Evidence for heterodimeric association of leukemia inhibitory factor (LIF) receptor and gp130 in the mouse uterus for LIF signaling during blastocyst implantation

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Abstract

Implantation failure in mice lacking leukemia inhibitory factor (LIF) establishes that this cytokine is crucial to this process. LIF transcripts are expressed in the uterus in a biphasic manner: LIF is expressed in the gland on the morning of day 4 and again in stromal cells surrounding the blastocyst with the onset of implantation in the evening of day 4 of pregnancy. However, it is not yet clear whether both phases of LIF expression are required for implantation, since the receptor usage by uterine LIF still remains elusive. Here we have provided evidence that major cell types expressing the LIF receptor (LIFR) and its signal-transducing partner gp130 are mostly disparate in the mouse uterus during the glandular LIF expression in the morning of day 4. In contrast, LIFR and gp130 expressions overlap in the luminal epithelium at the time of blastocyst attachment on the evening of day 4 when the second phase of LIF expression occurs in stromal cells surrounding the blastocyst, suggesting that LIF participates in implantation in a paracrine manner. Similar expression patterns for LIFR and gp130 were observed when a delayed implantation model was used. For example, a transient overlapping expression of LIFR and gp130 was evident at 12 h after estrogen-induced termination of delayed implantation. Co-immunoprecipitation experiments showed that LIFR and gp130 form heterodimers and are available for LIF signaling at the time of blastocyst attachment. We have also shown that an intra-peritoneal administration of recombinant LIF in LIF-deficient pregnant mice on the evening of day 4, close to the time when the second phase of LIF expression is normally observed, is sufficient to rescue implantation failure, and that there is no evidence of antagonistic action by soluble forms of the receptors. Collectively, our results have provided evidence that LIFR and gp130 form a functional heterodimer in the uterus during the attachment reaction to direct LIF signaling.


Introduction

Leukemia inhibitory factor (LIF) is a pleiotropic cytokine that regulates various cellular functions depending on its microenvironments (Hilton 1992, Metcalf 1992, 2003, Shellard et al. 1996, Dani et al. 1998). LIF exerts its multiple biological functions through heterodimerization of membrane-bound LIF receptor (LIFR) and gp130. LIF binds first to LIFR with low affinity and then recruits gp130 to form a high affinity functional receptor complex, leading to activation of downstream signal transduction pathways, such as signal transducer and activator of transcription (STAT) (Boulanger et al. 2003). LIF belongs to the interleukin (IL)-6 family which includes IL-6 itself, IL-11, oncostatin M (OSM), ciliary neurotrophic factor (CNTF), and cardiotophin-1 (CT-1). Each of these family members shares gp130 as a common receptor for signal transduction, implicating functional redundancy in their signaling pathways (Kishimoto et al. 1994, Barasch et al. 1999). OSM, CNTF, and CT-1 also use LIFR as part of their high affinity receptor complex (Heinrich et al. 2003). In addition to membrane-bound receptors for IL-6 family members, a number of soluble forms of receptors are formed to potentiate or dampen the biological activities of their ligands in humans and mice (Layton et al. 1992, 1994, Narazaki et al. 1993, Zhang et al. 1998, Jostock et al. 2001). While soluble receptors of IL-6, IL-11, CNTF, and CT-1 act like their membrane-bound receptors in mediating ligand-activated signaling, the soluble forms of...
LIFR and gp130 are known to function as antagonists, competing with their membrane-bound receptors for binding to ligands (Zhang et al. 1998, Jostock et al. 2001).

While overlapping actions and functional redundancy exist due to the sharing of gp130 and LIFR in signaling by IL-6 family members in vivo (Kishimoto et al. 1994), implantation failure in mice deficient in LIF clearly demonstrates that LIF plays an irreplaceable role in embryo implantation (Stewart et al. 1992, Escary et al. 1993). Blastocysts irrespective of their genotype fail to initiate implantation in the uterus lacking LIF, suggesting that maternal LIF is critical to implantation in mice (Stewart et al. 1992). Expression studies also suggest that uterine LIF is important for implantation in many other species, including humans (Yang et al. 1994, Song et al. 1998, Hirzel et al. 1999, Vogiagis & Salamonsen 1999). In addition, mice with gp130 knock-in mutation in which all STAT-binding sites in the carboxyl terminal are deleted show implantation failures similar to LIF-deficient mice (Ernst et al. 2001). This suggests that a functional LIFR–gp130 receptor complex for LIF signaling is critical for early events in implantation in mice.

Diffusible LIF, one of three alternatively spliced forms of LIF (membrane-associated, diffusible, and truncated) (Haines et al. 1999, Voyle et al. 1999), is predominantly induced in the uterus on the morning of day 4 of pregnancy (the day of implantation), suggesting that uterine LIF is a paracrine factor (Bhatt et al. 1991, Shen & Leder 1992, Stewart et al. 1992). We have demonstrated that LIF is expressed not only in uterine glands on the morning of day 4, but also in stromal cells surrounding the implanting blastocyst at the time of attachment reaction (2400 h on day 4), suggesting complex signaling of LIF with biphasic expression patterns during implantation in mice (Song et al. 2000). Suggested roles for LIF signaling in implantation include leukocyte trafficking (Schofield & Kimber 2005), regulation of decidualization (Fouladi Nashta et al. 2004, 2005), and differentiation of luminal epithelium (Fouladi-Nashta et al. 2005), and LIF seems to utilize STAT3 as a downstream mediator during this process (Cheng et al. 2001). However, the mechanism by which LIF executes its actions in implantation requires further investigation since receptor usage by uterine LIF during biphasic expression is not yet clearly understood. There is a report describing expression of LIFR and gp130 at the time of glandular LIF expression (Cheng et al. 2001), but no information is available regarding the status of receptor expression and dimerization during the second phase of stromal LIF expression.

Thus, to better understand the molecular basis of LIF signaling in implantation, we examined the expression of LIFR and gp130 and their functionality during the attachment reaction. Our results have shown that a functional association of LIFR and gp130 is present to mediate the signaling of stromal LIF necessary for the attachment reaction.

Materials and Methods

Mice

All the mice used in this study were housed in the Animal Care Facility according to NIH and institutional guidelines for laboratory animals. Adult CD1 female mice (Charles River Laboratories, Inc., Wilmington, MA, USA) were mated with fertile males of the same strain to induce pregnancy. LIF-deficient mice were kindly provided by Dr Story Landis (NINDS/NIH, Bethesda, MD, USA). These mice were originally generated by Philine Brulet (Pasteur Institute, Paris, France). The disruption of the LIF gene was achieved in (129/Sv) ES cells by homologous recombination as described (Escary et al. 1993). These mice were out-bred to mice on the CD1 background. PCR analysis of tail genomic DNA was used for the genotyping of LIF-deficient mice. The morning when a vaginal plug was found was designated day 1 of pregnancy. Recombinant LIF (rLIF; kindly provided by Serono S.A., Rockland, MA, USA) was dissolved in phosphate-buffered saline (PBS) and injected (i.p.) to pregnant LIF-deficient mice. Implantation sites at midnight on day 4 (2400 h) or the morning of day 5 (0900 h) were visualized by intravenous injection (0.1 ml/mouse) of 1% Chicago blue dye solution in saline (Paria et al. 1993).

Delayed implantation

To induce delayed implantation, adult CD1 female mice were ovariectomized on the morning (0900 h) of day 4 of pregnancy and maintained with daily injections of progesterone (P4, 2 mg/mouse) from days 5 to 7 (0900 h). To activate dormant blastocysts and initiate implantation, P4-primed delayed implanting pregnant mice were injected with estradiol-17β (E2, 25 ng/mouse) (Paria et al. 1998). Implantation sites were visualized by the blue dye method 24 h after E2 injection. All steroids were dissolved in sesame oil and injected subcutaneously (0.1 ml/mouse). Uterine tissues were processed for various analyses.

Hybridization probes

Specific regions from full-length mouse LIF, LIFR, and gp130 sequences were amplified by RT-PCR and subcloned into a riboprobe vector using TOPO cloning kit (Invitrogen, Carlsbad, CA, USA). For in situ hybridization, sense and antisense 35S-labeled cRNA probes were generated using appropriate polymerases. The probes were used at specific activities of 2 x 10⁹ d.p.m./ml.

In situ hybridization

In situ hybridization was performed as previously described (Das et al. 1994, Lim et al. 1997b). Small pieces of tissues were flash-frozen in liquid Histo-Freeze (Fisher Scientific, St Louis, MO, USA). Frozen sections (12 μm) were mounted onto poly-L-lysine-coated slides, fixed in cold 4% paraformaldehyde solution in PBS, acetylated,
and hybridized at 45°C for 4 h in hybridization buffer containing the 35S-labeled antisense cRNA probes. After hybridization, the sections were incubated with RNase A (20 μg/ml) at 37°C for 20 min. RNase A-resistant hybrids were detected by autoradiography using Kodak NTB-2 liquid emulsion (Eastman Kodak, Rochester, NY, USA). Sections hybridized with the corresponding sense probes served as negative controls. Slides were post-stained with hematoxylin and eosin. In situ hybridization experiments were repeated at least three times using independent samples.

Antibodies

Affinity-purified rabbit polyclonal antibodies raised against the carboxyl terminus of mouse gp130 (sc-656) and human LIFR (sc-659), which is identical to the mouse sequence (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), were used for Western blotting and co-immunoprecipitation. The affinity-purified goat polyclonal antibody raised against the extracellular domain of mouse gp130 (AF468; R&D Systems, Minneapolis, MN, USA) was used for Western blotting.

Western blotting

Western blot analysis was performed as previously described (Lim et al. 1997a). In brief, day-4 mouse uteri were collected into buffer A (10 mM Tris–HCl (pH 8.0), 250 mM sucrose, 2 mM EGTA, 10 μg/ml leupeptin, 20 μg/ml phenylmethylsulfonylfluoride (PMSF), and 10 μg/ml aprotinin), homogenized in the same buffer and centrifuged at 2000 r.p.m. for 10 min at 4°C. The supernatants were recentrifuged at 35,000 r.p.m. for 1 h at 4°C. The pellets were resuspended in the same buffer and spun again for 1 h at 35,000 r.p.m. at 4°C. The pellets were then resuspended in buffer B (10 mM Tris–HCl (pH 7.4), 0.15 mM NaCl, 1 mM EGTA, 10 μg/ml leupeptin, 20 μg/ml PMSF, and 10 μg/ml aprotinin). Protein concentrations of uterine extracts were measured using Bradford protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA). Uterine extracts (≈ 60 μg protein) were mixed with sample buffer, boiled for 5 min, and run on a 7.5% SDS-PAGE gel under reducing conditions. Separated proteins on the gel were transferred onto a nitrocellulose membrane. The membrane was preincubated with 5% non-fat dry milk in Tris–buffered saline (TBS) for 1 h to block non-specific binding, followed by incubation with antibodies to LIFR or gp130 overnight at 4°C. The membrane was washed three times (10 min each) in TBS and incubated with donkey anti-rabbit or rabbit anti-goat IgG conjugated (1:10,000) with horseradish peroxidase (Jackson Immunoresearch, West Grove, PA, USA) for 1 h. The membrane was again washed three times (10 min each) in TBS. Signals were detected with an ECL kit (Pierce, Rockford, IL, USA).

Co-immunoprecipitation

Membrane proteins (~200 μg) were solubilized by treatment with 0.1% Triton X-100 for 2 min on ice. After centrifugation, supernatant was mixed with a specific antibody (1–2 μg) used in Western blotting in Ab/Ag buffer (50 mM Tris–HCl (pH 8.0), 1 mM EDTA, 0.2 mM EGTA, and 10% glycerol) for 1 h with constant rotation at 4°C. About 50 μl protein A/G PLUS agarose beads (Santa Cruz Biotechnology, Inc.) was added to the reaction tubes. After 1 h of wheeling at 4°C, the protein–antibody–bead complex was washed with the same buffer and boiled in SDS-PAGE sample buffer for 5 min to elute the bound protein. After centrifugation at 10,000 r.p.m. for 5 min, the supernatant was collected and loaded onto 7.5% SDS-PAGE gels. Proteins were transferred onto nitrocellulose membrane that was subsequently blocked in 5% non-fat milk in TBS and incubated overnight at 4°C with the primary antibodies. After washing, membranes were probed with a 1:10,000 dilution of the respective secondary antibodies coupled to horseradish peroxidase for 1 h and developed with ECL reagents (Pierce).

RT-PCR analysis

RT-PCR was performed as previously described (Song et al. 2002) with primers specific to LIFR and gp130. LIFR primers were designed to detect the 3’-untranslated region (3’-UTR) region specific to short and long forms of soluble LIFR transcripts, nucleotides 2237–2320 (Tomida et al. 1993) and 2523–2679 (Tomida et al. 1994) in the published cDNA sequence respectively. gp130 primers were used to detect the region containing the transmembrane domain of gp130 for examination of gp130 alternative splicing in the uterus (Sharkey et al. 1995, Diamant et al. 1997).

Results

Cell type-specific expression of LIFR and gp130 in the peri-implantation mouse uterus

It is well established that LIF works via the heterodimeric receptor complex of LIFR and gp130. We therefore examined their expression patterns in the peri-implantation mouse uterus by in situ hybridization. LIFR mRNA was first detected in the luminal epithelium on day 3 of pregnancy (data not shown) followed by more intense signals on day 4 (Fig. 1). Following implantation on day 5, LIFR was highly expressed in the luminal and glandular epithelia. On days 6–8 of pregnancy, LIFR was predominantly localized in the primary decidual zone (PDZ) (Fig. 1).

In contrast, gp130 mRNA was first detected in the glandular epithelium on day 1 (Fig. 1), but the signal was very low to undetectable on days 2 and 3 (data not shown). On day 4, gp130 expression was primarily limited
to glands and was at a modest level in the stroma. A similar uterine expression pattern was present on day 5 (Fig. 1). On days 6–8, LIFR expression was mainly concentrated in the PDZ, close to the implanted embryo. gp130 was expressed in the stroma away from the implanting embryo on day 6. On days 7 and 8, gp130 was mainly expressed in the PDZ overlapping with LIFR expression. However, gp130 expression was more widely distributed than that of LIFR.

gp130 expression was observed in the PDZ similar to LIFR expression on these days. The results showed that while localization of gp130 overlaps with that of LIFR in the PDZ after implantation, they do not seem to exhibit overlapping expression pattern on the morning of day 4 of pregnancy when the first phase of LIF expression is high in uterine glands. These results led us to further examine the expression profiles of LIF, LIFR, and gp130 specifically around the time of initiation of implantation.

**Expression of LIFR and gp130 overlaps in the luminal epithelium at the time of the attachment reaction**

The attachment between the blastocyst trophoderm and the uterine luminal epithelium first occurs around midnight of day 4 of pregnancy (Das et al. 1994). Since the second phase of LIF expression coincides with the attachment reaction, we examined the expression of LIF, LIFR, and gp130 in the uterus at the time of the attachment reaction (day 4 2400 h). As shown in Fig. 2A, the second burst of LIF was induced focally in stromal cells surrounding the blastocyst at this time. LIFR was expressed at high levels in the luminal epithelium and at a low level in glands at the time of the attachment reaction, while gp130 expression was noted in the luminal epithelium, underlying stroma and in glands during this period (Fig. 2A). These results demonstrated that LIF and gp130 are co-localized in the luminal epithelium, providing a site of LIF action during the attachment reaction.

To examine more precisely the expression profiles of LIFR and gp130 in the uterus in relation to the initiation of implantation, we analyzed the expression profile of these two genes in the uterus using the delayed implantation model in mice in which the onset of the attachment reaction can be manipulated by ovariectomy and exogenous application of ovarian steroid hormones. For example, implantation can be delayed by ovariectomy before the preimplantation estrogen secretion on the morning of day 4 and this condition can be maintained for several days by P4 supplementation (Paria et al. 1993). Under this condition, blastocysts remained closely apposed to the uterine luminal epithelium without initiating the attachment reaction. A single injection of estrogen led to the resumption of the attachment reaction. We observed that the expression of LIFR and gp130 was low to undetectable in the P4-primed delayed implanting uterus. However, both LIFR and gp130 were concurrently expressed in the luminal epithelium 12 h after termination of the delayed implantation with resumption of implantation by estrogen (Fig. 2B). The expression pattern of these genes at 24 h after termination of the delayed implantation was similar to that seen on day 5 of normal pregnancy (compare Fig. 2B with Fig. 1, D5). Collectively, our results derived from normal and delayed implantation models suggested that LIF signaling is operative during the attachment reaction, when LIF-R and gp130 are concurrently expressed in the luminal epithelium.
LIFR and gp130 form heterodimers during implantation

Although the above results showed that LIFR and gp130 are expressed in the luminal epithelium at the time of blastocyst attachment, it is still not known whether these two receptors form heterodimers in response to LIF expressed in vivo in the uterus during implantation. We addressed this issue by using Western blotting and co-immunoprecipitation of LIFR and gp130 using day-4 uterine membrane protein preparation. We found that antibodies against the cytoplasmic regions of LIFR and gp130 detected protein bands of approximately 190 kDa and 130 kDa for LIFR and gp130 respectively (Fig. 3A). To determine whether LIFR forms a heterodimer with gp130 when LIF is present in the day-4 pregnant uterus, co-immunoprecipitation experiments using both LIFR (data not shown) and gp130 antibodies were performed using uterine membrane protein preparations obtained from day-4 uteri at 0900 h (whole uterus) and 2400 h (implantation sites only). We observed that a trace of LIFR–gp130 receptor complex was detected in the uterine preparations on the morning of day 4 when LIF was highly expressed in uterine glands (0900 h in Fig. 3B). However, more intense association between LIFR and gp130 was evident in preparations consisting of the separated implantation sites at the time of the attachment reaction when stromal LIF was induced (2400 h in Fig. 3B). This suggests that LIF-induced heterodimerization of LIFR with gp130 perhaps provides signaling information for blastocyst attachment with the luminal epithelium.

The second phase of LIF expression during the attachment reaction is critical for implantation

To further examine the relative importance of biphasic LIF expression, we attempted to induce implantation in LIF-null mice by delivering rLIF at two different times on day 4 of pregnancy (Table 1). Mice injected intraperitoneally with rLIF in the morning (0730 h) of day 4 showed implantation 24 h later as monitored by the blue dye injection. Surprisingly, a single injection of same doses of rLIF in LIF-deficient pregnant mice in the evening (1800 h) of day 4 only a few hours prior to the anticipated attachment

Figure 2 In situ hybridization of LIF, LIFR, and gp130 mRNAs at the time of attachment reaction and in uteri of P4-primed delayed implanting mice before and after E2 treatment. Darkfield photographs of representative longitudinal uterine sections are shown at 40 X except for the right panel in (A) (100 X). (A) In situ hybridization of LIF, LIFR, and gp130 mRNAs in sections of uteri collected at 2400 h on day 4 of pregnancy during the attachment reaction. (B) In situ hybridization of LIFR and gp130 mRNAs in uteri of P4-primed delayed implanting mice before and after E2 treatment. P4 24 h represents a uterine section from a delayed implanting mouse treated with P4 from days 5 to 7 and killed 24 h after the last injection. P4 + E2 12 h and 24 h represent sections of uteri from P4-treated delayed implanting mice killed 12 and 24 h after an E2 injection respectively. le, luminal epithelium; ge, glandular epithelium; s, stroma; myo, myometrium. Arrows indicate the location of blastocysts.

Figure 3 Western blotting and co-immunoprecipitation of LIFR and gp130 in the uterus. (A) Membrane proteins from day-4 pregnant uteri were immunoblotted using a rabbit polyclonal antibody to LIFR or gp130. Approximately 190 kDa LIFR and 130 kDa gp130 proteins were detected. (B) Uterine membrane proteins from day 4 at 0900 h and implantation sites at 2400 h were subjected to immunoprecipitation (IP) with a rabbit polyclonal antibody raised against gp130 (gp130). Samples were run on SDS-PAGE and subjected to Western blotting with either anti-LIFR or anti-gp130 antibodies as indicated. The right lane contains membrane protein sample without IP and serves as a control for antibody specificity.
reaction also induced implantation (blue bands) by 12 h post-injection, i.e. on the morning of day 5, similar to that observed for pregnant wild-type mice. This observation that LIF requirement for implantation was fulfilled by a single injection in the evening of day 4 strongly suggested that the second phase of LIF expression during the attachment reaction is critical for implantation. Collectively, these results reaffirm our previous and current findings that LIF signaling is crucial during the attachment reaction.

LIF signaling in the uterus is not negatively regulated by its soluble receptors during implantation

While membrane-associated LIFR and gp130 are required for LIF signaling, there are reports of negative regulation by soluble forms of these receptors in other systems. Alternative splicing and/or proteolytic cleavages produce the soluble forms that are believed to antagonize LIF action (Layton et al. 1992, 1994, Narazaki et al. 1993, Zhang et al. 1998, Jostock et al. 2001). Although we observed concomitant expression of LIF, LIFR, and gp130, and heterodimerization of LIFR and gp130 at the beginning of implantation, it is possible that soluble forms of LIFR and/or gp130 are present in the uterus as negative regulators of LIF signaling. Thus, we examined whether soluble LIFR and gp130 receptors are produced in the uterus during the peri-implantation period.

Different assortments of exons have classified LIFR transcripts as type I (membrane-associated), type II (short soluble), and type III (long soluble) forms (Michel et al. 1997). The LIFR probe that was used for in situ hybridization (Figs 1 and 2) does not distinguish alternatively spliced LIFR transcripts. Thus, we examined cell type-specific localization of soluble LIFR by using cRNA probe from 3'UTR specific to soluble LIFR transcripts. While both LIFR transcripts for soluble LIFR were detected in the liver (positive control), their expression was undetectable in all major uterine cell types on day 4 of pregnancy (Fig. 4A) by in situ hybridization. These results suggested that there is little soluble LIFR in the uterus to antagonize LIF signaling during implantation.

With respect to gp130, two different alternative splicings occur at the same region before the transmembrane domain of gp130 in humans (Sharkey et al. 1995, Diamant et al. 1997). To examine whether similar alternative splicing occurs in mice, we performed RT-PCR of gp130 with day-4 uterine RNA using the primers that distinguish both alternatively spliced forms from the membrane-associated form in humans. We detected a single

Table 1 LIF-deficient mice received an i.p. injection of rLIF (20–40 µg/0.1 ml PBS) at the times indicated on day 4 of pregnancy. Mice were killed 12–24 h after injection as indicated. Chicago blue B solution was given intravenously 3 min prior to killing to demarcate implantation sites, if any.

<table>
<thead>
<tr>
<th>Time of injection (day 4)</th>
<th>Time of death</th>
<th>No. of IS</th>
</tr>
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<tbody>
<tr>
<td>0730 h</td>
<td>12–15 h</td>
<td>100% (4/4*) 8.5 ± 4.0</td>
</tr>
<tr>
<td>1800 h</td>
<td>24 h</td>
<td>100% (9/9) 8.8 ± 2.3</td>
</tr>
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* Number of mice with implantation sites (IS)/number of mice in group (means ± S.E.M.).
RT-PCR product (323 bp) (Fig. 4B) and sequencing of this product confirmed that it was identical to that of the mouse membrane-associated gp130 as previously reported (Saito et al. 1992). To examine if a soluble form of gp130 produced by proteolytic cleavage (Montero-Julian et al. 1997) was present, we performed Western blot analysis using an antibody raised against the extracellular domain of gp130 that detects both the soluble and membrane-bound gp130 (Fig. 4C). While we detected a single band of immunoreactive gp130 in the membrane fraction using this antibody, we failed to detect any immunoreactive gp130 protein in the soluble fraction. Collectively, these results suggest that LIF signaling in implantation is not negatively regulated by antagonistic actions of its soluble receptors.

Discussion

LIF was originally identified through its ability to induce macrophage differentiation in the murine myeloid leukemia cell line M1 (Metcalf et al. 1988). Since then pleiotropic actions of LIF have been unveiled in many systems (Hilton 1992, Metcalf 2003). Intriguingly, gene-targeting experiments revealed that LIF plays an unequivocal role in blastocyst implantation in mice (Stewart et al. 1992, Escary et al. 1993). LIF is also associated with infertility in humans; reduced uterine concentration of LIF and point mutations of the LIF gene leading to aberrant interaction of LIF with LIFR have been described in infertile women (Laird et al. 1997, Giess et al. 1999). While the importance of LIF for implantation is well documented, information regarding its receptor utilization and its site of action in heterogeneous uterine cell types during the attachment reaction is limited. We have shown in the present investigation that LIFR and gp130 show co-expression during the attachment reaction in a manner suggestive of the paracrine nature of stromal LIF acting upon uterine epithelial cells. This is consistent with our result that delivery of rLIF to day-4 pregnant LIF-deficient mice in the evening, close to the time when the second phase of LIF expression is normally observed (Song et al. 2000), is sufficient to correct implantation failure. The importance of the second phase of LIF signaling was also previously demonstrated by us in that 3 ng estrogen which does not induce glandular LIF expression can still initiate implantation with LIF expression in stromal cells surrounding the blastocyst after termination of the delay in a delayed implantation model (Ma et al. 2003). Several genes that are critical to implantation, such as cPLA2, COX-2, and PPARδ, are induced by implanting blastocysts during the attachment reaction (Das et al. 1994, Lim et al. 1997b, 1999). Our previous and present studies showing similar induction of stromal LIF at the time of the attachment reaction present this cytokine also as a critical signaling player in establishing implantation (Song et al. 2000, Song et al. 2002). Future studies as to whether these various signaling pathways work independently, co-operatively, or converge to a final common pathway directing implantation are warranted.

Since LIF is a pleiotropic cytokine with a broad range of biological effects, the limited availability of a functional receptor complex could be one mechanism by which the LIF signaling is tightly modulated for implantation in the uterus. LIFR and gp130 are also co-expressed in other species during implantation. In humans, both of these receptors are expressed in the luminal epithelium during the secretory phase of the menstrual cycle (Cullinan et al. 1996). While the mechanism(s) by which LIF participates in implantation could be different among species, our present results have shown that the functional LIF receptor complex is available in the luminal epithelium for stromal LIF in the mouse uterus during the attachment reaction. The importance of stromal LIF expression during the attachment reaction is consistently correlated with complete failure of the initial attachment reaction and subsequent decidualization in LIF-deficient mice (Song et al. 2000, Fouladi-Nashta et al. 2005).

A number of soluble receptors for the IL-6 family of cytokines are produced to enhance or reduce the biological effects of their cognate ligands in mice and humans (Laty et al. 1992, 1994, Narazaki et al. 1993, Zhang et al. 1998, Jostock et al. 2001). While our data suggest that there is no evidence for negative control with soluble receptors on LIF signaling in the mouse uterus during implantation, there are other reports showing the presence of negative modulation with soluble receptors during pregnancy after implantation. For example, the concentration of soluble LIFR in the mouse serum is dramatically increased during pregnancy (Laty et al. 1992, Tomida et al. 1993). However, it still remains unknown as to whether this increased level of soluble LIFR during pregnancy controls the action of LIF or other LIFR ligands. While soluble gp130 is produced in human endometrium and its level is differentially regulated during the menstrual cycle (Sherwin et al. 2002), our present study has demonstrated that there is no apparent mechanism for the production of soluble gp130 in the uterus during implantation in mice (Fig. 4).

For many years before our finding of biphasic nature of LIF expression in the mouse uterus during implantation (Song et al. 2000), LIF induced in uterine glands on the morning of day 4 was considered crucial for implantation (Bhatt et al. 1991). In this regard, glandular LIF expression has been examined in several gene-targeted mice with peri-implantation defects to define the molecular interaction between these signaling pathways. It was found that the first phase of glandular LIF expression is normal in Hoxa10−/−, IL-11 receptor alpha−/−, or COX-2-deficient females with severe implantation defects (Benson et al. 1996, Lim et al. 1997b, Robb et al. 1998). In contrast, we observed that the second phase of LIF expression in stromal cells is not induced in uteri at the time of anticipated implantation in cPLA2-deficient mice with implantation defects (Song et al. 2002). It is possible that glandular LIF
expressed on the morning of day 4 prepares the uterus for the second phase of LIF expression in stromal cells surrounding the blastocyst which is critical for the attachment reaction. Collectively, our present findings have reinforced our notion that stromal LIF is involved in the attachment reaction.

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