Determining the LIF-sensitive period for implantation using a LIF-receptor antagonist

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Abstract

Uteri of *Lif* null mice do not support embryo implantation. Since deletion of some genes often prevents the survival of null mice to adulthood, we have used a proven inhibitor of leukaemia inhibitory factor (LIF) signalling to identify the precise window of time during which LIF is required *in vivo*, and assessed the cellular expression of several LIF-associated targets. On day 4 of pregnancy, mice were injected with hLIF-05 (inhibitor) into the uterine lumen, with corresponding volumes of PBS (vehicle) injected into the contralateral horn. On days 5 and 6, the number of implantation sites was recorded and the uteri processed for immunohistochemistry. Blockade of LIF on day 4 reduced embryo implantation by 50% ($P \leq 0.0001$) and was effective maximally between 0930 and 1230 h. Antagonism of LIF signalling was evidenced by a lack of phosphorylated STAT3 in the luminal epithelium (LE). Amphiregulin was absent from the LE on day 4 evening and H-type-1 antigen expression was retained in the LE on day 5 in inhibited uteri. Interleukin-1 α and oncostatin M expression were reduced in the stroma on day 6, following LIF inhibition. Unexpectedly, PTGS2 expression in stroma was unaffected by LIF inhibition *in vivo*, in contrast to *Lif* null mice. In summary, this suggests that LIF signalling is effective for implantation during a discrete time window on day 4 and antagonism of LIF signalling recapitulates many features exhibited in *Lif* null uteri. The data presented validates the use of antagonists to investigate tissue specific and temporal cytokine signalling in reproductive function.

Reproduction (2009) 138 827–836

Introduction

The initiation of pregnancy requires a precisely timed synchrony between endometrial development and the implanting embryo. Under the influence of ovarian steroids, the uterine endometrium undergoes profound modifications in cellular proliferation and differentiation in order to support the embryo during pregnancy. Although oestrogen and progesterone are essential for development of an appropriate endometrial environment necessary for successful embryo implantation, it is now evident that these effects are further modulated by cytokines and growth factors that are secreted by a variety of cell types within the uterine endometrium. Leukaemia inhibitory factor (LIF) is a 40–70 kDa protein with seven putative N-glycosylation sites in its primary structure, which allows extensive post translational modifications (Taupin *et al.* 1998). LIF belongs to the interleukin 6 (IL6) family of cytokines including, IL11, cardiotrophin-1 (CTF1), oncostatin M (OSM), ciliary neurotrophic factor (CNTF) and CTF like cytokine (CLCF1), of which only LIF and IL11 have been shown to be essential for embryo implantation. *Lif* transcripts are expressed in low levels in numerous tissues (Brown *et al.* 1994) and like most cytokines, LIF production is

highly inducible by various stimuli in a wide range of cell types (Fukada *et al.* 1997, Morel *et al.* 2000, Tofaris *et al.* 2002), its expression being particularly high in murine uterine tissue on day 4 of pregnancy (the time at which implantation occurs; Bhatt *et al.* 1991). Uterine LIF is expressed in two transient peaks during early pregnancy in the mouse. First, on day 1 of pregnancy LIF expression is stimulated by ovulatory oestrogen in both luminal and glandular epithelium. Second, on day 4 nidatory oestrogen stimulates expression of both *Lif* mRNA and protein in the glandular epithelium (Bhatt *et al.* 1991, Stewart *et al.* 1992, Fouladi-Nashta *et al.* 2005). This peak of LIF expression is essential for embryo implantation into the uterus on the evening of day 4 of pregnancy (Chen *et al.* 2000).

LIF acts on its target cells via the activation of the LIF-receptor (LIFR) complex. The LIFR complex requires heterodimerisation of the two low affinity components LIFR and glycoprotein 130 (gp130), where combination transforms a low-affinity binding complex into a high-affinity binding site (Gearing *et al.* 1992, Boulanger & Garcia 2004). Activation of the LIFR complex can trigger several signal transduction pathways; however, in the uterus activation occurs primarily via the JAK and STATs pathways (Auernhammer & Melmed 2000,

Cheng *et al.* 2001). The first insight into the crucial role of LIF in murine implantation came with the generation of mice with a null mutation of the gene encoding *Lif*. These mice were infertile because of the failure of blastocyst implantation (Stewart *et al.* 1992, Escary *et al.* 1993, Fouladi-Nashta *et al.* 2005). The importance of LIF for successful embryo implantation in the mouse may be of general significance to all mammals, as increased levels of LIF during pregnancy have been shown to be conserved.

A mutant form of human LIF, hLIF-05, has been shown to be powerful antagonist of the action of those cytokines which utilise the LIFR complex (Hudson *et al.* 1996, Vernallis *et al.* 1997). Experiments have shown that functional inactivation of LIFR by hLIF-05 is required for the arrest of murine rod differentiation *in vitro* (Neophytou *et al.* 1997) and previous work in our laboratory has shown that LIF induced effects can be antagonised by hLIF-05 in cultured murine uterine stromal cells (Fouladi-Nashta *et al.* 2004, 2008). However, this antagonist has not been used previously *in vivo*. In the present study, we set out to assess the effect of hLIF-05 during early pregnancy and whether abrogation of LIF signalling resulted in reduced implantation. Our results show that injections of hLIF-05 directly into the uterine lumen of day 4 pregnant MF1 mice resulted in 50% reduction in embryo implantation compared to vehicle control and that this inhibition is uterine stage dependent. Furthermore, we report that the cellular expression of a number of previously identified LIF-regulated molecules and novel targets are altered in LIF-inhibited uteri.

Results

LIF inhibition *in vivo* and its effects on implantation

Once the optimal injection volume had been established (see Materials and Methods), mice either on the evening of day 3 (1800 h; $n=3$) or on day 4 of pregnancy (between 0830 and 1800 h – divided into hourly time sets; $n=34$) were injected into the uterine horn with 5 μ l hLIF-05 or PBS (control vehicle). The number of implanted embryos was counted on days 5 or 6 of pregnancy. Uterine horns were normal in appearance and ovulation was confirmed in all animals by the presence of corpora lutea. A number of mice ($n=7$) were also used as non-surgical controls and the number of implantation sites recorded in parallel with those in experimental mice. The mean number of implanted embryos in non-surgical mice was 6.8 ± 0.4 compared with 6.3 ± 0.3 in PBS-injected horns. These results show that intrauterine injections of vehicle on day 4 of pregnancy did not significantly perturb embryo implantation.

Overall the mean number of implantation sites after injection on day 4 of pregnancy in the hLIF-05 group was 3.2 ± 0.3 compared with 6.3 ± 0.3 in the control horn

(Fig. 1A). This reduction of $\sim 50\%$ was statistically significant ($P=0.0001$). However, two animals exhibited 100% inhibition of implantation following interference of LIF signalling with hLIF-05. In other animals, two to four implantation sites were visible in inhibited horns, but the majority of these implantation sites were at the cervical end of the uterine horn, away from the site of injection.

When the data were broken down according to the time of injection it was clear that there was a specific period when LIF inhibition effectively inhibited embryo implantation (Fig. 1B). Maximal reduction of implantation was observed in mice injected between 1030 and 1129 h on day 4 of pregnancy where implantation sites were reduced to 36% of the number in control horns ($P=0.004$). Injections administered between 0930 and 1029 h also significantly decreased implantation to 50% of the number in control horns ($P=0.009$). However, injections on the evening of day 3 of pregnancy (1800 h) did not give a statistically significant difference in implantation in LIF inhibited compared to PBS-treated horns ($P=0.130$). Similarly injections between 0830 and 0929 h and between 1630 and 1729 h on day 4 of pregnancy did not give statistically significant differences in implantation between control and treated horns ($P=0.142$ and 0.3 respectively). For all further analyses uteri were selected from those in which implantation sites were reduced by at least 50% (primarily from mice that were injected between 0930 and 1130 h) and typically from implantation sites closest to the point of injection.

Figure 1 Comparison of uterine implantation sites following *in vivo* administration of LIF inhibitor on day 4 of pregnancy. (A) On day 4 of pregnancy between 0830 and 1730 h, mice were given intraluminal injections of hLIF-05 in one horn and PBS vehicle into the contralateral horn ($n=34$). On day 5 or day 6, the numbers of embryos in each uterine horn were recorded. Mean values are indicated by the lines. The reduction in implantation in treated uterine horns compared to control was significant ($P \leq 0.0001$). (B) Animals were injected within several 1 h time periods to examine temporal effects of LIF inhibition on implantation. The numbers in each group were as follows: day 3 ($n=3$); day 4, 0830–0929 h ($n=8$); 0930–1029 h ($n=7$); 1030–1129 h ($n=7$); 1130–1229 h ($n=4$); 1530–1629 h ($n=5$); 1630–1729 h ($n=3$). The effectiveness of the inhibitor to reduce implantation rate was maximal between 1030–1130 h. *Denotes $P \leq 0.05$ (1530–1629 h), ** $P \leq 0.01$ (0930–1029 h, 1030–1129 h and 1130–1229 h), where significant differences are relative to control. Values represent mean $\pm$ s.e.m.

Macroscopic and microscopic analysis of LIF-inhibited uteri

In order to gain further insight into the effects of the LIF inhibitor on embryo implantation and uterine morphology, uterine sections were stained with haematoxylin and eosin. A comparison of histological features of the mouse uterus on days 4 and 5 is shown for control and inhibited horns (Fig. 2C–F). A normal pregnancy phenotype was observed in control uteri on day 5

Figure 2 Analysis of implantation sites after LIF inhibition *in vivo*. (A and B) On day 5 of pregnancy, implantation sites were visualised by i.v. injections of pontamine blue dye prior to killing. Presence of vascular permeability indicating implanted embryos can be seen as blue bands (arrows). Note that, in the inhibited horn, an implantation site can be seen at the cervix end of the uterine horn, the furthest point from the site of injection (*). Scale = 1 mm. Following injection with hLIF-05 or vehicle on day 4 of pregnancy (0900 h), uterine tissue was harvested 1 h post injection and assessed for immunoreactive p-STAT3 ($n=3$). (C) In vehicle-injected uteri, positive immunoreactive nuclear p-STAT3 can be seen in the LE and embryo (inset shows negative rabbit IgG control). (D) In contrast, no p-STAT3 was detected in the uteri 1 h post injection of hLIF-05 (negative control inset). Histological examination on day 5 of pregnancy ($n=4$) shows, in control uteri (E), invasion of the LE by trophoblastic cells (arrows; higher magnification inset). (F) In LIF inhibited uteri, although the embryo is present in an implantation chamber, the trophoblast is only superficially apposed to the LE (arrows; higher magnification inset). Scale bars represent 100 μ m. LE, luminal epithelium; S, stroma; L, lumen; E, embryo; MP, mesometrial pole; AMP, antimesometrial pole.

(0900–1000 h) with the embryo in an implantation chamber and evidence of adhesion/penetration of the luminal epithelium (LE) by the trophoblastic cells/trophoblast (Fig. 2E arrows). In contrast, although the majority of day 5 inhibited embryos were evident in an implantation chamber and appeared to make intimate contact with the LE, no invasion of the LE by the trophoblast was observed (Fig. 2F arrows). No or very little decidualisation was also apparent concurrent with the lack of pontamine blue staining (Fig. 2A and B) and reduced stromal desmin immunoreactivity (see Fig. 4A and B later). In contrast, implantation sites that did form in inhibited horns adjacent to the cervix were analysed on day 5 and day 6 of pregnancy ($n=4$) and exhibited normal size and histology (data not shown).

STAT3 phosphorylation in response to LIF inhibition

To determine whether the reduction in implantation in LIF-inhibited uteri was due to antagonism of the LIFR complex and subsequent inactivation of STAT3 signalling, animals were killed at 1 h (1000 h) or 12 h (2200 h) post injection on day 4 of pregnancy. Immunohistochemical analysis of tyrosine phosphorylated STAT3 (p-STAT3) showed that at 1 h post-injection, strong nuclear expression of p-STAT3 was evident in the LE and in the embryo in PBS-treated uteri, but was absent from the LE in the LIF-inhibited horn (Fig. 2C and D). By 12 h post-injection, no immunoreactive p-STAT3 was detected in either control or treated horns or in the embryo (data not shown).

Response of molecules in the LE to LIF inhibition

To assess whether the reduction in implantation was attributable to the alteration of implantation-related proteins *in vivo*, the expression of a number of molecules known to be induced in the LE and potential downstream targets of LIF were examined by immunohistochemistry.

Amphiregulin expression

A comparison of amphiregulin (AREG) expression in the inhibitor treated and control uteri are shown in Fig. 3A and B. On the evening of day 4 (2100 h) of pregnancy, immunoreactive AREG was restricted to the LE cells at the site of implantation in control uteri. In contrast, AREG was not detected in the LE cells at the potential implantation site in inhibited uteri.

H-type-1 antigen expression

The H-type-1 antigen is associated with implantation and is misregulated in the *Lif* null uterus at the expected time of implantation (Fouladi-Nashta *et al.* 2005). On day 5 of pregnancy, no H-type-1 antigen was detected in the LE at the site of implantation in control uteri and it

was only detected in few areas of GE (Fig. 3E and F) indicating that it is downregulated at or just after implantation as shown previously. In contrast, H-type-1 expression in inhibited uterine horns remained strong and was localised to the apical border of the LE at the implantation site and GE.

Figure 3 Changes in LE expression in response to *in vivo* administration of LIF inhibitor on day 4 of pregnancy. Uteri were treated between 0930 and 1130 h and harvested at 2100 h on day 4 of pregnancy and uterine sections were stained with an antibody to amphiregulin ($n=3$). (A) In control uteri, amphiregulin (green) was localised to the LE exclusively at the site of embryo implantation. (B) In treated uteri, no LE staining was observed. Images C and D show negative goat IgG control in control and inhibited uteri respectively. Uterine sections on day 5 of pregnancy ($n=3$) from control (E) and treated (F) horns were stained for H-type-1 antigen (red). Negative controls show nuclei counterstained with DAPI alone (G and H). In control uteri, H-type-1 was only sparsely evident in the uterine glands. In contrast, LIF-inhibited horns retained H-type-1 epitopes as seen on the apical surface of cells in the LE (arrow) surrounding the embryo. Scale bars represent 100 µm. LE, luminal epithelium; E, embryo; S, stroma.

Analysis of stromal components in response to LIF inhibition

To examine whether the reduction in implantation was attributable to secondary changes in decidualisation, the expression of a number of molecules associated with the decidualisation process and thought to be regulated, if indirectly, by LIF were also examined.

Desmin expression

Cellular expression of the decidualisation associated protein, desmin, was examined in uterine sections from inhibited and control horns on day 5 of pregnancy (Fig. 4A and B). In control uteri, desmin filaments were detected in the subluminal stroma at the site of implantation. In contrast, in day 5 LIF inhibited uteri, immunoreactive desmin filaments were reduced compared to the control and expression appeared similar to that seen in preimplantation uteri and as reported previously in the *Lif* null uterus on day 5 of pregnancy (Fouladi-Nashta *et al.* 2005).

PTGS2 expression

PTGS2 expression was assessed by immunohistochemistry in inhibitor treated and control horns on day 5 and day 6 of pregnancy (Fig. 4C–F). On day 5, immunoreactive PTGS2 was localised in both the LE and subluminal stromal cells at the implantation site in both treated and control uteri. By day 6 of pregnancy, PTGS2 expression was observed deeper in the surrounding stroma in both control and treated horns, but appeared fainter and more restricted in treated uteri. It was also evident that even by day 6 of pregnancy in the inhibited horn; embryo implantation had not progressed as the embryo was clearly visible within the lumen, still in the implantation chamber and adjacent to the LE (Fig. 4F arrow).

OSM expression

OSM has been shown to be expressed at the implantation site in wild-type mice, but absent from the uteri of *Lif* null mice (Fouladi-Nashta *et al.* 2005). OSM immunolocalisation in control and LIF-inhibitor treated uterine horns on day 5 and day 6 of pregnancy is shown in Fig. 4G–J. On day 5 of pregnancy, there was no difference in OSM expression between the control and inhibited horns; expression mainly restricted to the LE and embryo. However, by day 6, OSM (Fig. 4I and J) was expressed in stromal cells surrounding the implanted embryos in control horns, but no OSM was detected in LIF-inhibited horns where embryos were still evident in the implantation chamber.

IL1A and IL1B expression

IL1-associated molecules are temporally regulated during the implantation period in wild-type mice and this regulation is aberrant in the uteri of *Lif* null mice (Fouladi-Nashta *et al.* 2008). To determine if hLIF-05 inhibition causes misregulation of IL1 during early pregnancy, immunohistochemical analysis of IL1A and IL1B was performed in control and LIF-inhibited uteri on days 5 and 6 of pregnancy. On day 5 of pregnancy, immunoreactive IL1A and IL1B were detected in a similar pattern in both control and treated uterine horns (Fig. 5A, B, E and F). However, by day 6 of pregnancy in vehicle treated horns intensely IL1A positive stromal cells were detected around the embryo, and to a lesser

extent throughout the control decidua (Fig. 5C and D). In contrast, in inhibited uteri, immunoreactive IL1A was detected in LE adjacent to embryos. No stromal IL1A was detected in LIF inhibited uteri. IL1B was detected on day 6 of pregnancy; its expression localised to both the LE and decidualising stroma in PBS-treated uteri. In contrast, its expression was restricted to the LE and GE and undetectable in undifferentiated stromal cells in LIF-inhibited uteri (Fig. 5G and H).

Discussion

Gene targeting in mice has revealed much about gene function in normal development and disease processes. Often, the genetic alteration prevents the survival of null mice to adulthood and thus studies on reproductive physiology are hampered, reviewed in Salamonsen *et al.* (2001). Previous work has centred upon identifying possible targets of LIF during early pregnancy from comparisons between wild-type and *Lif* deficient mice (Fouladi-Nashta *et al.* 2004, 2005, 2008, Sherwin *et al.* 2004). However, this neither permits the study of the temporal and spatial influence of LIF, particularly during pregnancy nor does it address the possibility that the phenotype may partly arise from the gene deletion construct causing *-cis* or *-trans* effects on adjacent genes. To address these points, we have utilised a proven inhibitor of LIF signalling to investigate the functionality of LIF on embryo implantation and to assess if inactivation of LIFR affects the expression of novel and key molecules involved during early pregnancy.

Injections of hLIF-05 on day 4 of pregnancy reduced implantation by 50% in MF1 mice compared to mice injected with the same volume of vehicle. This was attributed to the blockade of LIF signalling, since p-STAT3 expression was undetectable in the LE 1 h

Figure 4 Changes in decidualisation associated proteins in response to LIF inhibition. On day 5 of pregnancy, control and treated uteri were harvested and sections ($n=3$) were stained with an antibody to desmin (green). Nuclei were counterstained with DAPI (blue). Immunoreactive desmin was detected in the subluminal stromal cells at the site of embryo attachment in control uteri (A), but less extensive in treated uteri (B). On days 5 and 6 of pregnancy, LIF-inhibitor treated and control uterine sections were stained with an antibody to PTGS2 or OSM ($n=3$ for each antibody). On day 5 of pregnancy immunoreactive PTGS2 was detected in the LE and stromal cells in both control (C) and treated horns (D). By day 6 of pregnancy PTGS2 was also detected in the stroma surrounding the embryo in both controls (E) and treated horns (F) but staining was more restricted in the latter. Note that even by day 6 of pregnancy the embryo had not undergone implantation in the inhibited horns and was clearly visible in an implantation chamber (arrow). No difference in OSM expression was observed on day 5 of pregnancy between controls (G) and treated uteri (H). By day 6 OSM was detected in stromal cells at the implantation site in control uteri (I), but in LIF-inhibited uteri (J) staining was almost absent. Weak immunoreactive OSM can be seen only in LE adjacent to the embryo and the embryo itself. Scale bars represent 100 μm . LE, luminal epithelium; E, embryo; S, stroma.

Figure 5 Effect of LIF inhibition *in vivo* on IL1A and IL1B expression during early pregnancy. On days 5 and 6 of pregnancy ($n=3$ for each antibody), control and treated uteri were harvested and sections were labelled with antibodies to IL1A (green) and IL1B (brown). On day 5, IL1A immunoreactivity was detected throughout the uterus in control (A) and treated (B) uteri. On day 6, this expression was maintained in control uterine horns (C), but LIF-inhibited uteri (D) showed only residual IL1A staining around the embryo which is still present in an implantation chamber (arrow). On D5 of pregnancy no differences in IL1B expression were observed between controls (E) and treated uteri (F). On day 6, IL1B immunoreactivity was observed in the LE and decidualising stromal cells in control uteri (G), whereas in treated uteri (H) IL1B expression remained localised to the LE and GE, and was not detected in the undifferentiated stroma. Scale bars represent 100 μm . LE, luminal epithelium; S, stroma; E, embryo.

post LIF inhibition, in contrast to the nuclear staining in control injected horns. Our results confirm the localised activation of STAT3 in LE at the implantation site on day 4 of pregnancy as previously shown (Cheng *et al.* 2001). This illustrates the rapid effect of such inhibitors, in agreement with a separate study in which p-STAT3 was shown to be downregulated within 2 h following injection of a different LIFR antagonist *in vivo* (White *et al.* 2007). Since p-STAT3 expression in the LE 12 h post injection was absent in control and inhibited uteri,

STAT3 activation is tightly regulated early on day 4. Studies using a peptide inhibitor of STAT3 or a STAT3 decoy *in vivo* reduced embryo implantation by 70% (Catalano *et al.* 2005, Nakamura *et al.* 2006); further demonstrating that STAT3 is a key regulator of uterine maturation. Moreover, STAT3 also transduces the response to other IL6 family cytokines such as IL11, which is also involved in later events of implantation. IL11 receptor α knockout females undergo successful implantation but exhibit defective decidualisation (Robb *et al.* 1998). HLIF-05 is a powerful antagonist *in vitro* of the action of those cytokines that depend on the LIFR, including CNTF and CTF-1 (Boulay *et al.* 2003) and the effects of inhibiting these molecules in addition to LIF cannot be excluded. However, previous *in vivo* studies demonstrate that the activation of STAT3 in the LE on day 4 of pregnancy is induced by LIF alone and not by IL6 or CNTF (Cheng *et al.* 2001).

In this study, hLIF-05 was administered directly into the uterine lumen of day 4 pregnant mice because previous studies have shown that LIFR is predominantly expressed in the LE at this time (Cheng *et al.* 2001); suggesting that the LE is the primary target of LIF. This also avoided any possible systemic endocrine effects that may affect implantation indirectly. Furthermore, the use of paired uterine horns from the same animal ensured that in each horn the endometrium was exposed to an identical ovarian steroid environment. The possibility of the inhibitor just affecting LIF signalling in the embryo is unlikely since a previous study using a LIF antagonist showed no differences in pre-implantation embryo development or blastocyst outgrowth capability between treated and control groups (White *et al.* 2007). Furthermore, *Lif*-deficient embryos are viable and able to implant into pseudopregnant recipients (Stewart *et al.* 1992) and embryos deficient in *Lifr* are also able to undergo successful implantation (Ware *et al.* 1995).

To examine the temporal effects of LIF inhibition on embryo implantation, females were injected over a series of 1 h time periods. Maximal reduction of implantation was achieved between 0930 and 1230 h on day 4 of pregnancy. However, LIF inhibition prior to ~0930 h and later than 1630 h on day 4 of pregnancy had no significant effect on implantation, strongly suggesting that LIF-sensitisation of the uterus, required for successful embryo implantation, occurs between mid-morning and early afternoon on day 4. LIF signalling either side of this 'window' appears to be dispensable to implantation. Injections of recombinant LIF at 0730 and 1800 h into *Lif*-deficient females have been reported to restore implantation (Song & Lim 2006). It is quite possible that LIF given well after the normal period of essential action may be able to redress the lack (in a *Lif* null uterus) of LIF signalling events normally occurring in the morning of day 4 of pregnancy. However, our data show that the normal essential period for LIF action is the morning to middle of day 4 of pregnancy.

We next examined downstream targets of LIF to assess whether these molecules are misregulated after inhibitor action and contribute to the infertility of *Lif* null mice. AREG is transiently upregulated in wild-type murine uteri during the implantation period (Das *et al.* 1995), but undetectable in the uteri of *Lif* deficient mice at this time (Song *et al.* 2000) and exogenous injections of LIF into the uterus of *Lif* null mice on day 4 of pseudopregnancy increases levels of *Areg* mRNA (Sherwin *et al.* 2004). Thus, AREG appeared a good candidate molecule that could be used to help to substantiate the abrogation of LIF signalling by hLIF-05 within the uterus *in vivo*. AREG was detected in LE cells from control implantation sites, but absent in LE cells adjacent to the embryo in LIF inhibited horns. These results together confirm that AREG is regulated in the LE by LIF on day 4 of pregnancy. However, since mice simultaneously deficient for epidermal growth factor (*Egfr*), transforming growth factor α (*Tgfa*) and *Areg* are fertile (Troyer *et al.* 2001), AREG expression is not essential for LIF mediated uterine receptivity. The LE antigen fucosylated H-type-1 is stimulated by oestrogen and inhibited by progesterone (White & Kimber 1994, Sidhu & Kimber 1999) and temporally regulated during implantation; being downregulated between days 4 and 5 of pregnancy, reviewed in Kimber *et al.* (2001). We showed that H-type-1 is aberrantly retained in the apical cells of the LE in *Lif* null mice at least until day 6 of pregnancy at the implantation site (Fouladi-Nashta *et al.* 2005). Following injection of LIF inhibitor, H-type-1 expression was retained in the LE at the implantation site on day 5 of pregnancy as seen for *Lif* null mice, while in control horns expression was downregulated on schedule. These results suggest that LIF together with signals from the implanting embryo are likely to directly downregulate the expression of the H-type-1 epitope on the LE cells during implantation. Interestingly, both molecules (AREG and H-type-1 antigen) are progesterone responsive genes, reflecting a role for LIF in enhancing the effects of progesterone (Sherwin *et al.* 2004, Kimber 2005). H type-1 expression was also observed in the glandular epithelium on day 5 of pregnancy in agreement with previously published data demonstrating that the steroid hormonal environment in the peri-implantation uterus also regulates H type-1 expression in the glandular epithelium even if less dramatically than in the lumen (Kimber *et al.* 1988, Kimber & Lindenberg 1990). Overall, our results suggest that LIF plays a fundamental role in the molecular maturation of the LE for uterine receptivity. On the morning of day 4, uterine glands express high levels of LIF protein and to a lesser extent on the evening of day 4 (2200 h; Fouladi-Nashta *et al.* 2005). It has been shown that *Lifr* transcripts are localised predominantly to the LE late on day 4 (2400 h), whereas surprisingly *Gp130* mRNA expression is most highly expressed in the uterine glands (Cheng *et al.* 2001, Ni *et al.* 2002,

Song & Lim 2006). However, the data on STAT3 activation together with those from *Lif* null animals and LIF inhibition, all point to the sequence of LIF being secreted from the GE late on day 4 of pregnancy, binding to LIFR in the LE and triggering a signal cascade important for embryo implantation and LE maturation.

The hLIF-05 induced alteration in decidual differentiation was confirmed by changes in expression of desmin, extensively used as a marker of decidualisation in mice and rats (Glasser & Julian 1986, Oliveira *et al.* 2000). *Lif* null mice show little evidence of decidualisation either in response to an embryo or an artificial stimulus (Stewart *et al.* 1992). Fouladi-Nashta *et al.* (2005) demonstrated that few desmin intermediate filaments were detectable in the *Lif* null uterus on days 5 and 6 of pregnancy. Similarly desmin expression was also reduced in LIF inhibited uterine horns on day 5 of pregnancy compared to controls. It is widely accepted that prostaglandins (PGs) are involved in implantation and decidualisation (Kennedy 1985, Parr *et al.* 1988) and PTGS2 is a critical enzyme necessary for PG synthesis (Smith 2000). Since *Lif* null mice exhibit dysregulated stromal expression of *Ptgs2* transcripts and protein at the implantation site it has been postulated that the lack of a decidual response is associated with regulation of PG synthesis mediated through PTGS2 induction by LIF (Song *et al.* 2000, Fouladi-Nashta *et al.* 2005). Abrogation of LIF signalling (by hLIF-05) on day 4 of pregnancy (to a level that prevents implantation) does not completely abolish PTGS2 expression in LE or stroma. This suggests that PTGS2 expression in the LE is not directly regulated by LIF signalling and the limited expression of stromal PTGS2 observed in LIF inhibited compared to control horns on both days 5 and 6 of pregnancy may be a consequence of defective blastocyst-LE signalling. Studies on *Ptgs2* deficient mice are in keeping with this theory as *Ptgs2* null females exhibit delayed decidualisation, but are fertile (Cheng & Stewart 2003).

Previous work by us suggested that OSM was a likely downstream target of LIF during the implantation period since it is completely absent from implantation sites in *Lif* null mice (Fouladi-Nashta *et al.* 2005). Analysis of OSM expression in LIF inhibited uteri was similar to control uteri on day 5 of pregnancy. On day 6, OSM expression in control uteri was detected in stromal cells surrounding the implanting embryo as seen in wild-type mice, but no staining was seen adjacent to the embryo in the inhibited horns. As OSM is induced exclusively at the site of embryo attachment at implantation, it is not directly regulated by LIF signalling and likely to be reliant upon localised embryonic signals acting on a LIF primed uterus.

We have shown that members of the IL1 system are dysregulated during the implantation period in the uteri of *Lif* null mice compared to wild-type mice (Fouladi-Nashta *et al.* 2008). Examination of IL1A and IL1B protein in hLIF-05 treated and control uteri on day 5 of pregnancy revealed no differences in expression,

suggesting that abrogation of LIF signalling in the LE does not directly regulate IL1A or IL1B expression at this level. On day 6 of pregnancy, however, IL1A was detected throughout the implantation site in control uteri; but in contrast, was negligible in LIF inhibited uteri. Expression of IL1B on day 6 was localised to the LE and decidualising stroma in vehicle treated uteri, but was not observed in stroma after LIF inhibition (on day 4 am) suggesting LIF regulated secondary signals control stromal expression. Interestingly, *in vitro* studies on uterine LE cells have demonstrated that IL1A induces PG secretion (namely PGE₂ and PGF_{2α}) by uterine stromal cells, via stimulation of PTGS2 (Jacobs & Carson 1993, Jacobs *et al.* 1994). The disappearance of IL1A may therefore contribute to the restricted enhancement and spread of a PTGS2 response, which is initiated on queue. These results support our recent *in vitro* data showing that LIF stimulates IL1A and PGE₂ secretion in a co-culture system (Fouladi-Nashta *et al.* 2008) and that LIF signalling may both directly and indirectly regulate IL1A and IL1B expression in the peri-implantation uterus. IL1A (and IL1B) expression is not implantation site specific (Fouladi-Nashta *et al.* 2008) and therefore the absence of IL1A is unlikely to be due to lack of/or aberrant embryonic signals. Furthermore, it is important to note that inhibition of LIF signalling on day 4 of pregnancy resulted in blockade of IL1A expression only 48 h post inhibition. This is in keeping with an indirect inhibition of IL1A. However, it must also be noted that these effects could be attributed to a secondary consequence of defective or incomplete decidualisation.

In summary, these results suggest that antagonism of LIF signalling on day 4 of pregnancy using a peptide antagonist, recapitulates many of the uterine features of *Lif* null mice. The data presented validates the use of an antagonist, to instigate tissue specific and temporal disruption of cytokine signalling pathways to investigate reproductive function. Although this study has focussed on the use of hLIF-05 for research purposes there is a clear therapeutic application. Clinical trials of recombinant hLIF (Emfilermin) used to improve implantation in women with recurrent implantation failure has been not successful (Brinsden *et al.* 2009); however, a non-steroid based drug that inhibits implantation may have clinical relevance. In this context (White *et al.* 2007), have demonstrated 100% reduction in implantation in mice by i.p. injections of a polyethylene glycol tagged (PEGylated) LIFR antagonist and propose its use in clinical trials.

Materials and Methods

Animals

All mice were maintained under conditions in accordance with the UK Home Office and procedures conducted under a Home office licence. MF1 female mice (Harlan, Indianapolis, IN, USA) between 7 and 9 weeks of age were paired in the

evening with MF1 males and checked the following morning for vaginal plugs (considered day 1 of pregnancy). Implantation takes place during the evening/night of day 4.

Establishment of appropriate injection volume

Initial experiments using 20, 10 and 5 µl intrauterine injections of a nuclear fluorescent dye (Hoechst 33342) demonstrated that 5 µl showed positive staining throughout the injected horn with no evidence of reflux into the non-injected horn (as seen for 10 and 20 µl volumes). Subsequently, all further experiments were carried out using 5 µl volumes of hLIF-05.

Intrauterine injection of LIF inhibitor

Animals were anaesthetised with Fentanyl-Fluanisone (Janssen Animal Health, Bucks, UK) and Midazolam (Phoenix Pharma Ltd, Gloucester, UK) following UK Home Office guidelines. Upon sedation, animals underwent dorsal incision and the uterine horn was located. Mice on either the evening of day 3 of pregnancy (1800 h; *n*=3) or on day 4 (between 0830 and 1800 h – divided into hourly time sets; *n*=34) were injected with 5 µl hLIF-05 (51 ng/µl) into the uterine lumen. Intraluminal injections were made into the uterine horn distal to the utero-tubal junction, with the corresponding volume of vehicle (PBS) being injected into the contralateral horn in an identical manner. Uterine horn injections were alternated to avoid bias. In order to inject such small volumes accurately, a 50 µl HPLC syringe (Hamilton Ltd, Birmingham, UK) was used with a fine 30 gauge luer lock needle (Coopers Needleworks, Birmingham, UK). Preliminary experiments determined that 5 µl was the optimum injection volume sufficient to pass through the length of the uterine horn without reflux into the contralateral horn. In order to identify implantation sites in uterine horns, all animals on day 5 and 6 of pregnancy were injected with 100 µl of 0.4% (w/v) trypan blue dye (Sigma) into the tail vein (Psychoyos 1993). After 10 min, these animals were killed by cervical dislocation and the number of blue bands along the uterine horn was recorded prior to fixation. Uterine horns were dissected and fixed in either 4% (w/v) paraformaldehyde or Carnoy's fixative prior to paraffin embedding as previously described (Fouladi-Nashta *et al.* 2005). Analysis was made on tissue from a minimum of three animals, selected from those in which implantation was reduced by at least 50% (primarily from mice that were injected between 0930 and 1130 h) and typically from implantation sites closest to the point of injection.

Reagents

All reagents were purchased from Sigma unless stated otherwise. Primary antibodies were as follows; goat anti-IL1A (2 µg/ml) (R&D systems, Oxfordshire, UK), rabbit anti-IL1A (4 µg/ml), goat anti PTGS2 (4 µg/ml), goat anti-OSM (4 µg/ml), goat anti-AREG (4 µg/ml; all from Santa Cruz Biotechnology, Heidelberg, Germany), rabbit anti-desmin (1:40; Sigma), mouse anti-H-type-1 antigen (1:20; Monocarb, Lund, Sweden) as in Kimber *et al.* (1988), rabbit anti-tyrosine p-STAT3 (1:500; Cell Signalling Technology, Hertfordshire, UK). Alexa Fluor

488 or 586 conjugated secondaries were used (10 µg/ml; Invitrogen) or biotin conjugated secondary anti-goat, – rabbit or – rat IgG (7 µg/ml; Vector Labs, Oxford, UK). The LIFR antagonist, hLIF-05, was characterised and previously described (Hudson *et al.* 1996, Vernallis *et al.* 1997, Fouladi-Nashta *et al.* 2004, 2008).

Immunohistochemistry

Deparaffinised sections were either processed for antigen retrieval by microwave treatment with TEG buffer as previously described (Fouladi-Nashta *et al.* 2005). Or following exposure to 0.3% (v/v) hydrogen peroxide in methanol for 10 min, and antigen retrieved with 0.01 M citrate buffer (pH 6.0) as described in (Fouladi-Nashta *et al.* 2008).

Statistical analysis

Data are presented as mean $\pm$ s.e.m. and all statistical analysis was carried out using Statistical Package of the Social Sciences (SPSS; SPSS Inc., Chicago, IL, USA) to carry out a paired Student's *t*-test to analyse the difference between control and treatment. Normality of the data was tested using the Kolmogorov–Smirnov test. Differences were accepted as significant if $P < 0.05$.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research was funded by a BBSRC UK grant to S J Kimber and BBSRC UK studentship to L Mohamet. Further funding was received from The John Pinto Foundation (J K Heath).

Acknowledgements

We thank the staff at the Biological Services Facility at the University of Manchester for their help with the animals and we would like to particularly like to thank Dr Mary Birch for all her help with the *in vivo* experiments.

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Received 27 March 2009

First decision 1 July 2009

Revised manuscript received 14 July 2009

Accepted 27 July 2009