receptive phase. Oil infusion into the murine uterus induces decidualization following progesterone and oestrogen priming but it is notable that oil can induce Stat-3 phosphorylation as well as transient cyclo-oxygenase (Cox-2) expression independent of hormones (Curtis-Hewitt *et al.* 2002). So the temporal response to LIF in LE may be partly regulated at the level of sensitivity of Stat-3 to phosphorylation. Indeed mice homozygous for deletion of the STAT activation site in gp-130 show an apparently identical infertility defect to LIF-null females (Ernst *et al.* 2001). The lack of Stat-3 phosphorylation in pseudopregnant mice (Teng *et al.* 2004), together with the effect of oil, points to the role of an embryonic signal in facilitating activation of Stat-3 in LE. At the same time, Stat-3 can also transduce the response to other IL-6 family cytokines such as IL-11, which is also implicated in reproductive tract function. IL-11 transcripts are expressed at the murine implantation site and although implantation occurs in IL-11 receptor-α knockout animals, secondary decidualization is grossly defective and trophoblast giant cells appear in excess. After 7.5 days post coitum the majority of embryos was found to be necrotic (Robb *et al.* 1998).

Suppressor of cytokine signalling protein-3 (SOCS-3) is induced by LIF and acts as a feedback inhibitor, preventing phosphorylation of gp-130 and Stats. It has been implicated in regulating uterine LIF signalling and might curtail Stat-3 signalling. Embryos in which SOCS-3 has been deleted die *in utero* (Roberts *et al.* 2001) so are not informative for uterine implantation.

It is therefore likely that LIF acts co-operatively with blastocyst signals to induce decidualization on days 4–5 of pregnancy by activation of Stat-3 signalling and subsequent promotion of new gene expression and secondary signalling from LE. In addition, LIF-Rβ transcripts and activated Stat-3 are detected in stroma particularly after decidualization in both mice and humans (Yang *et al.* 1995, Ni *et al.* 2002, Fouladi-Nashta *et al.* 2004, Perrier d'Hauterive *et al.* 2004, Teng *et al.* 2004). Thus stromal cells are also capable of responding directly to LIF.

Ultrastructural changes in LE during the peri-implantation period in LIF-null mice

In wild-type animals, luminal epithelial cell polarity becomes less marked in the peri-implantation period when latero-basal markers become detectable in the apical membrane (Thie *et al.* 1996, Kimber 2000, Kimber & Spanswick 2000). Prior to implantation, LE cells normally become more cuboidal and microvilli are replaced by bulbous protrusions called pinopods (Nilsson 1966, Lopata *et al.* 2002, Murphy 2000a) which increase in number up to day 5 postcoitus (Bansode *et al.* 1998). In rodents, these apical

modifications of the uterine LE appear to mediate uptake of fluid (Enders & Nelson 1973) and macromolecules (Parr & Parr 1974). In contrast, in LIF-null animals on days 4–5 of pregnancy LE cells remain more columnar, similar to wild-type LE on days 2–3 of pregnancy but with a rather domed apical surface. Pinopods do not develop over the apical cell membranes which remain microvillous (Fig. 2) up to day 7 of pregnancy (Fouladi-Nashta *et al.* 2005). In the human, uterodomes, which develop at an equivalent stage in the menstrual cycle to rodent pinopods, have been associated with receptivity (Nikas *et al.* 1995). It has been suggested that they carry potential embryo-adhesion molecules (Bentin-Ley *et al.* 1999, Creus *et al.* 2002) although they are not pinocytotic (Adams *et al.* 2002). Our results have indicated that failure of apical maturation and pinopod formation by LE cells is a major reason for the inability of embryos to interact firmly and irreversibly with the uterus in LIF-null mice. By day 5 of pregnancy, wild-type LE cells make intimate association with the trophoectoderm (TE) cell surface via the pinopod membranes while in LIF-null animals more superficial contact is observed at the LE microvillar tips (Fouladi-Nashta *et al.* 2005). However, it should be noted that pinopods develop in rats in experimental delay of implantation so pinopod formation is insufficient for implantation in the absence of embryonic activation (Isychoyos & Mandon 1971). Hoxa-10 has also been shown to be required for pinopod formation: after Hoxa-10-antisense treatment uterodomes do not appear on the LE at the start of the period of receptivity (Bagot *et al.* 2001). Thus both Hoxa-10 and LIF play a role in apical LE differentiation.

Molecular changes in the LE of LIF-null animals

A number of other molecular defects have been detected in the day 4–5 LE in the absence of LIF. However, not all features of LE associated with implantation are affected, providing insight into the distinct stages in the preparation of LE for interaction with the trophoblast. Various molecules are normally downregulated or disappear around implantation including H-type-1 glycans (Kimber *et al.* 1988, Kimber & Spanswick 2000), which our previous data have implicated in implantation (Lindenberg *et al.* 1988, 1990). The mucin Muc-1, which has been postulated to form a barrier to embryo attachment, disappears from the rodent LE at the time of implantation (Braga & Gendler 1993). Desmosomal proteins are also reduced (Illingworth *et al.* 2000) and there are changes in tight junction proteins (Murphy 2000b, Orchard & Murphy 2002). However, in pregnant LIF-null uteri, Muc-1 is

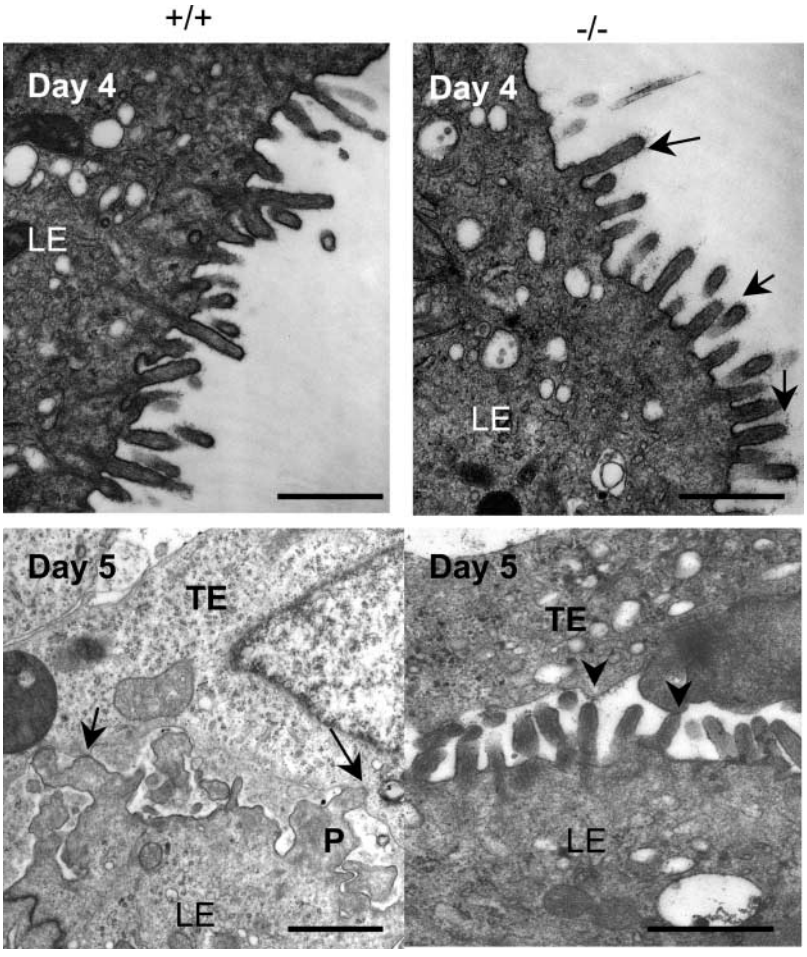

Figure 2 Ultrastructure of LE in LIF-null and wild-type mice on days 4 and 5 of pregnancy. On day 4, both the wild-type and null mice have LE bearing microvilli, but the microvilli of the wild-type mouse have less glycocalyx than those of the null animal which has a prominent filamentous covering over many microvillous processes (arrows). Scale bars = 0.5 μm. On the morning of day 5 of pregnancy in the wild-type mice, pinopods (P) of the LE make close contact, sometimes indenting the surface of the blastocyst trophoectoderm (TE) (arrows) whereas, in the null mouse, the blastocyst is only contacted by the tips of untransformed microvilli (arrow heads) and no intimate contact is made. Scale bars = (left) 1 μm and (right) 0.5 μm (Reprinted from *Developmental Biology*, vol 281, Fouladi-Nashta *et al.* Characterization of the uterine phenotype during the peri-implantation period for LIF-null, MF1 strain mice, pp 1–21, 2005, with permission from Elsevier).

removed on schedule (Chen *et al.* 2000, Fouladi-Nashta *et al.* 2005) nor have differences in junctional molecules been detected at least at the level of confocal microscopy.

Changes in LE glycosylation are well established in the pre- and peri-implantation period (Aplin 1991, Kimber 1994, Kimber *et al.* 2001) and these occur in parallel with a reduction in the LE glycocalyx (Chavez & Anderson 1985). Failure to develop pinopods in LIF-null mice is associated with aberrant retention of thick glycocalyx and glycosyl residues on the surface of the LE. This includes the continued presence on day 5 (especially adjacent to the embryo) and day 6 of pregnancy of H-type-1 antigen (Fig. 3) and fucosylated molecules bound by Ulexeuropaens agglutinin 1 (UEA-1) and Tetragonolobus purpureus agglutinin (LTA) lectins (Fouladi-Nashta *et al.* 2005). Thus LIF, either directly or indirectly in conjunction with embryonic signalling, downregulates fucosylated oligosaccharides as the uterus becomes refractory. However, embryos null for the fucosyl transferase responsible for H-type-1 epitope formation do not have an implantation phenotype, so this saccharide epitope is not indispensable for implantation (Domino *et al.* 2001). Interestingly, it appears that a similar mis-regulation occurs for transcripts of the homeodomain protein Msx-1 (Daikoku *et al.* 2004). In wild-type mice, transcripts are absent on day 1 then strongly expressed in LE and glands on the morning of day 4 of pregnancy. They are dramatically downregulated by the evening of day 4 in both pregnancy and pseudopregnancy. However, in LIF-null mice, Msx-1 downregulation does not occur. Whether this is related in any way to the aberrations in the apical LE cell surface observed in these mice is not yet clear.

Observation of the implantation site in LIF-null mice also suggests various molecular abnormalities associated with the lack of decidualization. Many of these aberrations may be secondary to a lack of proper signalling between the blastocyst and LE when LIF is not available to trigger normal changes in LE phenotype. For example, at the implantation site, transcript expression for Cox-2 in stroma and Heparin-binding-epidermal growth factor (HB-EGF) in the LE is lost (Song *et al.* 2000). Prostaglandins and prostacyclins, products of Cox enzyme activity, are important in initiating decidualization (Kennedy & Ross 1993, Lim *et al.* 1997) while local membrane-anchored HB-EGF on LE is a suggested attachment factor for the blastocyst (Raab *et al.* 1996, Chobotova *et al.* 2002). Two other members of the EGF family, amphiregulin and epiregulin (but not TGF-α), are also greatly reduced or not expressed in LIF-null LE (Song *et al.* 2000) both before and at the time of implantation. This points to their co-ordinated control or

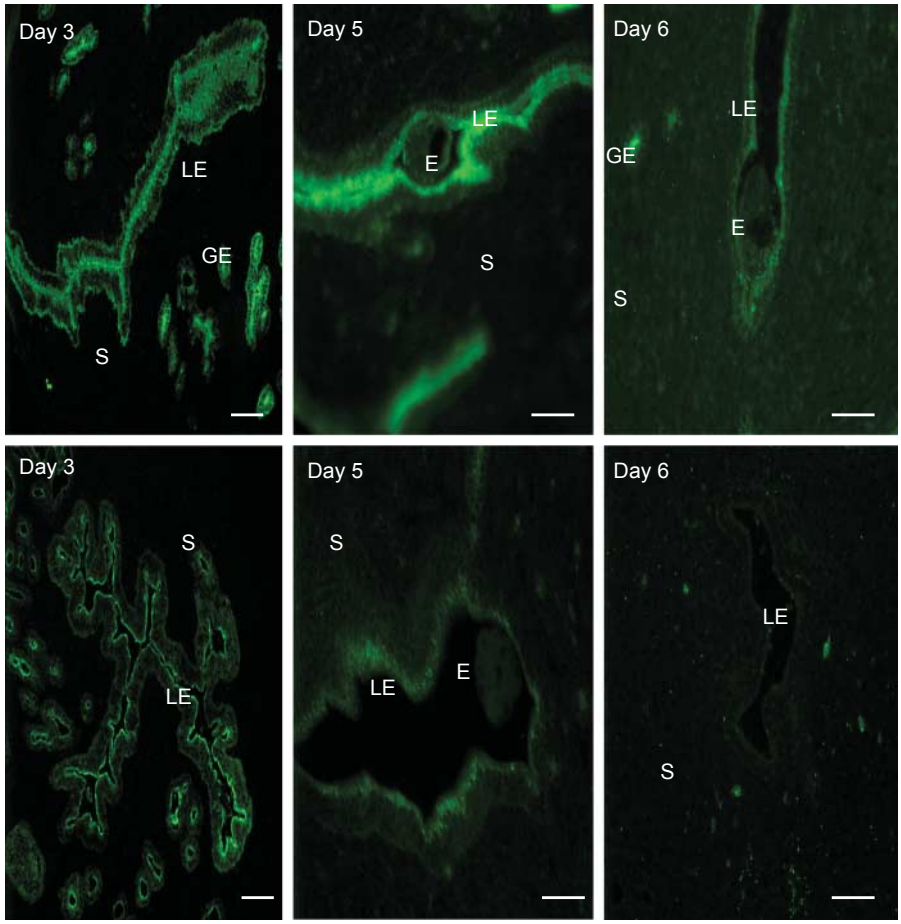

Figure 3 Immunofluorescence staining for H-type-1 antigen in LIF −/− and wild-type mouse uterus. Frozen sections from days 3, 5 and 6 after mating were stained, using mouse monoclonal 667/9E9, for H-type-1 antigen which is expressed in the LE. Note the retention of H-type-1 staining on days 5 and 6 of pregnancy. GE = glandular epithelium and S = stroma. Scale bars = 50 μm (Reprinted from *Developmental Biology*, vol 281, Fouladi-Nashta *et al.* Characterization of the uterine phenotype during the peri-implantation period for LIF-null, MF1 strain mice, pp 1–21, 2005, with permission from Elsevier).

indicates that their expression is interdependent. EGF family receptors appear not to be affected. Interestingly, amphiregulin is inducible by progesterone injection of ovariectomized LIF-null females even though progesterone levels are normal in these animals (Song *et al.* 2000). Therefore the threshold for progesterone stimulation of amphiregulin, or a regulatory factor, might be altered in LIF-null mice.

Use of microarray and subtractive hybridization to identify LIF targets

Several potential LIF-regulated molecules have been discovered by subtractive hybridization or microarray approaches. Cochlin, an extracellular matrix protein containing von Willebrand factor A-binding domains, has recently been identified as restricted to LE on day 4 of pregnancy and lacking in LIF-null mice (Rodriguez *et al.* 2004). Once again, gene deletion of this molecule does not give an implantation phenotype, suggesting that loss of cochlin alone cannot explain the lack of implantation in the absence of LIF. IGF-binding protein-3 (IGFBP-3) expression was recently identified to be increased by LIF (Sherwin *et al.* 2004). IGFBP-3 is expressed mainly in the LE before implantation and then at the implantation site, but *in situ* hybridization revealed no difference in distribution or intensity of signal between LIF-null and heterozygote or wild-type animals. Thus upregulation by LIF must occur above a detectable basal level, presumably that induced by progesterone. The spread of IGFBP-3 to the stroma around the embryo in pregnant wild-type animals on day 5 was not seen in LIF-null animals. Two other progesterone-responsive genes, immune response gene 1 (IRG1) and amphiregulin were also induced in uteri by LIF injection although this was not found for several other progesterone-regulated molecules. Knockout data have suggested that amphiregulin and IGFBP-3 are not indispensable for implantation (Luetteke *et al.* 1999, Pintar 2001). IRG1 is stimulated by progesterone but upregulated to a greater extent by both progesterone and oestrogen (Chen *et al.* 2003, Cheon *et al.* 2003, Sherwin *et al.* 2004). It has been found to be an essential factor for implantation (Cheon *et al.* 2003). So deficiency in IRG1 expression may, at least partly, explain the implantation defect in LIF-null mice. Paradoxically, in another study, IRG1 did not appear to be affected by the absence of LIF (Chen *et al.* 2003) so future work is needed to clarify the relationships between these molecules.

LIF and decidualization

Although decidualization in normal mice requires signals from the blastocyst, in humans it occurs without the involvement of the embryo, so regulatory routes may differ. Stromal cells at the implantation site show marked signs of differentiation by day 5 of murine pregnancy (Abrahamsohn & Zorn 1993). They enlarge, take on a more epithelial appearance with deposition of glycogen in the cytoplasm and some become binucleate. By days 5–6 loss of extracellular matrix and close cell–cell apposition is apparent in the cells of the primary decidual zone. In LIF-null mice, however, even by days 6–7 of pregnancy stromal cells adjacent to the blastocyst appear fibroblastic with none of the ultrastructural changes indicative of decidualization (Chen *et al.* 2000, Fouladi-Nashta *et al.* 2005).

In culture we have shown that LIF induces IL-1α secretion by semi-polarized LE cells, an effect blocked by a competitive inhibitor of LIF (A A Fouladi-Nashta, L Mohamet, N Nijjar, J K Heath & S J Kimber, manuscript in preparation). Cox-2 is aberrantly expressed in the absence of LIF at both protein (Fouladi-Nashta *et al.* 2005) and mRNA (Song *et al.* 2000) levels. Protein is expressed in the LE adjacent to the embryo but there is extremely limited expression in adjacent stroma (Fig. 4). IL-1 has previously been shown to induce stromal expression of Cox-2, as well as inducing prostaglandin E_2 (PGE_2) synthesis in stromal cells *in vitro* (Jacobs & Carson 1993, Jacobs *et al.* 1994, Bany & Kennedy 1995). Prostacyclin, acting via the nuclear receptor peroxisome proliferator-activated receptor δ (PPARδ) is a key mediator of decidualization *in vivo* (Lim *et al.* 1999, Lim & Dey 2000). PGE_2 is also a trigger for the vascular oedema, angiogenenesis and stromal cell differentiation during the decidual response to embryonic signals in rodents (Sananes *et al.* 1976, Kennedy 1977, Kennedy & Ross 1993). Therefore it is possible that transduction by LE of the LIF signal combined with a blastocyst signal(s) stimulates production of IL-1 sufficient to trigger local decidual changes. This, together with the loss of most Cox-2 expression at the implantation site in LIF-null females, supports a signalling cascade involving IL-1 induction of prostaglandins in the decidualization response. All the same, although implantation was reported to be blocked by repeated intraperitoneal injection of the receptor antagonist IL-1ra (Simon *et al.* 1994) it occurred normally in mice lacking the IL-1 type-1 receptor (Abbondanzo *et al.* 1996). Thus even if IL-1 is downstream of LIF, signalling through this receptor may not be essential for implantation.

Stromal phenotype in the presence or absence of LIF

We have examined the expression of proteins suggested to be direct or indirect targets of LIF in the stroma, or with expression patterns fitting that role (Fouladi-Nashta *et al.* 2005). We found mis-expression of a number of decidual markers such as desmin (Glasser *et al.* 1987), bone morphogenetic factor-2 (BMP-2) and -4 (Paria *et al.* 2001) and tenascin which is associated with stromal cells immediately around the implanting embryo (Julian *et al.* 1994). Tenascin has been suggested to be regulated by progesterone via IL-1α and prostglandins (Noda *et al.* 2000); both the latter are clearly also influenced by LIF. This concurs with true synergy between progesterone and LIF.

Stromal cells maintain LIF-Rβ expression in primary culture and we have shown that LIF has a moderate

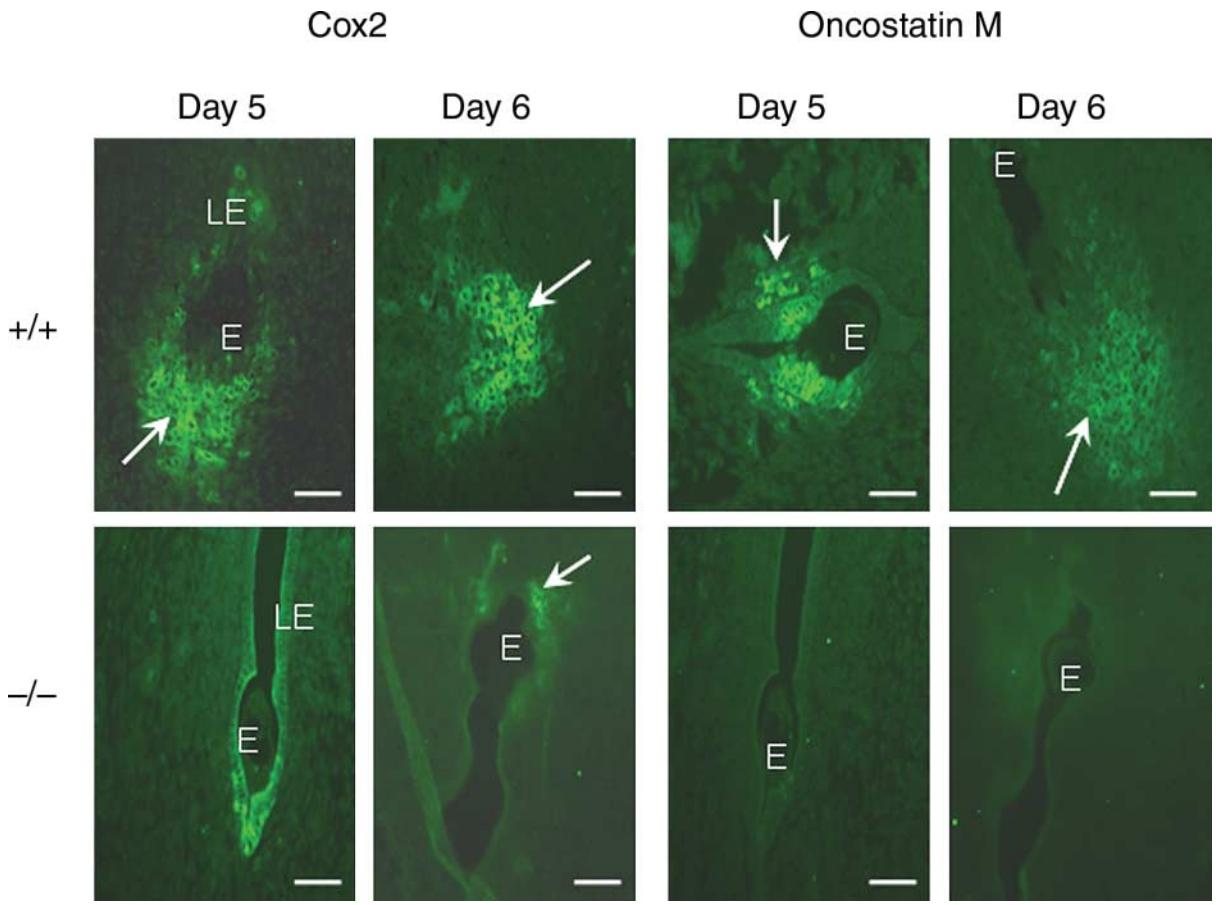

Figure 4 Immunofluorescence staining for Cox-2 and oncostatin M (OsM) in LIF-null and wild-type mouse uterus on days 5 and 6 of pregnancy. Cox-2 protein is strongly expressed in the LE and underlying stromal cells (arrows) at the implantation site on day 5 and expression extended deeper into the stroma by day 6. In LIF-null mice, expression was limited to the LE cells and only a few stromal cells expressed Cox-2 in the day-6 uterus. The pattern of expression for OsM protein in wild-type mice was similar to Cox-2. In LIF-null animals, OsM was completely absent around the embryo on days 5 and 6 of pregnancy. E = embryo, scale bar = 100 μm (Reprinted from *Developmental Biology*, vol 281, Fouladi-Nashta *et al.* Characterization of the uterine phenotype during the peri-implantation period for LIF-null, MF1 strain mice, pp 1–21, 2005, with permission from Elsevier).

dose-dependent inhibitory effect on stromal decidualization *in vitro* (Fouladi-Nashta *et al.* 2004). The repression of cellular differentiation was not simply a result of inhibition of PGE-2 secretion by stromal cells which was unaffected by LIF. One possibility is that a direct effect of LIF on stroma may limit decidualization to the implantation site and prevent it spreading to uterine segments lacking an implanting embryo. This inhibition may only be over-ridden in the presence of a distinct LE signal at the implantation site. Reduction or loss of bone marrow-derived cell populations in stromal culture (e.g. loss of natural killer (NK) cells) might also affect the response of stromal fibroblasts to LIF. The expression of high levels of LIF in human decidua suggests a role in decidualization, but receptor localization and functional studies have suggested that a major target in humans is the trophoblast and placenta (Kojima *et al.* 1994, 1995, Sawai *et al.* 1995, Nachtigall *et al.* 1996, Ren *et al.* 1997, Sharkey *et al.* 1999, Kayisli *et al.* 2002, Chen *et al.* 2004).

One of the most distinct aspects of the LIF-null phenotype is the complete absence at the implantation site of expression of oncostatin M (OsM), another member of the IL-6 family (Fouladi-Nashta *et al.* 2005) (Fig. 4). OsM is a 28 kDa protein which binds to a heterodimeric receptor consisting of the OsM receptor-β (OsM-Rβ) and gp-130, although human OsM (but not murine) can also signal through the LIF-R (Ichihara *et al.* 1997, Wang *et al.* 2000). Signalling leads to activation of both Stats (3 and/or 5) and the ERK pathway. Although showing overlapping functions, LIF and OsM also have distinct independent functions (Hara *et al.* 1998). OsM protein shows exquisitely defined temporal and spatial regulation in the murine uterus, which is quite distinct from the glandular expression seen for LIF. We have been unable to detect any LIF protein adjacent to the implanting embryo on late day 4 or day 5 of pregnancy (Fouladi-Nashta *et al.* 2005) in contrast to the report of transient transcript expression (Song *et al.* 2000). OsM is expressed only adjacent to the

implanting mouse embryo, first in the LE and then in adjacent stroma at the implantation site (Fouladi-Nashta *et al.* 2005) (Fig. 4). The role of OsM has not been previously investigated in the mouse uterus, but the in human it is reported to reduce proliferation and induce differentiation in uterine stroma in the secretory phase (Ogata *et al.* 2000, Ohata *et al.* 2001) and has been implicated in tissue remodelling in other systems. It has been shown to upregulate both matrix metalloproteinases (MMPs) and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) in diverse cell types (Richards *et al.* 1993, Korzus *et al.* 1997, Sanchez *et al.* 2004) as well as tissue plasminogen activator and plasminogen activator inhibitor (Macfelda *et al.* 2002, Spence *et al.* 2002), while in human vascular smooth muscle cells it induces alkaline phosphatase (Shioi *et al.* 2002) and in astocytes Cox-2 (Repovic *et al.* 2003). All of these are present in decidualizing stroma, a tissue undergoing intense remodelling at the time of implantation. There is evidence that TIMP-1 regulates trophoblast protease activity (Behrendtsen *et al.* 1992). However, the OsM-Rβ knockout mouse apparently does not have an implantation or fertility-related phenotype (Tanaka *et al.* 2003), indicating that OsM signalling may not be indispensable for implantation.

LIF and the pre-implantation uterus

Although the major research emphasis has been on the role of LIF at the time of implantation, it may also affect uterine cells in the pre-implantation period since it is transiently expressed after ovulation on day 1 of pregnancy, mainly by the LE (Bhatt *et al.* 1991). Seminal fluid has been shown to induce increased LIF in the human uterus (Gutsche *et al.* 2003). As well as epithelial cells and stromal fibroblasts, other cell populations such as leukocytes may be adversely affected in the absence of LIF. We have identified differences between the LIF-null and wild-type uterus in several uterine cell populations, including overall stromal cell dynamics, the proportions and distribution of various leukocyte subsets and the density of uterine glands with a peak effect on day 3 of pregnancy (Schofield & Kimber 2005).

Since LIF appears to be a downstream mediator of oestrogen action in the murine peri-implantation uterus, one possible role might be regulation of cell proliferation. During the reproductive cycle, preovulatory oestrogen stimulates luminal and glandular proliferation, which is curtailed by the rise in progesterone on days 2–3 of pregnancy. A transient rise in oestrogen early on day 4 of pregnancy then stimulates stromal proliferation. It has been reported that absence of LIF has no effect on proliferation in either LE or stroma during early pregnancy in inbred C57Bl6 mice (Chen *et al.* 2000). However, detailed comparison of different regions of the uterus between LIF-null and wild-type outbred animals after labelling with the nucleotide precursor bromodeoxyuridine revealed alterations in cell proliferation in the absence of LIF on days 4 and 5 of pregnancy. The overall percentage of proliferative cells in stroma was found to be reduced in the LIF-null uterus compared with wild-type and regional differences were also evident (G Schofield & S J Kimber, manuscript in preparation).

LIF and uterine glands

In the absence of LIF, we also found greater numbers of uterine glandular profiles from day 3 of pregnancy, suggesting that it may have a cytostatic effect on glandular proliferation or branching morphogenesis. Interestingly the *Hoxa-11* knockout mouse does not express LIF in the uterus but shows a deficit of uterine glands, suggesting the complexity of their regulation. Oestrogen exposure at the correct time is well established to be critical for regular glandular morphogenesis. Diethylstilboesterol given *in utero* perturbs proper reproductive tract morphogenesis including inhibiting uterine gland development, an effect mimicked by loss of Wnt7a (Miller *et al.* 1998, Carta & Sassoon 2004). Wnt7a −/− mice are sterile and in adults uterine cell death is enhanced in response to diethylstilboestrol, while proliferation is unaffected; the adult uterus also shows a range of molecular mis-regulations. Any interaction between the LIF and wnt pathways in this context is as yet unknown nor have we yet determined if differences in apoptosis between LIF-null and wild-type glands are evident.

Leukocyte populations in the LIF-null uterus

Macrophages, NK cells and eosinophils are present in the pregnant uterus and are thought to be beneficial. Alterations in the proportions of NK cells and macrophages can adversely affect pregnancy (Pollard *et al.* 1991, Guimond *et al.* 1997, Ashkar & Croy 1999). Strikingly, the percentage of macrophages is reduced by more than a half in LIF-null mice on day 3 of pregnancy and their distribution is also altered (Schofield & Kimber 2005), suggesting that LIF is a chemokine for these cells. However, by day 4, macrophage density appeared similar to that in the wild-type. NK cells were detected as early as day 3 of pregnancy in wild-type and LIF-null mice but the LIF knockout uteri had double the wild-type percentage of NK cells at day 3, with particularly high levels at the anti-mesometrial side of the uterus. So it is possible that LIF restricts migration of NK cells into the uterus. The increase in eosinophils on days 3 and 4 in specific regions of the LIF-null stroma suggests similar control for these cells. Thus the absence of LIF leads to different relative proportions of cells on day 3 of pregnancy compared with wild-type uterus. Alterations in the uterine leukocyte subpopulations in LIF knockout mice may give rise to a less robust pregnancy and contribute to failure of implantation at least on an MF1 background. Since several injections of LIF early on day 4 of pregnancy are enough to correct the implantation defect in the null mice, a supply of

LIF on day 4 may be sufficient to redress the effects of leukocyte and glandular aberrations present on day 3 and allow normal uterine function. Alternatively, these abnormalities may not be sufficient to interfere with implantation and, once threshold levels of particular bone marrow-derived cells are acquired, their contribution to uterine function may be sufficient for implantation and establishment of the placenta. All the same, the role of the high levels of LIF seen on day 1 of pregnancy still requires further investigation.

Role of LIF in peri-implantation and later development of the embryo

Mammalian blastocysts express LIF-R (Charnock-Jones *et al.* 1994, Nichols *et al.* 1996, Chen *et al.* 1999) with reciprocal expression of LIF by trophoblast and its receptor by inner cell mass (ICM) in mouse blastocysts (Nichols *et al.* 1996). LIF enhances blastocyst development and differentiation *in vitro* (Lavranos *et al.* 1995, Dunglison *et al.* 1996). Furthermore, it has been reported that the proportion of morulae and/or blastocysts is reduced after microinjection of LIF antisense at the two-cell stage (Cheng *et al.* 2004). As noted above, LIF-null, gp-130-null and LIF-R-null embryos are all able to progress through the pre-implantation period and implant, suggesting that LIF has no function in the embryo during this time. However, gp-130 is required for reactivation of the blastocyst to implant after the diapause-like condition caused by experimental delay of implantation (Nichols *et al.* 2001).

Trophoblast and placenta are also important targets for LIF in both the mouse and human (Harvey *et al.* 1995, Sharkey *et al.* 1999). Murine blastocyst trophoblast out-

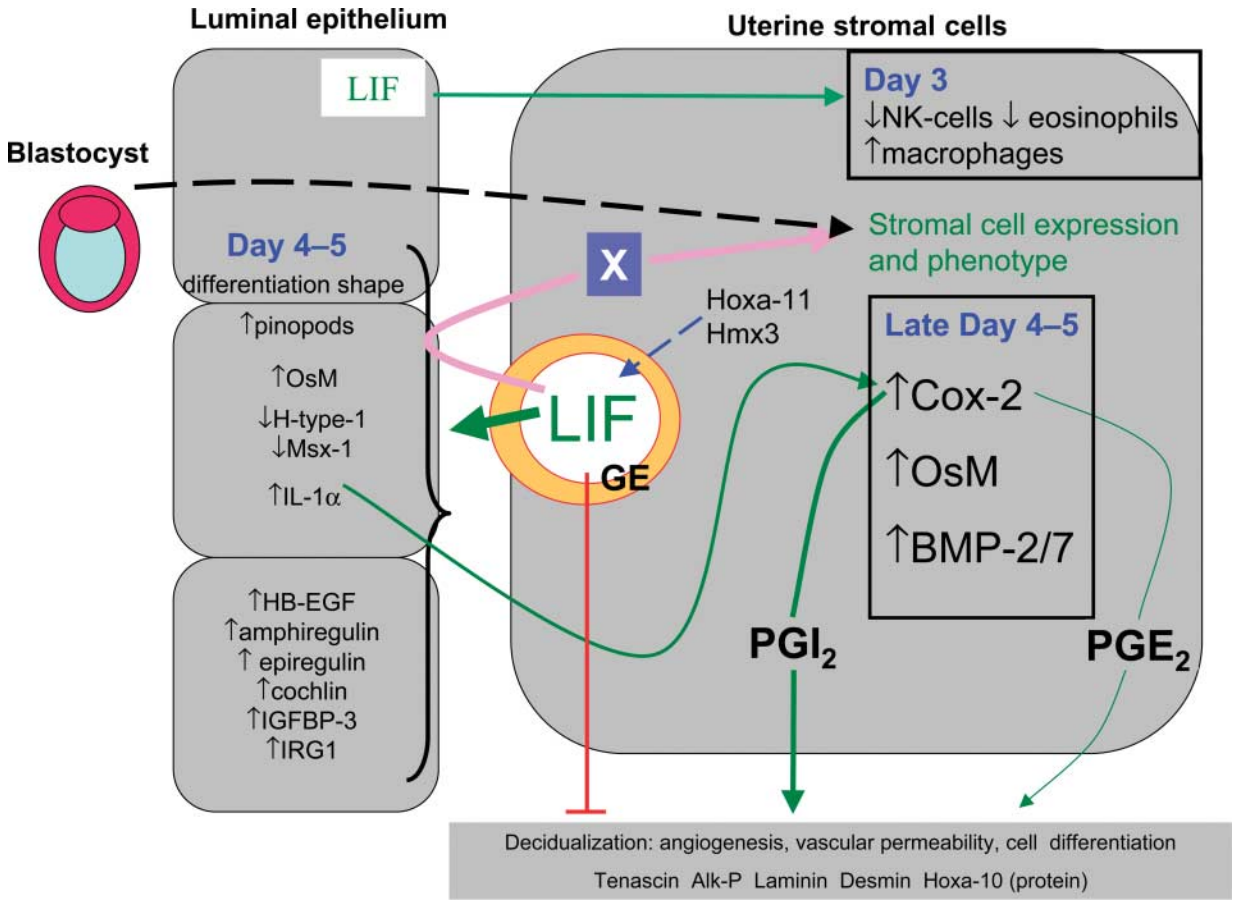

Figure 5 Diagram showing some of the interactions of LIF in the uterus around the time of implantation. Sequential changes over Days 3–5 detailed. Black dashed arrow indicates influence of unknown blastocyst signal on stromal phenotype at implantation site. The blastocyst sends signals which are transduced by the LE in conjunction with LIF from the glandular epithelium (GE) and LIF induced molecules (shown by blue boxed X and pink arrow) contribute to the various stromal cell expression and phenotype changes detailed. Green arrows indicate positive effects. Blue dashed line indicates possible regulation of LIF by Hoxa-11 and Hmx3. LIF is not required for expression of Cox-2 protein or transcripts in LE but is required for significant stromal expression at the implantation site. LIF is needed for both LE and stromal OsM protein expression. Blastocyst signals are also needed for expression of Cox-2 in LE and underlying stroma. In LE, LIF expresses H-type-1 antigen, promoting its downregulation on the evening of day 4. LIF stimulates expression of a number of LE proteins, some already associated with the implantation process and others with unknown function. Morphological pinopods do not develop in LE in the absence of LIF. The regulation of trophoblast-derived LIF is not shown. The likely route of indirect induction of decidualization is illustrated and direct repression of decidualization by LIF is also indicated.

growths upregulate MMP-9 and urokinase-type plasminogen activator in response to LIF (Harvey *et al.* 1995) and MMP-9 plays a role in trophoblast invasion into the uterus (Salamonsen 1999). LIF stimulates hCG and oncofetal fibronectin production by human trophoblast and its induction of human trophoblast differentiation is hCG dependent (Nachtigall *et al.* 1996, Yang *et al.* 2003, see above).

In our analysis of the progeny of matings involving animals with a deletion of the gene encoding LIF we found that the number of nulls was 58–68% that expected for a Mendelian ratio, for both males and females. The lower proportion of LIF-null offspring indicates some embryo loss in the MF1 strain of mouse. It has been reported that on an inbred C57BL6/J background there is no loss of null offspring *in utero* and Mendelian frequencies are obtained (Stewart *et al.* 1992). LIF has a variety of effects on different cell types *in vitro*, inhibiting the differentiation of embryonic stem cells and promoting the survival and/or proliferation of neurons, primitive haematopoietic precursors and primordial germ cells (Hilton 1992). The breadth of influence is reflected in the defects reported in LIF-R-null fetuses (Ware *et al.* 1995). As well as placental defects, LIF-deficient mice have dramatically decreased numbers of stem cells in spleen and bone marrow, while heterozygous animals are intermediate in phenotype, implying that LIF has a dosage effect. Deficiency in the stem cell population can be prevented by exogenous LIF (Escary *et al.* 1993).

Summary

It is indisputable that LIF is essential for implantation in mice and important in other animals including humans. However, LIF has complex regulatory roles. Working out the LIF-generated sequence of molecular interactions which are critical for implantation, rather than merely secondary repercussions of the loss of primary LIF targets (see Fig. 5), will require further research. Recent evidence has focused on the co-operative action of LIF and progesterone in gene regulation. The molecular cascades controlling implantation of the embryo require strict temporal and spatial regulation. In normal development, a remarkable degree of synchrony is needed between differentiation of the epithelial and stromal compartments of the uterus and the developing embryo if implantation is to be successful. This is perhaps most evident in species like the mouse where the period during which implantation can occur is so short. Even a brief delay in maturation of the uterus can result in asynchrony with the blastocyst. Asynchronous embryo transfer studies indicate that the embryo can normally wait while the uterus differentiates to the receptive state and that, experimentally, the embryo may have some influence over the time of implantation (see Aplin & Kimber 2004). One may speculate that in the absence of LIF, lack of uterine maturation may be equivalent to an asynchrony between embryo and uterus which is never resolved by the development of the appropriate trophoblast-interactive epithelium. Cellular and molecular analysis shows this is not just equivalent to experimental delay of implantation (Fouladi-Nashta *et al.* 2005). The blastocyst remains apparently quiescent in the lumen, is never activated and the pregnancy fails. Therefore, timeliness of expression of LIF and its targets in the uterus is likely to be particularly important.

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The author declares that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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