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Leukemia Inhibitory Factor and Human Endometrial Receptivity

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Introduction

Embryo implantation requires both an activated blastocyst and a receptive endometrium. The human endometrium undergoes extensive remodeling in each menstrual cycle to prepare the endometrium for implantation, should conception occur in that cycle. For most of the cycle, the endometrium is retained in a non-receptive or refractory state and it will support implantation of the blastocyst for only about 4 days commencing around 6 days after the luteinizing hormone (LH) peak (during the mid secretory phase) of a normal menstrual cycle, a time known as the 'window of implantation' (1). The preparation for pregnancy includes differentiation of the glands into a highly secretory state and changes in cell surface proteins and adhesion molecules on the apical and basolateral surfaces of the luminal epithelium. In the stromal compartment, changes are initiated in the fibroblasts close to the spiral arterioles, that lead to differentiation (decidualization) of these cells into decidual cells – this process continues as pregnancy is established to form the decidua of pregnancy.

Leukaemia inhibitory factor (LIF)

The pleiotropic cytokine LIF was initially identified by its ability to induce the macrophage differentiation of the myeloid leukaemic cell line M1 (2). It is now known as a member of the IL-6 family of cytokines that includes interleukin (IL)-11, IL-6, oncostatin M, cardiotrophin (CT)1, ciliary neurotrophic factor (CNTF) and cardiotropin-like cytokine/cytokine-like factor (CLC-CLF). A number of these (LIF, OSM, CT-1 and CNTF) bind to the LIF receptor a chain (LIFR), which triggers dimerisation with gp130, forming a high affinity receptor and leading to activation of the janus-kinase/signal transducer and activator of transcription (JAK/STAT) signal transduction pathway (3). Both membrane-bound and soluble forms of the receptor components have been identified and these may act as inhibitors of cytokine action by competing with cell surface receptors to limit dimerisation with gp130 (4). Signal transduction from cytokines acting through the JAK/STAT pathway is attenuated by the suppressors of cytokine signaling (SOCS) family of cytoplasmic proteins that complete a negative feedback loop (5).

Discovery of the importance of LIF for embryo implantation

LIF was one of the first molecules discovered to be essential for implantation in mice: deletion of the LIF gene resulted in complete failure of implantation resulting from lack of a peak of LIF expression in the uterine glands, which coincided with early embryo attachment (6). Interestingly, mice with a null mutation for gp130, phenocopy mice deficient for LIF (7). Other gp130 family members, particularly IL-11 and IL-6, are also important for implantation in the mouse, but these have different expression patterns and actions (8).

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Expression of LIF and its receptor at the human embryo-maternal interface.

LIF mRNA and protein are expressed in the glandular and luminal epithelium of the human endometrium, specifically between days 18-28 of a normalized 28 day menstrual cycle (9-11) with maximal immunoreactive protein seen during the mid-secretory phase (12). LIF protein has also been detected in the stroma (11, 13-15) and LIF mRNA is present in leukocytes in the very early implantation site (16). LIF is also expressed in the human Fallopian tube: its mRNA is detectable throughout the cycle (17). Endometrial LIFR mRNA is restricted to glandular and luminal epithelium in the mid-secretory phase along with the mRNA for gp130 (12). LIFR transcripts are also expressed in blastocysts produced by in vitro fertilization and cultured to the peri-implantation stage (18) (Fig. 1A).

In the later stages of implantation, LIF is highly expressed in first trimester decidua, in the chorionic villi of first trimester and term placenta and in decidual leukocytes (16, 19) while LIFR mRNA and immunoreactivity are localized on trophoblast cells (both villous and extravillous trophoblast) and in endometrial cells of the foetal villi (16, 20) (Fig. 1B, C). Thus it is likely that LIF may mediate actions between decidual cells and leukocytes within the decidua and the invading trophoblast.

Polarised secretion of LIF

Determination of the direction of secretion of LIF from endometrial epithelial cells and whether this might change during the receptive phase provides an opportunity to understand its major functions at this time. Epithelial cells, with their prominent apical and basolateral domains are well known to sort membrane proteins to specific domains. Less is known about the origin of secreted proteins. Recent studies suggest that at least in MDCK a renal

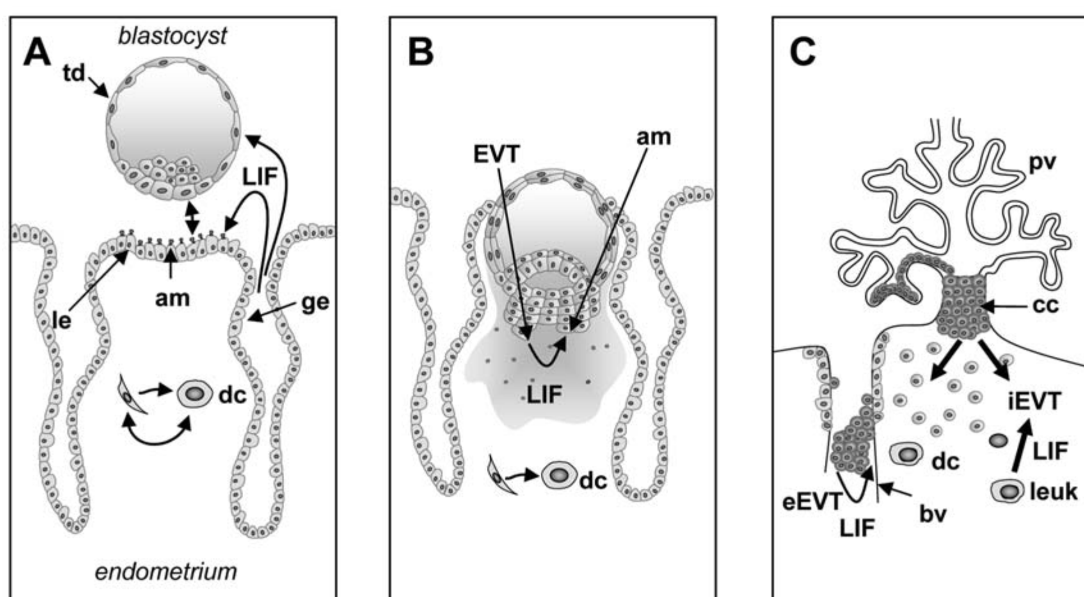


Fig. 1 : Sites of LIF expression and action at the implantation site. A. During the peri-implantation phase LIF is produced by the endometrial epithelium while receptors are present on the trophectoderm and luminal epithelium. B and C. As implantation proceeds, LIF is produced by extravillous trophoblast and by decidual leukocytes while LIF receptor is present on endovascular and interstitial villous trophoblast. td, trophectoderm; le and ge, luminal and glandular epithelium respectively; dc, decidual cell; am, adhesion molecules; pv, placental villus; cc, bv, maternal blood vessel; cc, cell column; iEVT and eEVT, interstitital and endovascular extravillous trophoblast respectively.

cell line, LIF is predominantly secreted in an apical direction regardless of expression level whereas in intestinal epithelial Caco-2 cells, only ~40% of the LIF was secreted apically. This was maintained following apical stimulation by IL-1β but following basolateral stimulation only 20% of the LIF was secreted apically (21). Such information is not currently available for endometrial epithelial cells. However, LIF is clearly secreted apically from the endometrial glands and luminal epithelium into the uterine lumen as it is detected in pg-ng/ml concentrations in uterine washings (22-24) from the mid-secretory phase to a maximum 12 days after the LH surge. Whether it is also secreted basally, and whether the ratio of apical/basal secretion changes in relation to physiological changes in the endometrium, is not known.

Regulation of endometrial LIF expression

The precise mechanisms regulating LIF production during the secretory phase of the menstrual cycle are not fully understood. Human chorionic gonadotrophin (hCG), is a product of the blastocyst and trophoblast in the human (25), and of endometrial epithelium (26), while full-length hCG/LH-receptor mRNA is expressed across the cycle (27). Regulation of LIF production in women by hCG was elegantly demonstrated by Licht et al. (23): delivery of hCG directly into the uterine lumen during the secretory phase induced a rise in LIF concentrations. The tumour suppressor p53 also appears to regulate LIF expression. In p53-deficient female mice which have low fertility, expression levels of uterine LIF are reduced by about 4-fold, while appropriately timed injection with LIF significantly increases the number of embryos that develop to term, suggesting that p53 contributes to control of uterine LIF expression (28). In women a polymorphism in p53 correlates with women having recurrent implantation failure (29). More recently, prokineticin 1, which along with its receptor is maximally expressed in the epithelium of non-pregnant endometrium during the secretory phase of the cycle, has been demonstrated to regulate expression of LIF mRNA and protein and to act as an intermediate in the hCG regulation of LIF (30).

LIF expression is also regulated by pro-inflammatory cytokines including IL-1 and TNF-a and this fits the concept that inflammatory-like processes are important at implantation. In vitro, IL-1β stimulates LIF secretion by endometrial epithelial cells (31) and LIFR is upregulated by both IL-1β and leptin (32, 33). The increased uterine LIF stimulated by seminal fluid (34) may be part of the 'inflammatory-type' reaction induced by factors in seminal fluid (35). Thus, the in vivo situation leading to regulation of LIF is clearly complex, depending on tightly regulated local microenvironments and the balance between individual positive and negative regulators. Further work is required to establish which factors are of most importance, since regulation of these may present an opportunity for manipulation of the 'window of implantation'.

What is known of gene regulation by LIF

A number of potential LIF-regulated molecules have been identified in the endometrium of mice by subtractive hybridization or microarray approaches (Table I). The extracellular protein cochlin, was found to be lacking in the luminal epithelium in LIF-null mice but subsequent gene deletion of cochlin did not result in an implantation

Table I. Implantation-regulated genes known to be regulated by LIF*.

Endometrial Epithelium		*Human Trophoblast*
Mouse	*Human*	
cochlin	integrin α2	integrin β4
IGFBP3	TIMP-1	TIMP-1
amphiregulin	TGFβ1	TIMP-2
IRG-1	SCD44	hCG
		oncofetal fibronectin
		ERBB4

*See text for references

phenotype (36). Conversely, expression of IGF-binding protein 3 (IGFBP3), amphiregulin and immune response gene 1 were upregulated in the luminal epithelium by LIF (37). While IGFBP3 and amphiregulin are not essential for implantation in mice, there is still dispute as to whether IRG1 is essential (38, 39). There is clearly a need for global analysis of LIF-regulated genes in appropriate models of receptive human endometrium and of trophoblast and decidua in early pregnancy.

Actions of LIF at the embryo-maternal interface

Actions on the blastocyst and first trimester trophoblast

The cytokine content (including LIF) of uterine fluid is likely to provide an optimal environment for preimplantation blastocyst development. LIF is also expressed in the Fallopian tube with only slight variation during the menstrual cycle – if secreted this would be available to modulate early embryo development even prior to its entry into the uterine cavity. Importantly LIF promotes human blastocyst development in vitro (40).

Effects of LIF and other growth factors on early cell fate decisions in human embryos cultured to the blastocyst stage, have recently been examined (41). Although the proportion of embryos reaching blastocyst stage was not altered, culture in the presence of LIF facilitated the number of genes expressed per embryo including *ERBB4* which encodes the HBEGF receptor. When HBEGF was also present, gene expression was further modified and in particular, LIFR transcripts were increased, demonstrating a clear mechanism for synergy between these two factors (41). This data underscores the importance of growth factor regulation of human embryo development and raises the need to understand the basis of such influence, particularly since some commercially–available embryo culture media already contain growth factors.

LIF also induces changes in human first trimester extravillous trophoblast (EVT), related to their invasion (42). Initially it was shown that LIF stimulates the phosphorylation of STAT3 protein, indicating signal transduction following receptor binding and dimerisation, an action that was inhibited by a specific LIF inhibitor that blocks such transduction (Fig. 2A). Subsequently, LIF was shown to increase EVT adhesion to the ECM components, fibronectin, vitronection and laminin (Fig. 2B): such action in vivo is needed to allow traction and movement of the EVT through the decidua and/or to transmit signals between the ECM and the cell interior. LIF also decreased integrin b4 mRNA levels in the EVT by 50% and stimulated secretion of tissue inhibitors of metalloproteinases (TIMP)-1 and -2 (Fig. 2C), although it was without effect on MMP2 and MMP9 secretion (42). Thus LIF may contribute to the regulation of EVT invasion during early placental development.

Furthermore, LIF has the potential to stimulate hCG and oncofoetal fibronectin production by human trophoblast (43): this was shown in term trophoblast cells and is presumed to also apply in the first trimester. Synergy between LIF and hGC is demonstrated by the finding that induction of human trophoblast differentiation is hCG dependent (44, 45).

Actions of LIF on the endometrium

Given that LIFR are expressed on human endometrial epithelium and that LIF is upregulated during the receptive phase and secreted into the uterine lumen, it is likely that LIF will act on the luminal epithelium to facilitate blastocyst attachment and implantation. Among the important changes for implantation are those in the adhesive properties of the luminal epithelium. In support of this, LIF treatment increased adhesion of primary endometrial epithelial cells to fibronectin and collagen IV (46) (Fig. 3A) which are present on the surface of blastocysts (47) and human first trimester trophoblast (48) respectively. LIF also increased

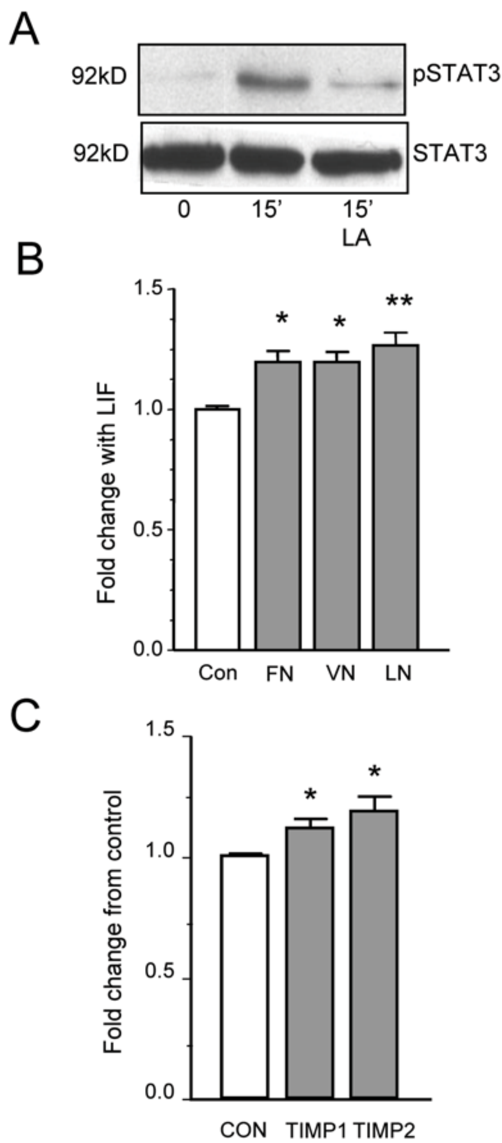


Fig. 2 : Actions of LIF on human extravillous trophoblast. A. LIF stimulates phosphorylation of STAT3 (pSTAT3) an action reduced in the presence of specific LIF antagonist (LA). B LIF increases adhesion to fibronectin (FN) vitronectin (VN) and laminin (LN). C. LIF stimulates secretion of TIMP1 and TIMP2. [data derived from (42)].

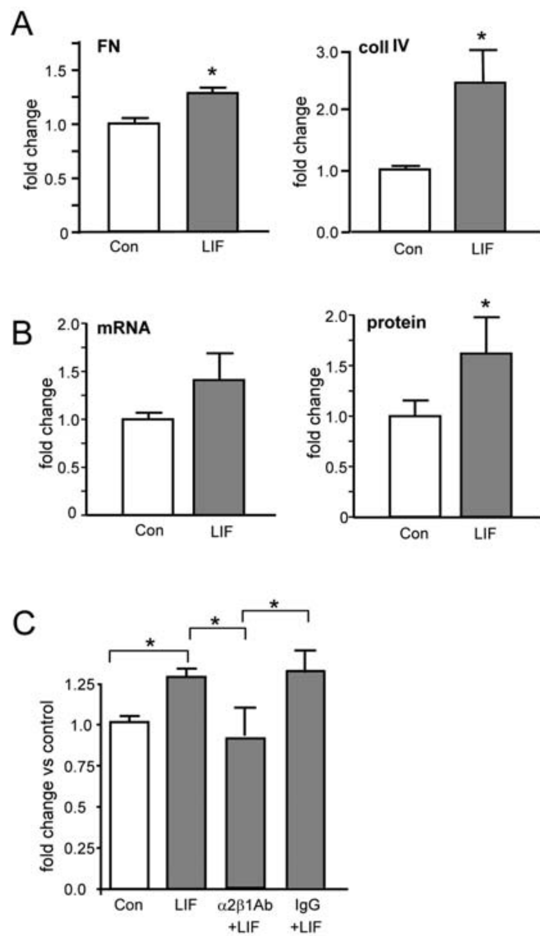


Fig. 3 : Actions of LIF on human endometrial epithelial cells. A. LIF increases adhesion to both fibronectin (FN) and collagen IV (.oll IV). B, LIF increases integrin α2 mRNA and protein. C. LIF regulates adhesion to collagen, an action inhibited by an antibody (Ab) to integrin α2β1 but not by non-immune IgG. [data derived from (46)].

integrin α2 mRNA and protein expression by endometrial epithelial cells (Fig. 3B) and regulated adhesion of the cells to collagen via the heterodimer integrin α2β1 (Fig. 3C). This is in contrast to its action on human placental trophoblast cells in which LIF does not regulate integrin α2β1 but does regulate integrin b6 mRNA (42). Inhibition of STAT3 reversed the effects of LIF on the epithelial cells while application of a LIF antagonist abolished LIF-induced STAT3 phosphorylation (46). In addition to its actions on integrin α2, LIF also increased TIMP-1 and TGFβ1 mRNA in the epithelial endometrial cells and synergized with IL-11 to increase TGFβ1 secretion and sCD44 secretion (46).

Does disturbance of LIF affect fertility in women

One in eight couples have difficulty in conceiving naturally, resulting in assisted reproductive technologies (ARTs). Implantation failure is a key factor limiting success in ART; typically 70% of embryos selected for transfer fail to implant and at least 50% of this is likely to result from the endometrium not being fully receptive (49). Inadequacy of the early steps of implantation leads to early pregnancy loss (30% of pregnancies) whereas placental insufficiency is implicated in recurrent miscarriage, low birth weight, preeclampsia and pre-term delivery. The long term consequences of growth impairment in utero are increased susceptibility to chronic disease in adult life (50).

The importance of LIF for fertility in women is still under debate, despite the promising results in animal models. However, evidence is emerging that disturbance of endometrial LIF may result in infertility in at least some women: what is also clear is that many other factors, particularly other cytokines, probably provide redundancy and that loss of any one cytokine may be compensated by other factors playing synergistic roles.

Changes in LIF expression in clinically important conditions

LIF expression is down-regulated in the endometrial epithelial glands of some but not all infertile women, including those also with endometriosis (51-53). Phosphorylated STAT3 is also lower in glandular epithelium in women with unexplained infertility although pSTAT3 levels did not correlate with LIF levels (51), probably because other factors (such as IL-11) are also activating STAT3. If the reduced STAT3 levels are indicative of reduced STAT3 abundance, this could result in inadequate LIF action.

LIF levels in the uterine secretome are suggested to be predictive of embryo implantation (54) and are reduced in women with primary fertility in the late secretory phase (10 days following the LH peak) compared with normal women (22, 24). These data support other studies showing that cultured endometrial explant tissue from infertile women produced less LIF than tissue obtained from fertile women (55, 56).

Disturbances of LIF secretion or action may also contribute to unexplained recurrent abortion. Withn this context is the decreased expression of LIF mRNA by decidual T cells (57). Furthermore, an abnormally persistent expression of LIF receptor on EVT cells in placental bed biopsies has been demonstrated in patients with early onset pre-eclampsia combined with intra-uterine growth retardation (58).

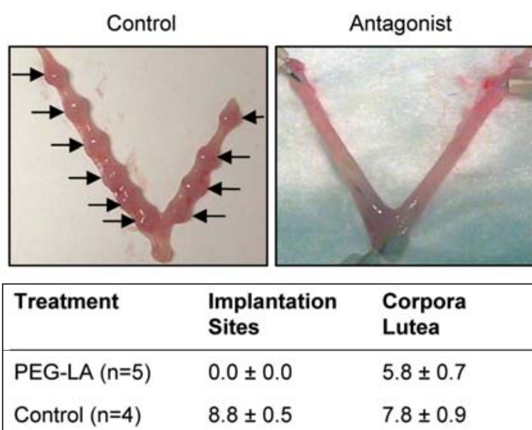
Potential for endometrial LIF as a target for contraception

New contraceptive strategies for women are urgently required. Given that LIF is indispensable for implantation in mice, blocking LIF expression or action locally in the human endometrium may provide an environment in which implantation cannot occur. First steps towards this outcome have involved reproducing the LIF-null phenotype in the uteri of non-human primates or mice by the use of neutralizing antibodies or inhibitors against LIF or by blocking transduction of its signal. Blockade of LIF's action by administering polyclonal antibody or monoclonal antibody against LIF into the uterus of the monkey during the peri-implantation phase (59, 60) significantly reduced implantation in rhesus macaques. However, both timing and route of administration were important. As an alternative strategy, functional blockade of Stat3 activation by injection into the uterine lumen of a cell-permeable Stat3 peptide inhibitor blocked implantation in mice by 70%: the effectiveness of the inhibitor on blocking Stat3 activation was in the luminal epithelium and was not due to non-specific effects or to actions on the embryo (61).

We have taken a different but parallel approach to prove the principle that blocking LIF could provide a non-hormonal contraceptive strategy (62). A highly potent LIF antagonist (referred to as LA) was produced by mutating human LIF at regions that bind the LIFR and gp130. Relative to wild-type human LIF, LA has a >1000-fold higher affinity for binding to LIFR but does not bind gp130 as required for productive signaling. LA is species cross-reactive and is also a potent inhibitor of murine LIF-induced bioactivity. However, this inhibitor had a very short half-life as does the parent molecule LIF, so we conjugated the inhibitor to polyethylene glycol (PEGLA) which increased its serum half-life. Administration of this inhibitor to pregnant mice by three intraperitoneal injections between day 2-5 – 3.5 of pregnancy (day 0 = day of plug) completely blocks implantation (62) (Fig. 4). This PEGLA now needs testing in a primate model and its safety, reliability and reversibility in women determined.

Conclusion

Our understanding of LIF expression and actions in the human endometrium has markedly expanded since the discovery more than fifteen years ago that LIF is essential for implantation in mice and the subsequent identification of LIF as one of proteins produced and secreted from the endometrial epithelium during the receptive phase of the menstrual cycle. Unfortunately, there has been no or very limited translation of this information to problems of human fertility. It is however, now clear that there is a plethora of such regulatory factors that contribute to the attainment of uterine receptivity in women and that there is considerable redundancy; hardly surprising given the key role of implantation in species survival of placental mammals. It is to be hoped that the more global approaches of systems biology and the '*omics*' will enable us to further understand the interactions between systems that lead to successful implantation and that these findings can be translated for the benefit of humankind.



Treatment	Implantation Sites	Corpora Lutea
PEG-LA (n=5)	0.0 ± 0.0	5.8 ± 0.7
Control (n=4)	8.8 ± 0.5	7.8 ± 0.9

Fig. 4 : Administration of a pegylated LIF antagonist (PEG-LA) completely blocked implantation in mice. [data derived from (62)].

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