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 periimplantation 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.

Reproduction (2008) 135 41–53

Introduction

The hormonal requirements for uterine receptivity and implantation differ among species. The preparation of a receptive uterus for supporting embryo implantation in mice and rats is achieved by sequential effects of ovarian progesterone (P₄) and estrogen (E; Yoshinaga & Adams 1966, Psychoyos 1973, Paria *et al.* 1993). However, uterine receptivity is regulated only by ovarian P₄ in hamsters (Prasad *et al.* 1960, Orsini & Meyer 1962, Harper *et al.* 1969, Wang *et al.* 2002, 2004, Zhang & Paria 2006). Studies in the mouse, rat, and agricultural species suggest that cytokines and growth factors produced by the uterine cells in response to ovarian steroids help in preimplantation embryo development and implantation (Carson *et al.* 2000, Modric *et al.* 2000, Paria *et al.* 2002). Among the cytokines studied, leukemia inhibitory factor (Lif) has been established as an essential molecule for uterine receptivity and implantation in mice (Stewart *et al.* 1992, Song *et al.* 2000).

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 periimplantation 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 ($P < 0.05$).

Figure 2 Cross-sections of hamster uteri from days 1 to 8 (D1–D8) of pregnancy were processed for *in situ* hybridization to demonstrate cell-specific *Lif* mRNA expression. Insert in D4 shows a higher magnification of *Lif* mRNA in the luminal epithelium of day 4 uterus. High magnification inserts in D4 pm show no expression of *Lif* mRNA in subluminal stromal (right insert) and myometrial (left insert) cells. Bar = 250 μ m. am, antimesometrial side; bl, blastocyst; D4 pm, Day 4 1600 h; em, embryo; ge, glandular epithelium; le, luminal epithelium; m, mesometrial side; myo, myometrium; s, stroma.

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).

Figure 3 Cross-sections of day 5 implantation sites from mouse and hamster were processed for *in situ* hybridization to demonstrate species-specific differences in *Lif* mRNA expression. Implantation sites were collected on the morning of Day 5 (0900 h) after an i.v. injection of blue dye as described in Materials and Methods. Bar = 100 μ m. am, antimesometrial side; bl, blastocyst; le, luminal epithelium; m, mesometrial side.

Cell type-specific *Lif* expression in the day 4 uterus of hypophysectomized P_4 -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 P_4 -treated hypophysectomized hamsters occurs similar to normal pregnancy. Hypophysectomized hamsters treated with P_4 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 P_4 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-17 β (E_2) 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 P_4 alone had little effect on *Lif* mRNA expression except at 24 h (Fig. 5A and C).

Since 1 μ g E_2 stimulated *Lif* expression in Northern blot hybridization by 2 h, we next determined the minimum dose of E_2 required to stimulate the uterine *Lif* expression by *in situ* hybridization. We treated

Figure 4 *In situ* hybridization of *Lif* mRNA in hypophysectomized (hyx) progesterone-treated pregnant (days 4 and 5) hamster uterus. Whole uterine tissues were collected on the morning of day 4 (0900 h). Implantation sites were collected on the morning of day 5 (0900 h) after an i.v. injection of blue dye as described in the Materials and Methods. Day 5 implantation sites obtained from sham operated pregnant hamsters treated with oil were used as negative control. Uterine sections were cut either longitudinally (day 4) or in cross (day 5). Bar=250 μ m. D4, day 4; D5, day 5; bl, blastocyst; ge, glandular epithelium; le, luminal epithelium; s, stroma.

ovariectomized hamsters with three different doses of E_2 (0.01, 0.10, and 1.00 μ g/hamster) and observed that, while the lower doses of E_2 were unable to induce uterine *Lif*, the highest dose (1 μ g) clearly stimulated epithelial *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 *Lif* in the adult ovariectomized hamster. Uterine *Lif* expression in the luminal epithelium was upregulated by E_2 as compared with the oil-treated control at 2 h followed by a gradual decrease by 24 h (Fig. 7). The expression of *Lif* mRNA was not seen in the glandular epithelium at any time point after an injection of E_2 . *Lif* mRNA expression was not observed in any cell type after an injection of P_4 even at 24 h (data not shown). However, uterine induction of *Lif* mRNA was noted both in the glandular and the luminal epithelia when ovariectomized animals received E_2 in P_4 -primed uterus (Fig. 8).

Cell type-specific expression of *Lif* receptor (*Lif-r* and *gp130*) mRNAs in periimplantation uteri

With respect to cell type-specific *Lif*-receptor expression, we noticed no clear expression of *Lif-r* (Fig. 9) and *gp130* (Fig. 10) in any uterine cell types on days 1 and 2 of pregnancy. *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

Figure 5 Northern blot analysis of uterine *Lif* mRNA in the uterus of ovariectomized hamsters treated with vehicle (sesame oil) or a single injection of P_4 or E_2 . (A) Uterine *Lif* mRNA in the uterus of ovariectomized hamsters treated with P_4 (1 mg/hamster). (B) Uterine *Lif* mRNA in the uterus of ovariectomized hamsters treated with vehicle (sesame oil) or E_2 (1 μ g/hamster). (C) Bar diagram to show relative changes among normalized *Lif* band intensities (each *Lif* band intensity/corresponding *Rpl7* band intensity). Vehicle-treated animals were killed at 2 h. E_2 - or P_4 -treated animals were killed at 2, 6, 12, and 24 h. Total uterine RNA (6 μ g/lane) samples from each treatment group were separated by formaldehyde-agarose gel electrophoresis, transferred, and u.v. cross-linked to nylon membranes and hybridized sequentially to 32 P-labeled *Lif* probes. The blot was also hybridized with *Rpl7*, a house keeping gene, to confirm integrity, loading, and blotting of RNA samples. Different letters over bars indicate significant differences ($P < 0.05$).

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 *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, *Lif-r* and *gp130* mRNAs mainly accumulated in the antimesometrial decidual cells adjacent to the implanting embryo. *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 *gp130* but its

Figure 6 *In situ* hybridization of *Lif* mRNA in uteri of ovariectomized hamster after various doses of estradiol-17 β treatment. Ovariectomized hamsters were killed 2 h after an injection of three doses of estradiol-17 β (E_2 ; 10, 100, and 1000 ng/hamster). Control ovariectomized hamsters that received an injection of oil were killed 2 h later. Bar=200 μ m. le, luminal epithelium; myo, myometrium; s, stroma.

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, E_2 , P_4 , or a combination of P_4 and E_2 . The results indicated that the expression of both *Lif-r* (Fig. 12) and *gp130* (Fig. 13) in the uterine epithelium was induced by P_4 , but not by E_2 (data not shown). The induction of *Lif-r* mRNA by P_4 was maximal in the uterine luminal and glandular epithelia by 2 h of P_4 injection as compared with oil-treated controls. The P_4 -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 P_4 treatment showed only modest but gradual upregulation of *gp130* mainly in the luminal epithelium (Fig. 13). When P_4 and E_2 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 μ g/12 μ l *Lif* antibody in one uterine horn. The contralateral horn of these hamsters received an equal amount of goat IgG (2.4 μ g/12 μ l). When compared with IgG-treated control

Figure 7 Cross-sections of ovariectomized hamster uteri treated with vehicle (sesame oil) or a single injection of estradiol-17 β (E_2 ; 1000 ng/hamster) were processed for *in situ* hybridization to demonstrate cell-specific *Lif* mRNA expression. Vehicle-treated animals were killed at 2 h. E_2 -treated animals were killed at 2, 6, 12, and 24 h. Sections from day 1 uterus were used as controls. Bar=250 μ m. le, luminal epithelium; myo, myometrium; s, stroma.

Figure 8 Cross-sections of ovariectomized hamster uteri treated with vehicle (sesame oil) or a combination of P_4 (1 mg/hamster) and E_2 (1000 ng/hamster) were processed for *in situ* hybridization to demonstrate cell-specific *Lif* mRNA. Animals treated with vehicle or P_4 for 2 days and a combination of P_4 and E_2 on the third day were killed at 6 h after the last hormone injection. Bar = 200 µm. Sections from day 1 uterus were used as controls. ge, glandular epithelium; le, luminal epithelium; s, stroma.

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 E_2 , *Lif-r* and *gp130* are regulated by P_4 , but not by E_2 , 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 E_2 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 P_4 and E_2 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 P_4 and E_2 in ovariectomized hamsters, suggesting a possible

Figure 9 Sections of hamster uteri from days 1 to 8 (D1–D8) of pregnancy were processed for *in situ* hybridization to demonstrate cell-specific *Lif-r* mRNA expression. Day 4 uterine sections were taken longitudinally. Bar = 250 µm. am, antimesometrial side; bl, blastocyst; cm, circular muscle; D4 am, Day 4 0900 h; D4 pm, Day 4 1600 h; em, embryo; ge, glandular epithelium; le, luminal epithelium; lm, longitudinal muscle; m, mesometrial side; pdz, primary decidual zone; s, stroma; sdz, secondary decidual zone.

Figure 10 Sections of hamster uteri from days 1 to 8 of pregnancy were processed for *in situ* hybridization to demonstrate cell-specific *gp130* mRNA expression. Day 4 uterine sections were taken long-itudinally. Bar=250 μ m. am, anti-mesometrial side; bl, blastocyst; cm, circular muscle; D4 am, Day 4 0900 h; D4 pm, Day 4 1600 h; em, embryo; ge, glandular epithelium; le, luminal epithelium; lm, longitudinal muscle; m, mesometrial side; pdz, primary decidual zone; s, stroma; sdz, secondary decidual zone.

mutual effect of steroids on glandular *Lif* expression. However, when day 4 uterine sections from the P_4 -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 P_4 -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 P_4 -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-r* and *gp130* were expressed in the luminal epithelium on

Figure 11 Cross-sections of hamster day 6 blastocyst-induced deciduum and suture-induced deciduoma were processed for *in situ* hybridization to demonstrate cell-specific expression of *Lif-r* and *gp130*. Sections from day 5 implantation site were used as controls. Bar=200 μ m. am, anti-mesometrial side; bl, blastocyst; em, embryo; le, luminal epithelium; m, mesometrial side; pdz, primary decidual zone; s, stroma; sdz, secondary decidual zone.

Figure 12 Cross-sections of ovariectomized hamster uteri treated with vehicle (sesame oil) or a single injection of progesterone (P_4 ; 1 mg/hamster) or progesterone (P_4 ; 1 mg/hamster) plus estradiol-17 β (E_2 ; 1 μ g/hamster) were processed for *in situ* hybridization to demonstrate cell-specific *Lif-r* mRNA expression. Vehicle-treated animals were killed at 2 h. P_4 -treated animals were killed at 2, 6, 12, and 24 h. Bar=200 μ m. le, luminal epithelium; ge, glandular epithelium; s, stroma.

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

Figure 13 Cross-sections of ovariectomized hamster uteri treated with vehicle (sesame oil) or a single injection of progesterone (P_4 ; 1 mg/hamster) or progesterone (P_4 ; 1 mg/hamster) plus estradiol-17 β (E_2 ; 1 μ g/hamster) were processed for *in situ* hybridization to demonstrate cell-specific *gp130* mRNA expression. Vehicle-treated animals were killed at 2 h. P_4 -treated animals were killed at 2, 6, 12, and 24 h. Bar=200 μ m. le, luminal epithelium; ge, glandular epithelium; s, stroma.

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 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 E_2 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 periimplantation 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 P_4 or E_2 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-inke *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 P_4 during this period, suggesting the possibility that expression of these molecules in the monkey and human uterus may be regulated by P_4 . In this study, for the first time, we report that *Lif-r* and *gp130* are most likely regulated by P_4 , but not E_2 , in the ovariectomized hamster uterus. E_2 also had no additive effects on the expression of these two genes when P_4 and E_2 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 preimplantation 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 proestrous 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 ovariectomized 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 ovariectomized 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). Ovariectomized 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 normal embryo-induced implantation and decidualization. Animals were killed on day 6 after an i.v. blue dye injection and the embryo-induced decidual and the suture-induced decidual tissues were collected separately. The expression of *Lif-r* and *gp130* was compared between the decidual and the decidual tissues by *in situ* hybridization.

To determine the effects of steroids on uterine *Lif*, *Lif-r*, and *gp130*, female hamsters were ovariectomized 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 ovariectomized 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 published 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'-CTGGTTCTGCACTGGAAACA-3' (sense) and 5'-AGTGGGGTTCAGGACCTTCT-3' (antisense)); *gp130* (GenBank accession number NM_010560, spanning nucleotides 1015–1476, size 462, 5'-ACAGCTGCAGCTGATTAGCC-3' (sense) and 5'-CAATATAGGACCAAAGATGC-3' (antisense)); and *Lif-r* (GeneBank accession number NM_013584, spanning nucleotides 2077–2446, size 370, 5'-GGAGAAAGGTTCTTCAAAC-3' (sense) and 5'-TCTCAGTGTCTTCTGGGATA-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 $\mu\text{g}/\text{ml}$) 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 $\mu\text{g}/12 \mu\text{l}$) 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 $\pm$ 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.

Acknowledgements

We acknowledge the support from all members of the Division of Reproductive and Developmental Biology of the Department of Pediatrics. This work was supported by National Institutes of Health (NIH) grants (HD044741 and UO1 HD042636) to B C P. The support of the National Cooperative program on Trophoblast–Maternal Tissue Interactions is gratefully acknowledged. The authors would like to give special thanks to Dr S K Roy for providing uterine tissues from hypophysectomized pregnant hamsters. Our thanks to S K Dey and S K Das for allowing free access to their laboratories and Hehai Wang for statistical analysis. Dr H Song was a graduate student of Dr S K Dey. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 8 January 2007

First decision 6 February 2007

Revised manuscript received 28 September 2007

Accepted 16 October 2007