Leukemia inhibitory factor ligand-receptor signaling is important for uterine receptivity and implantation in golden hamsters (*Mesocricetus auratus*)

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Abstract

Blastocyst implantation occurs in the progesterone-primed uterus of hamsters, but not in mice where the progesterone-primed uterus requires estrogen influence. Leukemia inhibitory factor (LIF), an estrogen-regulated gene in mice, is an absolutely needed cytokine for uterine receptivity and implantation in this species. This study aimed to evaluate the importance of LIF ligand-receptor signaling during uterine receptivity and implantation in hamsters. We investigated whether or not the uterine expression patterns of LIF and its receptors, LIF-r and gp130, during the perimplantation period of pregnancy and its hormonal regulation in the ovariectomized hamster correlate with some of the vital phases of uterine changes during early pregnancy. Uterine LIF, LIF-r, and gp130 mRNA expressions were examined by Northern and in situ hybridization. During the uterine preparatory phase for implantation, LIF, LIF-r, and gp130 were expressed either in the gland, luminal epithelium or both. As the implantation process began, LIF expression was minimal, but LIF-r and gp130 extended to the decidual areas. This decidual expression of LIF-r and gp130 was not dependent on the presence of the embryo since these genes were expressed in the suture-induced deciduomata. We also observed that, while the uterine LIF was induced by estrogen, LIF-r and gp130 were induced by progesterone in ovariectomized hamsters. Additionally, we show that a LIF antibody when instilled intraluminally on day 3 of pregnancy reduced the number of implantation sites. Taken together, these data suggest that LIF signaling is important for uterine receptivity and implantation in hamsters.


Introduction


LIF is a secreted glycoprotein that exhibits pleiotropic activities in varieties of cellular systems both in vivo and in vitro (Rathjen et al. 1990, Hilton 1992, Gearing 1993). It belongs to a family of ligands that includes interleukin-6 (IL-6), IL-11, oncostatin M, ciliary neurotrophic factor, and cardiotropin (Callard & Gearing 1994). Signal transduction of LIF depends on its binding to a low affinity LIF receptor (LIF-r) subunit, gp190, which causes association of the ligand/receptor complex with another membrane-bound protein, gp130 (Gearing et al. 1992). LIF is mainly expressed in the glandular epithelium of the uterus of mice on day 4 of pregnancy, suggesting its role in the preparation of the receptive uterus for implantation (Stewart et al. 1992, Song et al. 2000). Localized expression of LIF specifically in stromal cells surrounding the implanting mouse blastocyst on day 4 at midnight and the morning of day 5 of pregnancy suggests its involvement in the initiation of the embryo-uterine attachment reaction and subsequent initiation of stromal cell decidualization (Song et al. 2000). The complete failure of blastocyst implantation and uterine stromal cell decidualization in LIF null mice, and the reversal of these processes by exogenous LIF treatment, substantiated the
importance of this maternal molecule in the process of implantation and decidualization (Stewart et al. 1992). Uterine Lif may also be important for implantation in humans. Significant Lif expression was noted in the endometrium of fertile women rather than that of infertile women (Tsai et al. 2000). The peak expression of Lif and its receptor (Lif-r) was observed in the endometrium at the mid-secretory phase of the menstrual cycle in humans (Cullinan et al. 1996) with maximum expression between days 19 and 25 (Nachtigall et al. 1996). Lif immunostaining in the luminal and glandular epithelial cells was lowest in the follicular phase, moderate in the periovulatory phase, and strongest in the luteal phase (Cullinan et al. 1996). Thus, Lif occupies a central position among many uterine signaling events during uterine receptivity and implantation.

The hormonal requirements for making the uterus receptive and the timing of initiation of implantation are defined in mice (Paria et al. 1993). In contrast, dating of the time of ovulation, chronological development of the secretory endometrium, and the timing of implantation cannot be accurately predicted in humans. It has been demonstrated that in the majority of species, such as the hamster, rabbit, pig, guinea pig, monkey, and human, luteal estrogen is not required to initiate the process of implantation (Deanesly 1960, Prasad et al. 1960, Orsini & Meyer 1962, Harper et al. 1969, Meyer et al. 1969, Perry et al. 1973, George & Wilson 1978, Heap et al. 1981, Hoversland et al. 1982, Ghosh et al. 1994, Zegers-Hochschild & Altieri 1995, Wang et al. 2002, 2004, Zhang & Paria 2006). A comprehensive study has not been done on the relative involvement and functions of uterine Lif in species where implantation is dependent on ovarian P4, but not E. Thus, the present experiments were undertaken to study the expression of Lif and its receptors during the perimplantation period and their hormonal and embryonic regulation in hamsters. The relative importance of Lif in the hamster uterus during early pregnancy may point to a potential correlation between endometrial Lif expression and the extent of its involvement in maternal–embryo interactions in P4-dependent implantation processes. These studies will also help to distinguish the differences, if any, in the involvement of Lif in the process of implantation across species depending on their steroid requirements to initiate the process of implantation.

Results

Uterine Lif expression during the periimplantation period in hamsters

Northern blot analysis of Lif mRNA expression in periimplantation uteri from days 1 to 8

As demonstrated in the mouse uterus (Bhatt et al. 1991), the hamster uterus also showed the presence of a single ~4.2 kb Lif mRNA transcript from days 1 to 8 of pregnancy. Higher levels of Lif mRNA expression were observed on days 1 and 4 (Fig. 1A). When the intensities of hybridized Lif mRNA bands were normalized with the corresponding Rpl7 bands, the trend of Lif mRNA levels followed closely with the trend of band intensities showing significantly higher levels on days 1 and 4 as compared with its levels on other days of pregnancy (Fig. 1B). Next, in situ hybridization was employed to determine the accumulation of mRNA in specific uterine cell types on various days during early pregnancy.

Cell type-specific expression of Lif mRNAs in periimplantation uteri

As reported in mice (Bhatt et al. 1991, Stewart et al. 1992, Song et al. 2000), Lif was expressed in the luminal epithelium on day 1, but not in day 2 of pregnancy in hamsters. Lif expression started to appear in the luminal epithelium on day 3 and showed distinct accumulation in glandular epithelium, but not in stromal and myometrial cells, on the morning and afternoon of day 4 (Fig. 2). However, a low level of accumulation was maintained in the luminal epithelium (Fig. 2, insert). No implantation-specific accumulation of Lif surrounding the implanting blastocyst was observed on the evening (1700 h) of day 4 in hamsters. Thereafter, Lif mRNA expression remained at

Figure 1 Northern blot analysis of uterine Lif mRNAs in the uterus of hamsters from days 1 to 8 of pregnancy. (A) Representative Northern blot analysis; (B) bar diagram to show relative change in normalized Lif band intensities (each Lif band intensity/corresponding Rpl7 band intensity). Total uterine RNA (6 μg/lane) samples from each day of pregnancy were separated by formaldehyde-agarose gel electrophoresis, transferred, and u.v. cross-linked to nylon membranes and hybridized to 32P-labeled Lif probe. The blot was hybridized with Rpl7, a housekeeping gene, to confirm integrity, loading, and blotting of RNA samples. Acridine orange-stained gel showing 28s and 18s rRNAs are also presented. Mean values are plotted. The different letters over bars indicate significant differences (P<0.05).
low levels in all uterine cell types of the implantation site from days 5 to 8 of pregnancy (Fig. 2).

Cell type-specific Lif expression at the day 5 implantation site of hamsters and mice

Since previous studies demonstrated that Lif is expressed in the stromal cells situated just underneath the luminal epithelium of the day 5 implantation site of mice (Song et al. 2000), we hybridized sections placed on the same glass slide from the day 5 implantation sites of both the mouse and the hamster using a hamster-specific Lif cRNA probe. While Lif expression was at the background level in any uterine cell type of the day 5 implantation site of the hamster, its expression was above the background level in the subluminal stromal cells surrounding the implantation chamber of the mouse (Fig. 3).

Cell type-specific Lif expression in the day 4 uterus of hypophysectomized P₄-treated pregnant hamsters

Since uterine preparation for implantation in hamsters occurs in the absence of circulating estrogen, we decided to examine whether uterine Lif expression in the day 4 uterus of P₄-treated hypophysectomized hamsters occurs similar to normal pregnancy. Hypophysectomized hamsters treated with P₄ showed Lif mRNA expression only in the luminal epithelial cells compared with the control where Lif expression was observed both in the luminal and the glandular epithelia (Fig. 4). The absence of Lif expression in the day 5 implantation site was studied as a negative control. These results suggest that in the absence of ovarian estrogen, either the P₄ or the embryo is responsible for the induction of uterine Lif expression.

Hormonal regulation of uterine Lif mRNA expression (Northern blot and in situ analysis)

As described above, Northern blot analysis showed a ~4.2 kb transcript of Lif mRNA in total uterine RNA samples (Fig. 5A and B). Low levels of Lif mRNA were detected in ovariectomized hamsters treated with oil. However, a single injection of estradiol-17β (E₂) rapidly increased the levels of Lif mRNA. The levels peaked at 2 h, and gradually declined to basal levels by 24 h (Fig. 5B and C). A single injection of P₄ alone had little effect on Lif mRNA expression except at 24 h (Fig. 5A and C).

Since 1 μg E₂ stimulated Lif expression in Northern blot hybridization by 2 h, we next determined the minimum dose of E₂ required to stimulate the uterine Lif expression by in situ hybridization. We treated
ovariectomized hamsters with three different doses of E2 (0.01, 0.10, and 1.00 μg/hamster) and observed that, while the lower doses of E2 were unable to induce uterine \( \text{Lif} \), the highest dose (1 μg) clearly stimulated epithelial \( \text{Lif} \) expression by 2 h as compared with the oil-treated control uterus (Fig. 6). Next, we examined whether estrogen and progesterone and their combinations differentially regulate cell type-specific expression of \( \text{Lif} \) in the adult ovariectomized hamster. Uterine \( \text{Lif} \) expression in the luminal epithelium was upregulated by E2 as compared with the oil-treated control at 2 h followed by a gradual decrease by 24 h (Fig. 7). The expression of \( \text{Lif} \) mRNA was not seen in the glandular epithelium at any time point after an injection of E2. \( \text{Lif} \) mRNA expression was not observed in any cell type after an injection of P4 even at 24 h (data not shown). However, uterine induction of \( \text{Lif} \) mRNA was noted both in the glandular and the luminal epithelia when ovariectomized animals received E2 in P4-primed uterus (Fig. 8).

**Cell type-specific expression of \( \text{Lif} \) receptor (\( \text{Lif-r} \) and \( \text{gp130} \)) mRNAs in perimplantation uteri**

With respect to cell type-specific \( \text{Lif} \)-receptor expression, we noticed no clear expression of \( \text{Lif-r} \) (Fig. 9) and \( \text{gp130} \) (Fig. 10) in any uterine cell types on days 1 and 2 of pregnancy. \( \text{Lif-r} \) started to appear in the luminal epithelium on day 3 and showed distinct accumulation in the luminal, but not in the glandular, epithelium on days 4 and 5 (Fig. 9). The expression of \( \text{gp130} \) also started to appear on day 3, but its expression was not restricted to only the luminal epithelium; a low level of expression was also noticed in the glandular epithelium and stroma. While very low levels of \( \text{gp130} \) mRNA persisted in stromal cells, its expression was clear and distinct in the epithelium of glands and lumen on days 4 to 5 (Fig. 10). With the progression of implantation on days 6–8, \( \text{Lif-r} \) and \( \text{gp130} \) mRNAs mainly accumulated in the antimesometrial decidual cells adjacent to the implanting embryo. \( \text{Lif-r} \) expression was strong in the primary decidual zone (PDZ) of days 6 and 7 (Fig. 9). In contrast, the PDZ of day 6 implantation sites showed weak expression of \( \text{gp130} \) but its...
expression was stronger on day 7 (Fig. 10). Particularly on day 8, both the Lif-r and the gp130 showed almost comparable levels of expression, but their expression also extended to the mesometrial bed of the implantation site (Figs 9 and 10). Embryonic cells showed only a basal level of expression of Lif-r and gp130 mRNAs by in situ hybridization.

Expression of Lif-r and gp130 mRNAs in the implantation site is not regulated by the presence of blastocysts

Since Lif-r and gp130 mRNAs were both expressed in the PDZ area of the implantation site of hamsters, we explored the possibility of regulation of these genes by the implanting blastocyst. In situ hybridization studies using uterine sections obtained from day 6 decidual areas induced by either the blastocyst or the suture, demonstrated the presence of mRNAs of both Lif-r and gp130 in decidual areas induced by both methods (Fig. 11). These results suggest that decidual expression of Lif-r and gp130 mRNAs was not unique to the blastocyst stimulus and was mimicked by a physical stimulus.

Hormonal regulation of uterine Lif-r and gp130 mRNA expression

To examine whether ovarian steroids influence the induction of uterine Lif receptors, Lif-r and gp130 mRNA expressions in the uterus were examined by in situ hybridization on uterine sections obtained from ovariectomized mice treated with either oil, E2, P4, or a combination of P4 and E2. The results indicated that the expression of both Lif-r (Fig. 12) and gp130 (Fig. 13) in the uterine epithelium was induced by P4, but not by E2 (data not shown). The induction of Lif-r mRNA by P4 was maximal in the uterine luminal and glandular epithelia by 2 h of P4 injection as compared with oil-treated controls. The P4-induced Lif-r mRNA levels were decreased by 6 h and remained at the same level until 24 h (Fig. 12). However, the same dose of P4 treatment showed only modest but gradual upregulation of gp130 mainly in the luminal epithelium (Fig. 13). When P4 and E2 were given together, Lif-r and gp130 mRNA expressions were noticed only in the luminal epithelial cells.

Effect of a Lif antibody on implantation

Five pregnant hamsters were injected with 2.4 μg/12 μl Lif antibody in one uterine horn. The contralateral horn of these hamsters received an equal amount of goat IgG (2.4 μg/12 μl). When compared with IgG-treated control
horns, horns which received Lif antibody showed significant \( P < 0.05 \) reduction in the number of implantation sites (Fig. 14A and B) on day 5. These results demonstrate an involvement of Lif during the process of implantation in hamsters.

Discussion

In this study, the importance of the Lif ligand-receptor signaling system for preparatory changes of the uterus for receptivity and implantation was investigated in hamsters. The clear expression of Lif, Lif-r, and gp130 in the uterine luminal epithelium on days 3 and 4 suggests that the luminal epithelium is the main target of uterine Lif action during the preparatory phase of the uterus for implantation. Our results also provide a view that uterine Lif is a conserved molecule during the time of uterine receptivity in mice and hamsters irrespective of their differences in ovarian hormonal requirements for implantation. In addition, the decidual expression of Lif receptors, but not Lif, at the site of implantation from days 6 to 8 of pregnancy suggests the potential role of circulating Lif at the site of decidualization. Our results also demonstrate here that, while Lif is most likely regulated by E₂, Lif-r and gp130 are regulated by P₄, but not by E₂, in the hamster.

During the preimplantation period, the expression of Lif in the luminal epithelium of the day 1 pregnant uterus could be attributed to the effects of preovulatory ovarian E secretion (Baranczuk & Greenwald 1973). This is consistent with the induction of Lif in the uterine luminal epithelium in ovariectomized hamsters after an E₂ injection. On days 3 and 4 of pregnancy, low levels of Lif expression were observed in the luminal epithelium. This is consistent with the gradual increase in the levels of circulating estrogen from day 2 of pregnancy in hamsters (Leavitt & Blaha 1970, Baranczuk & Greenwald 1974). However, Lif expression was much more prominent in the glandular epithelium on day 4 as compared with the luminal epithelium. This glandular Lif expression on day 4 could be due to either the combined effect of ovarian P₄ and E₂ or the presence of an embryo inside the uterus on these days. Indeed, Lif expression in the glandular epithelium was induced, albeit at low levels, by a combined treatment of P₄ and E₂ in ovariectomized hamsters, suggesting a possible

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mutual effect of steroids on glandular Lif expression. However, when day 4 uterine sections from the P₄-treated hypophysectomized hamsters were processed for in situ hybridization to locate Lif expression, we observed its accumulation only in the luminal, but not in the glandular, epithelial cells. These results suggest that in the absence of circulating estrogen, the presence of an embryo inside the uterus can also induce an Lif mRNA expression that is restricted to the luminal epithelium. Since developing embryos enter into the uterus on day 3 of pregnancy in hamsters (Wang et al. 2002), an accumulation of Lif in the luminal epithelium could be influenced by the presence of embryos inside the uterus. The identification of this embryonic factor(s) remains a challenging question. There is evidence that the embryo of certain species including hamsters have the capacity to produce estrogen (Perry et al. 1973, Dickmann et al. 1976, George & Wilson 1978, Heap et al. 1981, Hoversland et al. 1982, Sholl et al. 1983). We have preliminary evidence that hamster preimplantation embryos express the aromatase protein (unpublished observation). Since (1) Lif is an important maternal factor for implantation (Stewart et al. 1992), (2) the uterine luminal epithelium expresses Lif during the time of uterine receptivity, and (3) normal implantation occurs in P₄-treated hypophysectomized hamsters (Prasad et al. 1960), it is possible that the embryo plays a critical role in regulating the uterine environment during the time of implantation in hamsters. Furthermore, normal blastocyst implantation in P₄-treated hypophysectomized hamsters also suggests that luminal epithelial Lif expression could be adequate to initiate the process of implantation in this species. Since both Lif- and gp130 were expressed in the luminal epithelium on Days 4 to 8 of pregnancy (Wang et al. 2002), we speculated that the embryo expresses Lif in the luminal epithelium on Day 4 of pregnancy in hamsters.
days 3 and 4 of pregnancy, Lif action on luminal epithelium is likely to be important for uterine receptivity and implantation. The specific purpose and function of Lif accumulation in the glandular epithelium on day 4 of pregnancy remains to be determined.

The importance of uterine Lif in the implantation process has been confirmed in mice by a gene knockout study (Stewart et al. 1992). The uterine expression pattern of Lif around the time of implantation in mice suggests that Lif may play dual functions in initiating implantation, first in the preparation of a receptive uterus and subsequently in the attachment reaction (Song et al. 2000). Lif mRNA expression in the gland as well as in the luminal epithelium prior to implantation in hamsters suggests its possible role during preparation of the receptive uterus. However, the absence of Lif expression in stromal cells surrounding the implanting embryo on the afternoon of day 4 and onwards indicates that stromal production of Lif is not necessary for uterine changes associated with implantation such as stromal cell proliferation and differentiation. This discrepancy between mice and hamsters could possibly be attributed to differences in hormonal regulation of implantation between these two rodents. We next studied the importance of Lif in implantation by neutralizing intrauterine Lif using an antibody of Lif. These results showed that intraluminal injection of a Lif antibody partially prevented implantation suggesting an important role of Lif during the time of implantation in hamsters.

Figure 12 Cross-sections of ovariectomized hamster uteri treated with vehicle (sesame oil) or a single injection of progesterone (P4; 1 mg/hamster) or progesterone (P4; 1 mg/hamster) plus estradiol-17β (E2; 1 μg/hamster) were processed for in situ hybridization to demonstrate cell-specific Lif-r mRNA expression. Vehicle-treated animals were killed at 2 h. P4-treated animals were killed at 2, 6, 12, and 24 h. Bar= 200 μm. le, luminal epithelium; ge, glandular epithelium; s, stroma.

Figure 13 Cross-sections of ovariectomized hamster uteri treated with vehicle (sesame oil) or a single injection of progesterone (P4; 1 mg/hamster) or progesterone (P4; 1 mg/hamster) plus estradiol-17β (E2; 1 μg/hamster) were processed for in situ hybridization to demonstrate cell-specific gp130 mRNA expression. Vehicle-treated animals were killed at 2 h. P4-treated animals were killed at 2, 6, 12, and 24 h. Bar = 200 μm. le, luminal epithelium; ge, glandular epithelium; s, stroma.

Figure 14 An intraluminal application of Lif antibody has partially inhibited implantation in pregnant hamsters. (A) Bar diagram showing the mean number of implantation sites in each uterine horn. (B) Representative uterine horns from one out of the five hamsters. Pregnant hamsters received a single intraluminal injection of Lif antibody (Lif Ab) in one uterine horn and goat IgG (control) in the contralateral horn on day 3 of pregnancy when embryos were still inside the oviducts. Implantation sites were determined by blue dye injection on day 5 at 0900 h. Number in parentheses indicates the number of uterine horns. Arrowheads indicate the location of implantation sites. *P<0.05 (Student’s t-test).
Preimplantation embryos themselves express certain cytokines or growth factors and their receptors. Several studies have reported that Lif and Lif-r are expressed in preimplantation blastocysts of mice and humans (Conquest & Brulet 1990, Murray et al. 1990, Sharkey et al. 1995, Nichols et al. 1996, Chen et al. 1999). Since Lif and Lif-r are also expressed in the uterus of these species, both an autocrine and a paracrine role of Lif in embryogenesis have been suggested. Exogenous Lif increases the number of human embryos developing to the blastocyst stage in vitro (Dunglison et al. 1996). A contradictory result also exists demonstrating that supplementing culture medium with Lif does not improve human blastocyst formation (Jurisicova et al. 1995). However, Lif is not critical to the formation of mouse blastocysts because fertilized Lif (−/−), Lif-r (−/−), and gp130 (−/−) embryos can develop normally to the blastocyst stage and implant (Stewart et al. 1992, Ware et al. 1995, Yoshida et al. 1996). Since Lif, Lif-r, and gp130 expression patterns in the pre-implantation embryos of hamsters are unknown, whether or not Lif produced by the uterus can interact with the embryo is unknown at this point.

The Lif-r–gp130 heterodimer is implicated in diverse functions of Lif in many processes including blastocyst implantation in mice (Song & Lim 2006). In our study, Lif-r and gp130 signals were clearly detected in the luminal epithelium on days 4 and 5 of pregnancy in hamsters. This suggests that the uterine luminal epithelium may be the main target of uterine Lif action because the expression of both the receptors in this uterine cell type. After the initiation of implantation, however, both Lif-r and gp130, but not their ligand, were expressed in decidual cells surrounding the implanting embryo. This pattern of uterine Lif-r and gp130 localization around the time of implantation was very similar to that reported in the mouse (Cheng et al. 2001, 2002, Ni et al. 2002), human (Cullinan et al. 1996), and monkey (Yue et al. 2000). The co-localization of Lif-r and gp130 in periimplantation decidual cells suggests that Lif action is important for decidualization. Since Lif was not expressed in decidual cells of hamsters, we suggest a possible role for circulating Lif on decidual cells. Contrary to this finding in hamsters, Lif expression in decidual cells and its regulation by decidual cytokines and E2 has been reported in humans (Kojima et al. 1994, Sawai et al. 1995, 1997). It is also possible that other members of the IL-6-type cytokine family that share the use of glycoprotein gp130 may be involved in decidualization. These cytokines are IL-6 itself, IL-11, oncostatin M, ciliary neurotrophic factor, and cardiotropin-1 (Gearing et al. 1992). It has been reported that IL-11 receptor α-null mice were infertile due to defective post-implantation decidua formation at the implantation site (Robb et al. 1998). Both IL-11 and IL-11 receptor α are highly expressed in the decidua on days 6–9 of pregnancy (Bilinski et al. 1998, Robb et al. 1998). Similar temporal and spatial pattern of IL-11 and IL-11 receptor α was observed in the perimplantation rat uterus (Li et al. 2001). Since gp130 is required for IL-11 receptor α to bind IL-11 with high affinity (Hilton et al. 1994), its expression in decidual cells is important for implantation.

The expression of Lif-r and gp130 on days 3 and 4 of pregnancy suggests possible hormonal regulation of these genes in the uterus. In this regard, it has been demonstrated in mice that, while P4 or E2 alone was not effective in stimulating Lif-r and gp130 mRNAs in the ovariectomized animal, a combination of these steroids did stimulate the expression of these genes in the uterine epithelium (Ni et al. 2002). Recently, another study has demonstrated E-induced Lif-r expression in the trabecular bone (Lindberg et al. 2002). Using immunocytochemical localization of proteins, several studies have demonstrated in the human and monkey uterus that Lif-r and gp130 localization during the luteal phase were stronger than their localization during the proliferative phase (Yue et al. 2000, Classen-linke et al. 2004). The most intense immunostaining of IL-11 was also seen in decidualized stromal cells in the mid- and late-secretory phases in human uterus (Dimistriadis et al. 2000). This strong expression of Lif-r, gp130, and IL-11 during the luteal phase coincided with the period of uterine receptivity, stromal cell decidualization, and increasing levels of maternal P4 during this period, suggesting the possibility that expression of these molecules in the monkey and human uterus may be regulated by P4. In this study, for the first time, we report that Lif-r and gp130 are most likely regulated by P4, but not E2, in the ovariectomized hamster uterus. E2 also had no additive effects on the expression of these two genes when P4 and E2 were given together.

Embryo–uterine interaction during the initiation of implantation is a multi-event cascade involving cell-specific alterations in the expression of various genes. The identification of such genes and evaluation of the consequences of their altered expression is essential for attempts to halt implantation or to correct implantation problems to improve the rate of pregnancy. One critical problem to be considered in evaluating such genes is the differences between species. In this study, the pre-implantation expression pattern and hormonal regulation of uterine Lif in hamsters were generally similar to mice. However, the Lif expression pattern during the time of embryo–uterine attachment reaction in hamsters was different from that in mouse. Thus, it is more likely that there is an intrinsic difference between mice and hamsters in the expression of Lif. In conclusion, although differences in hormonal requirements for uterine receptivity and initiation of implantation across species exist, the temporal specificity of epithelial Lif expression before implantation is a requirement for the preparation of the receptive uterus for initiation of maternal–embryo interactions for implantation.
**Materials and Methods**

**Animal and tissue preparation**

Adult virgin male and female golden hamsters (*Mesocricetus auratus*) and CD1 mice were purchased from Charles River Laboratory, Raleigh, NC, USA. They were housed on a 12 h light:12 h darkness cycle and fed commercial chow *ad libitum*. All surgical procedures and the killing of the animals were approved by the appropriate Institutional Animal Care and Use Committee. Female hamsters that showed three consecutive estrous cycles were placed with fertile males on the evening of the proestrus day for mating. Microscopic finding of spermatozoa in the vaginal discharge the next morning was designated day 1 of pregnancy (Wang *et al.* 2002, 2004, Zhang & Paria 2006). Female mice were mated with fertile male to induce pregnancy (day 1 of pregnancy = vaginal plug; Paria *et al.* 1993, Zhang & Paria 2006). Hamsters and mice on days 1–3 were killed between 0830 and 0900 h, and whole uteri were flash-frozen. Pregnancy on these days was confirmed by recovering embryos from oviducts. Whole pregnant uteri of hamsters and mice were collected on the morning of day 4 (0900 h) without flushing. Implantation sites from the pregnant hamsters on the afternoon (1600 h) of day 4 and the morning (0900 h) of days 5 and 6 were collected 15 min after an i.v. injection of Chicago Blue B dye solution (0.25 ml of 1% dye in saline). Implantation sites from the pregnant mice on the morning (0900 h) of day 5 were collected 5 min after an i.v. injection of Chicago Blue B dye solution (0.10 ml of 1% dye in saline). On days 6–8, implantation sites are distinct and their identification does not require blue dye injection. Uterine tissues were immediately flash-frozen for extraction of total RNA later or for use in *in situ* hybridization experiments (Paria *et al.* 1993, Wang *et al.* 2002, 2004).

Implantation occurs without delay in hamsters ovarioectomized or hypophysectomized on day 2 of pregnancy and given P₄ daily (Prasad *et al.* 1960, Harper *et al.* 1969, Wang *et al.* 2002, 2004). Thus, to address the issue of the regulation of implantation-specific gene expression by either P₄, blastocysts or both, a group of pregnant hamsters were either hypophysectomized or ovarioectomized on day 2 (0900 h). Hypophysectomized hamsters were given P₄ subcutaneously (Sigma Chemical Company; 1 mg in 0.1 ml sesame seed oil/hamster) on days 2 and 3 of pregnancy. Control animals underwent sham operations and were injected with oil. Whole uteri were collected on day 4 morning (0900 h) for *Lif* mRNA detection by *in situ* hybridization (Wang *et al.* 2002). Ovarioectomized hamsters were given an s.c. injection of P₄ on days 2, 3, 4, and 5 to maintain pregnancy. A small piece of silk suture was threaded through the lumen of one uterine horn on day 3 of pregnancy to induce deciduoma experimentally in this horn. The contralateral horn of this animal remained intact for pregnancy to induce deciduoma experimentally in this horn. Midpregnancy to induce deciduoma experimentally in this horn. Whole pregnant uteri of mice were flash-frozen. Pregnancy on these days was confirmed by microscopic detection of spermatozoa in the vaginal discharge the next morning was designated day 1 of pregnancy (Wang *et al.* 2002, 2004). The contralateral horn of this animal remained intact for pregnancy to induce deciduoma experimentally in this horn.

To determine the effects of steroids on uterine *Lif*, *Lif*-r, and gp130, female hamsters were ovarioectomized and rested for 12 days (Zhang & Paria 2006). One group of these hamsters received an s.c. injection of sesame oil (0.2 ml/hamster) or P₄ (1 mg/0.2 ml oil/hamster) or E₂ (1.0 µg/0.2 ml oil/hamster). Hamsters were killed at 2, 6, 12, and 24 h after injection of either P₄ or E₂. Animals treated with 2 days of P₄ and a combination of P₄ and E₂ on day 3 were killed at 6 h after last injection. Their uteri were collected for RNA extraction and *in situ* hybridization. To determine the minimum amount of E₂ required for the induction of *Lif* expression, the second group of ovarioectomized hamsters was given a single injection of 10, 100, or 1000 ng E₂. Animals in this group were killed at 2 h after an injection of E₂ and uteri were processed for *in situ* hybridization of *Lif*.

**Total RNA preparation**

Uterine RNA was extracted by applying TRIZOL reagent (Gibco Life Technologies). Briefly, tissues were homogenized in TRIZOL reagent (1 ml/50 mg tissue) and mixed with 0.2 ml chloroform/ml TRIZOL and centrifuged at 12 000 g for 15 min at 4 °C. The aqueous phase was measured, collected, and treated with isopropyl alcohol (0.5 ml/ml TRIZOL reagent) for precipitation of RNA. After an incubation period of 10 min at room temperature, the samples were centrifuged at 12 000 g for 10 min to precipitate the RNA. The precipitated RNA was suspended in 75% (v/v) ethanol (1 ml of 75% ethanol/ml of TRIZOL) and centrifuged at 7500 g for 5 min to reprecipitate RNA. At the end of the procedure, the RNA pellet was vacuum dried (Wang *et al.* 2002, 2004).

**Cloning of the hamster specific partial cDNAs for *Lif*, *Lif*-r, and gp130**

RT PCR was employed to generate the hamster-specific cDNA clones for *Lif*, *Lif*-r, and gp130. The sequences used to design the primers were of mouse origin. The primers were: *Lif* (GenBank accession number AF065918, spanning nucleotides 40–390; size 351, 5'-CTGGTCTCTGACCTGAAAAC-3' (sense) and 5'-AGTTGGGGTCCAGCTCTTCT-3' (antisense); gp130 (GenBank accession number NM_010560, spanning nucleotides 1015–1476, size 462, 5'-ACAGCTGACGCTTAGGCA-3' (sense) and 5'-CAATTTAGGACCAAGATGCT-3' (antisense); *Lif*-r (GenBank accession number NM_135584, spanning nucleotides 2077–2446, size 370, 5'-GGAGAAAGGTTCTTCCAAC-3' (sense) and 5'-TTCAGTGCTCTTGGATA-3' (antisense)). The uterine total RNA (1 µg) from day 4 pregnant hamster was reverse-transcribed in a total volume of 20 µl using antisense primer. RT products (3 µl) were amplified by PCR for 35 cycles using the following cycle parameters: 94 °C, 45 s; 55 °C, 30 s; 72 °C, 1 min 30 s. The RT-PCR products were cloned into a pCR-II-TOPO cloning vector (3.9 kb) using TOPO TA Cloning kit, Version K2 (Invitrogen Corporation) and nucleotide sequences of the clone were determined to verify the identity and orientation of the clones. The GenBank accession numbers for the resulting hamster *Lif*, *Lif*-r, and gp130 cDNA fragments are AF327897, AF492475, and EF442778 respectively. Nucleotide sequences of these partial cDNA clones showed more than 80% sequence similarities with those of the GenBank nucleotide database for rats, mice, and humans.
RNA probe preparation

Plasmids bearing hamster cDNA were exacted, purified, and linearized (Lif: T7/HindIII for antisense, SP6/NotI for sense; Lif-r: T7/BamHI for antisense, SP6/XhoI for sense; gp130: SP6/XhoI for antisense, T7/HindIII for sense) to generate antisense and sense riboprobes, which were transcribed using appropriate RNA polymerases and labeled with either $^{32}$P or $^{35}$S for Northern or in situ hybridizations respectively. A partial clone of mouse Rpl7 cDNA was also used as a template for synthesis of $^{32}$P-labeled antisense cRNA probe with T7 polymerase. All labeled sense and antisense cRNA probes used for hybridizations had specific activities of $\sim 2 \times 10^{9}$ dpm/µg (Wang et al. 2002, 2004).

Northern blot hybridization

Total RNA (6 µg) was separated by formaldehyde-agarose gel electrophoresis, blotted to nitrocellulose membranes, and cross-linked to the membrane by u.v. irradiation. Membranes were prehybridized, hybridized, and washed as described previously (Wang et al. 2002, 2004). The blots were then hybridized with $^{32}$P-labeled Lif and Rpl7 probes, and the hybrids were detected by autoradiography. Each Northern blot hybridization experiment was repeated to ensure reproducibility of results. The optical density of each hybrid band was determined using the NIH Image J program (NIH, Bethesda, MD, USA). For normalization, the intensity of each band was divided by the value of the corresponding Rpl7 band intensity.

In situ hybridization

The protocol was followed as described previously by our group (Wang et al. 2002, 2004). Briefly, frozen uterine sections were mounted onto poly-L-lysine-coated slides and fixed in cold 4% paraformaldehyde solution in PBS for 15 min on ice. After prehybridization, sections were hybridized to $^{35}$S-labeled antisense probes at 45 °C for 4 h in 50% formamide hybridization buffer. Sections were also hybridized with $^{35}$S-labeled sense probes as negative control. After hybridization and washing, 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 Company). The slides were then stained with hematoxylin and eosin. Because of the involvement of multiple variable steps in this lengthy process of in situ hybridization, quantitation of autoradiographic grains was not performed.

Analysis of blastocyst implantation after intraluminal treatment of Lif antibody in pregnant hamsters

The ability of the Lif antibody to inhibit implantation was determined in pregnant hamsters. An affinity-purified anti-Lif goat polyclonal antibody (Goat polyclonal IgG (M-19); cat. no. SC1767; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used in this study. Cross-reactivity of this anti-Lif antibody with hamster Lif was tested by Western blotting using day 4 hamster uterine tissues. Pregnant hamsters were anesthetized with avertin and an incision was made in each side of the lower back right above the ovarian fat pad. The ovarian end of each uterine horn was then exteriorized by pulling the ovarian fat pad out of the abdominal cavity. A single intraluminal injection of this Lif antibody (2.4 µg/12 µl) was administered with a Hamilton glass syringe attached to a 31-gauge steel needle in one uterine horn close to the utero-tubal junction on the morning of day 3 when embryos were still in oviducts. The injection site was held shut after withdrawal of the needle for at least 20–30 s to minimize reflux. The contralateral horn received an intraluminal injection of an equal volume and concentration of Goat IgG (cat. no. F-7381; Sigma) that was purified from normal goat serum by ion exchange chromatography and is essentially free from other goat serum proteins. Hamsters were killed on the morning of day 5 (0900 h) 15 min after blue dye injection. The number of blue bands present in each horn was visually counted.

Statistical analysis

Each experiment was replicated three times. All quantitative Northern blot data are presented as the mean ± S.E.M. To compare the difference in band intensities, results obtained in Northern blot hybridization experiments were subjected to statistical analysis (SAS 9.1 program from SAS Institute Inc., Cary, NC, USA) using one-way ANOVA. If overall ANOVA exhibited significant differences, then comparisons among groups were performed using Tukey’s Studentized range test. For other experiments, the difference between two groups was analyzed by Student’s t-test. P<0.05 was considered statistically significant.

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