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# Progesterone and interferon tau regulate leukemia inhibitory factor receptor and IL6ST in the ovine uterus during early pregnancy

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## Abstract

**The actions of leukemia inhibitory factor (*LIF*) via LIF receptor (*LIFR*) and its co-receptor, IL6 signal transducer (*IL6ST*), are implicated in uterine receptivity to conceptus implantation in a number of species including sheep. The present study determined the effects of the estrous cycle, pregnancy, progesterone (P4), and interferon tau (IFNT) on the expression of *LIFR* and *IL6ST* in the ovine uterus. *LIFR* mRNA and protein were localized to the endometrial luminal (LE) and superficial glandular epithelia (sGE), whereas *IL6ST* mRNA and protein were localized primarily in the middle to deep GE. Both *LIFR* and *IL6ST* mRNAs and protein were more abundant in pregnant than cyclic ewes and increased from days 10 to 20 of pregnancy. Treatment of ovariectomized ewes with P4 and/or infusion of ovine IFNT increased *LIFR* and *IL6ST* in endometrial LE/sGE and GE respectively. Co-expression of *LIFR* and *IL6ST* as well as phosphorylated STAT3 was observed only in the upper GE of the endometrium as well as in the conceptus trophectoderm on days 18 and 20. In mononuclear trophectoderm and GE cells, LIF elicited an increase in phosphorylated STAT3 and MAPK3/1 MAPK proteins. Collectively, these results suggest that *LIFR* and *IL6ST* are both stimulated by IFNT and regulated by P4 in a complex stage- and cell-specific manner, and support the hypothesis that LIF exerts effects on the endometrial GE as well as conceptus trophectoderm during early pregnancy in sheep. Thus, LIF and STAT3 may have biological roles in endometrial function and trophectoderm growth and differentiation.**

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## Introduction

Leukemia inhibitory factor (*LIF*), a member of the interleukin 6 (IL6, also known as interferon beta 2 (IFNB2)) cytokine family, is a secreted glycoprotein that acts as a polyfunctional cytokine in a variety of tissues and cells types (see (Auernhammer & Melmed 2000) for a review). LIF binds to the LIF receptor (*LIFR*) with low affinity and subsequently recruits IL6 signal transducer (*IL6ST*, also known as *GP130*) as a co-receptor. This heterodimer complex with high affinity for LIF activates janus kinase 1 or 2 (JAK1/2) and tyrosine kinase 2 (TYK2), and then signal transducer and activator of transcription (STAT), particularly STAT3, to regulate target gene expression (see (Shields *et al*. 1995, Auernhammer & Melmed 2000) for a review). In addition to the JAK–STAT signaling pathway, LIF also activates the MAP kinase (MAPK), protein kinase C (PKC), and phosphatidylinositol 3-kinase (PI3K) pathways. In mice, *Lif* is expressed exclusively in endometrial GE and plays an essential role in blastocyst implantation via the effects on endometrial LE (Bhatt *et al*. 1991, Stewart *et al*. 1992). In the mouse uterus, *Lifr* and *Il6st* are co-expressed in the LE, and LIF activates LIFR and then STAT3 at the time of blastocyst attachment (Cheng *et al*. 2001, Song & Lim 2006). In *Lifr* null mice, embryos attach to the endometrial LE successfully, but perinatal death occurs (Ware *et al*. 1995), whereas *Il6st* null embryos die *in utero* due to myocardial and hematological defects (Yoshida *et al*. 1996). In mice, LIF regulates the onset of uterine receptivity to blastocyst implantation and embryogenesis (see (Vogiagis & Salamonsen 1999, Cheng *et al*. 2002, Kimber 2005) for a review). Of particular note is that LIF regulates trophoblast giant cell differentiation in the mouse placenta (Takahashi *et al*. 2008), and human extravillous trophoblast invasion is stimulated by both LIF and STAT3 (Poehlmann *et al*. 2005).

LIF, LIFR, and IL6ST are implicated in uterine receptivity to implantation in a number of species including humans, primates, rodents, pigs, cattle, mink, and sheep (see (Auernhammer & Melmed 2000, Kimber 2005) for a review). In sheep, endometrial *LIF*


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mRNA decreased from days 12 to 14 and then was most abundant between days 16 and 20 (Vogiagis *et al.* 1997*a*). Immunoreactive LIF protein was most abundant in the caruncular and intercaruncular LE, but was also present in GE and intercaruncular stromal cells, as well as in the trophoblast cells of day 17 conceptuses (Vogiagis *et al.* 1997*a*). Although LIF may not be obligatory for implantation in ruminants, it does appear to have a role during the establishment of pregnancy (Vogiagis *et al.* 1997*b*). During early pregnancy in sheep, hatched blastocysts begin to elongate on day 12 to ultimately form an elongated filamentous conceptus (embryo and associated extraembryonic membranes) of ~19 cm in length by day 16 (Guillomot 1995, Spencer *et al.* 2004*a*). Blastocyst elongation is accompanied by the onset of trophoblast giant binucleate cell differentiation that begins on day 14 (Wooding 1992). Those cells migrate and fuse with the endometrial LE, form the basis of the cotyledonary area of the placentomes, and synthesize a number of hormones such as chorionic somatomammotropin hormone 1 (CSH1 or placental lactogen) and progesterone (P4). Blastocyst elongation in sheep requires the uterus and, specifically, secretions from the endometrial epithelia (Gray *et al.* 2001). Endometrial functions and epithelial secretions during the peri-implantation period of pregnancy in sheep are largely regulated by P4 from the corpus luteum (CL) and cytokines/hormones from the conceptus, such as interferon tau (IFNT; Spencer *et al.* 2004*b*, 2007). IFNT is the signal for maternal recognition of pregnancy in ruminants and is produced between days 10 and 21 of pregnancy in sheep by the mononuclear trophectoderm cells of the conceptus (Spencer *et al.* 1996, 2007). The anti-luteolytic actions of IFNT are required for the maintenance of a functional CL and continued secretion of P4, the essential hormone of pregnancy (Bazer *et al.* 1997). IFNT also induces or stimulates the expression of a number of genes, termed IFNT-stimulated genes (ISGs), in a cell-specific fashion within the endometrium, with emerging biological roles in uterine receptivity to conceptus development, differentiation, and implantation (Spencer *et al.* 2008).

Available results support the working hypothesis that LIF from the endometrium and conceptus regulate endometrial function and conceptus development via LIFR and IL6ST. The expression of *LIF* mRNA and protein in the ovine uterus during early pregnancy has been reported (Vogiagis *et al.* 1997*a*, 1997*b*), but the expression and hormonal regulation of *LIFR* and *IL6ST* in the ovine uterus and conceptus during early pregnancy has not been investigated. These studies were conducted to determine effects of 1) the estrous cycle and early pregnancy on the expression of *LIFR* and *IL6ST* as well as phosphorylated STAT3 in the ovine uterus, 2) P4 and IFNT on *LIFR* and *IL6ST* expression in the ovine uterus, and 3) LIF signaling in ovine trophectoderm and endometrial GE cells.

## Results

### *Effects of the estrous cycle and early pregnancy on the expression of LIFR and IL6ST mRNAs in ovine endometria (study 1)*

Slot-blot hybridization analyses assessed the steady-state levels of ovine *LIFR* and *IL6ST* mRNAs in endometria from cyclic and pregnant ewes (Fig. 1). In both cyclic and pregnant ewes, endometrial *LIFR* mRNA levels increased after day 10, and the increase was greater in pregnant than cyclic ewes from days 10 to 16 (day × status, $P<0.01$). In pregnant ewes, endometrial *LIFR* mRNA levels increased 2.7-fold between days 10 and 14 and remained abundant thereafter (quadratic effect of day, $P<0.01$). Overall, *IL6ST* mRNA levels were higher in pregnant than cyclic ewes (status, $P<0.04$) and increased 2.8-fold between days 10 and 20 of pregnancy (linear effect of day, $P<0.05$).

*In situ* hybridization analyses determined that *LIFR* mRNA (Fig. 2) was most abundant in endometrial

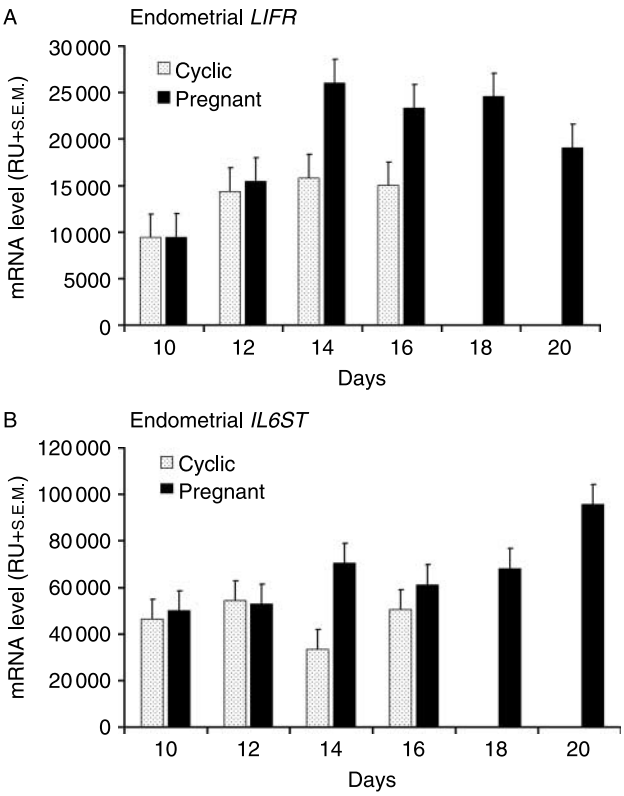


**Figure 1** Steady-state levels of *LIFR* and *IL6ST* mRNA in endometria from cyclic and early pregnant ewes as determined by slot-blot hybridization analysis. In both cyclic and pregnant ewes, endometrial *LIFR* mRNA levels increased after day 10, and the increase was greater in pregnant than cyclic ewes to day 16 (day×status, $P<0.01$). (A) In pregnant ewes, endometrial *LIFR* mRNA levels increased 2.7-fold between days 10 and 14 and then declined to day 20 (quadratic effect of day, $P<0.01$). (B) *IL6ST* mRNA levels increased 2.8-fold between days 10 and 20 of pregnancy (linear effect of day, $P<0.05$). Data are expressed as LSM relative units (RU) with S.E.M.

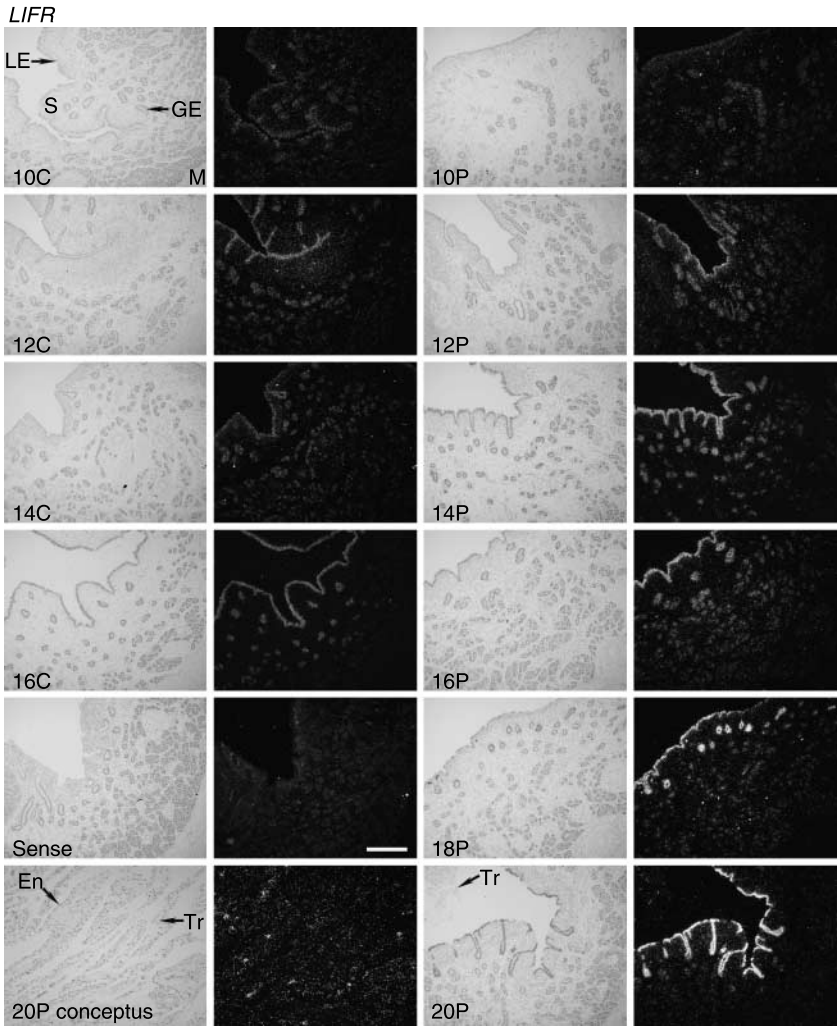


**Figure 2** *In situ* hybridization analysis of *LIFR* mRNA in the uteri of cyclic and pregnant ewes. Cross sections of the uterine wall from cyclic (C) and pregnant (P) ewes were hybridized with radiolabeled antisense or sense ovine *LIFR* cRNA probes. *LIFR* mRNA was present predominantly to the endometrial luminal (LE) and superficial glandular epithelia (sGE). In pregnant ewes, *LIFR* mRNA increased in endometrial LE/sGE between days 10 and 16 and remained abundant to day 20. *LIFR* mRNA was also detected in the trophectoderm of conceptuses on days 18 and 20 of pregnancy. LE, luminal epithelium; GE, glandular epithelium; S, stroma; M, myometrium; Tr, trophectoderm; En, endoderm. Scale bar, 25 μm.

LE/superficial glandular epithelia (sGE) and GE in the upper stratum compactum stroma, whereas *IL6ST* mRNA (Fig. 3) was most abundant in the middle to deep GE and present at lower abundance in the stroma and LE. In pregnant ewes, *LIFR* and *IL6ST* mRNAs increased in endometrial LE/sGE and GE respectively, between days 10 and 16 and remained abundant to day 20. Both *LIFR* and *IL6ST* mRNAs were also detected in the trophectoderm of conceptuses on days 18 and 20 of pregnancy. Of particular note is that both *LIFR* and *IL6ST* mRNAs were present in the conceptus trophectoderm with *IL6ST* mRNA being particularly abundant in trophoblast giant binucleate cells (BNC).

### *Early P4 treatment induces LIFR and IL6ST mRNAs in endometrial epithelia (study 2)*

This study used a sheep model in which exogenous P4 is administered from day 1.5 post-mating, thereby eliciting a premature increase in the circulating levels of P4 that is correlated with a larger blastocyst size on day 9 and the presence of elongated and filamentous conceptuses on day 12, which produce more IFNT (Satterfield *et al.* 2006). As shown in Fig. 4A and B, for day 9 ewes, endometrial *LIFR* mRNA abundance was increased twofold by early P4 (CO versus P4, $P<0.03$), while *IL6ST* mRNA was unaffected by this treatment (CO versus P4, $P>0.10$). In day 12 ewes, *LIFR* mRNA was also increased (1.6-fold) by P4 (CO versus P4, $P<0.05$) and *IL6ST* was not affected (CO versus P4, $P>0.10$). However, treatment of ewes with the antiprogestin RU486 from days 9 to 12 reduced endometrial *LIFR* mRNA levels by 2.5-fold (P4 versus P4+RU486, $P<0.005$; Fig. 4A) and *IL6ST* mRNA by 2.8-fold (P4 versus P4+RU486, $P<0.02$; Fig. 4B) compared with P4-treated ewes. *In situ* hybridization analyses revealed that early P4 increased *LIFR* mRNA in LE and GE (Fig. 4C) and that RU486 treatment reduced both *LIFR* and *IL6ST* mRNA in LE/GE and GE of the endometrium respectively (Fig. 4D).

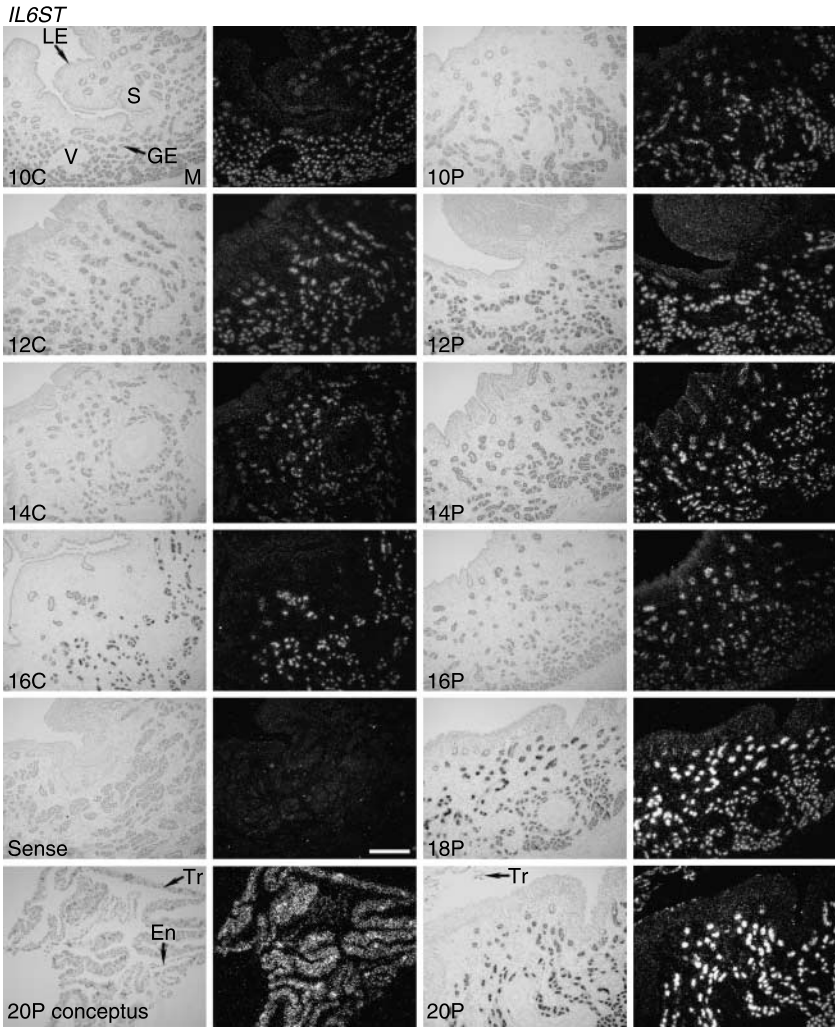


**Figure 3** *In situ* hybridization analysis of *IL6ST* mRNA in the uteri of cyclic and pregnant ewes. Cross sections of the uterine wall from cyclic (C) and pregnant (P) ewes were hybridized with radiolabeled antisense or sense ovine *IL6ST* cRNA probes. *IL6ST* mRNA was present primarily in uterine glandular epithelium (GE). In pregnant ewes, *IL6ST* mRNA increased in endometrial GE between days 10 and 16 and remained abundant to day 20. Interestingly, on days 18 and 20 of pregnancy, *IL6ST* mRNA levels were higher in the GE of upper than the deep GE of the endometria. *IL6ST* mRNA was also detected in the trophectoderm of conceptuses on days 18 and 20 of pregnancy. LE, luminal epithelium; GE, glandular epithelium; S, stroma; M, myometrium; V, blood vessels; Tr, trophectoderm; En, endoderm. Scale bar, 25 μm.

### *IFNT stimulates endometrial LIFR and IL6ST expression in ewes treated with P4 (study 3)*

In order to determine whether the expression of *LIFR* and *IL6ST* in endometria was regulated by P4 and/or IFNT, cyclic ewes were ovariectomized and fitted with intrauterine (i.u.) catheters on day 5, treated with exogenous P4 from days 5 to 16 and given intrauterine infusions of serum proteins as a control (CX) or roIFNT from days 11 to 16. As illustrated in Fig. 5A, *LIFR* mRNA levels in the endometrium was not affected by P4 (P4+CX versus P4+ZK+CX, $P>0.10$), whereas IFNT increased *LIFR* mRNA levels 1.7-fold (P4+CX versus P4+IFN, $P<0.04$) in the ewes treated with P4, but not in the ewes treated with P4+ZK. Endometrial *IL6ST* mRNA levels were increased 2.3-fold by P4 (P4+CX versus P4+ZK+CX, $P<0.03$) and an additional 1.5-fold (P4+CX versus P4+IFN, $P<0.05$) and 1.4-fold (P4+IFN versus P4+ZK+IFN, $P<0.04$) respectively in both P4- and P4+ZK-treated ewes (Fig. 5B). *In situ* hybridization found that IFNT increased *LIFR* mRNA primarily in LE and sGE (Fig. 5C) and *IL6ST* mRNA in GE and stroma (Fig. 5D) of the endometrium respectively. In P4+ZK ewes, IFNT increased *IL6ST* mRNA in the deep GE of the endometrium (Fig. 5D).

### *Localization of immunoreactive LIFR and IL6ST proteins in the ovine uterus (study 1)*

Immunohistochemical analysis indicated that LIFR protein was localized predominantly to endometrial LE/sGE, whereas IL6ST protein was localized primarily in uterine LE and GE (Fig. 6). In early pregnant ewes, both LIFR and IL6ST proteins in endometrial epithelia increased after day 14 and were abundant on day 20 of pregnancy (data not shown). Both LIFR and IL6ST proteins were detected in conceptus trophectoderm with IL6ST protein, particularly abundant in trophoblast giant BNC.

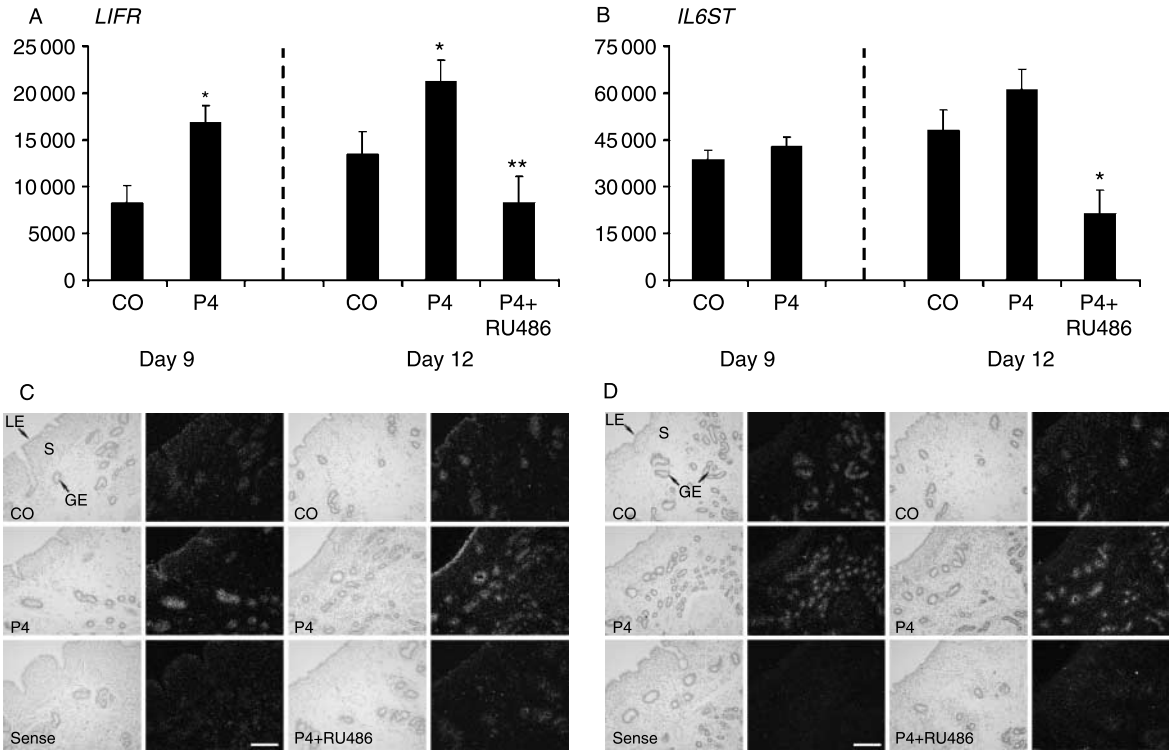


**Figure 4** Effects of progesterone (P4) during early pregnancy on *LIFR* and *IL6ST* mRNAs in the ovine uterus. (A and B) Steady-state levels of *LIFR* and *IL6ST* mRNA in endometria determined by slot-blot hybridization analysis. In ewes treated to day 9, endometrial *LIFR* mRNA abundance was increased twofold by P4 (CO versus P4, $P<0.03$), while *IL6ST* mRNA was unaffected (CO versus P4, $P>0.10$). In ewes treated to day 12, *LIFR* mRNA was increased 1.6-fold by P4 (CO versus P4, $P<0.05$), while *IL6ST* was not affected (CO versus P4, $P>0.10$). Treatment of ewes with the antiprogestin RU486 from days 9 to 12 reduced endometrial *LIFR* mRNA levels by 2.5-fold (P4 versus P4+RU486, $P<0.005$) and *IL6ST* mRNA by 2.8-fold (P4 versus P4+RU486, $P<0.02$) compared with P4-treated ewes. Asterisk denotes an effect of treatment (*$P<0.05$, **$P<0.01$). (C and D) *In situ* hybridization analyses of *LIFR* and *IL6ST* mRNAs. Cross sections of the uterine wall from treated ewes were hybridized with radiolabeled antisense or sense ovine *LIFR* and *IL6ST* cRNA probes. *LIFR* and *IL6ST* mRNAs were present at low levels in LE/sGE and in GE respectively of uteri from P4-treated ewes on day 9 or 12 respectively. LE, luminal epithelium; GE, glandular epithelium; S, stroma. Scale bar, 10 μm.

### *Localization of phosphorylated STAT3 protein in ovine endometrium and conceptus*

Immunohistochemical analyses revealed that phosphorylated STAT3 (p-STAT3) protein was present in most endometrial cell types in cyclic and pregnant ewes, but was particularly abundant in the nuclei of endometrial epithelia (Fig. 7A). In uterine LE, p-STAT3 protein increased between days 10 and 12, remained maximal from days 12 to 16, and declined thereafter in pregnant ewes. In the upper GE, p-STAT3 protein increased substantially between days 16 and 18 of pregnancy (Fig. 7A and B). The p-STAT3 protein was also abundant in conceptus trophectoderm and detected in the endoderm (Fig. 7C).

### *IFNT increase LIFR and IL6ST proteins in ovine trophoblast cells*

In oTr1 cells, IFNT increased amounts of LIFR (190 kDa) and IL6ST (130 kDa) proteins by 1.9-fold ($P<0.05$) and 2.2-fold ($P<0.01$) respectively over basal levels at 24-h post-treatment (Fig. 8A and B). In oGE cells, both LIFR and IL6ST proteins were present at all time points, but not affected ($P>0.10$) by IFNT (Fig. 9A and B).

### *LIF activates STAT3 and MAPK3/1 MAPK phosphorylation in ovine trophoblast and glandular epithelial cells*

The oTr1 cells were untreated or treated with LIF for 15 min and then phosphorylated and total STAT3 and MAPK3/1 (p42/p44) MAPK proteins detected in cell lysates by immunoblotting. As shown in Fig. 8C and D, LIF increased the abundance of both p-STAT3 and p-MAPK3/1 MAPK proteins. In response to treatment with 100 ng/ml of recombinant LIF, p-STAT3 abundance increased 5.3-fold ($P<0.01$) over basal levels within 5 min and then decreased to basal levels by 60 min (Fig. 8E). Meanwhile, LIF stimulated a rapid 5.6-fold ($P<0.01$) increase in p-MAPK3/1 MAPK abundance within 15 min that decreased to basal levels between 30 and 60 min (Fig. 8F). In oGE cells, roIFNT did not affect the abundance

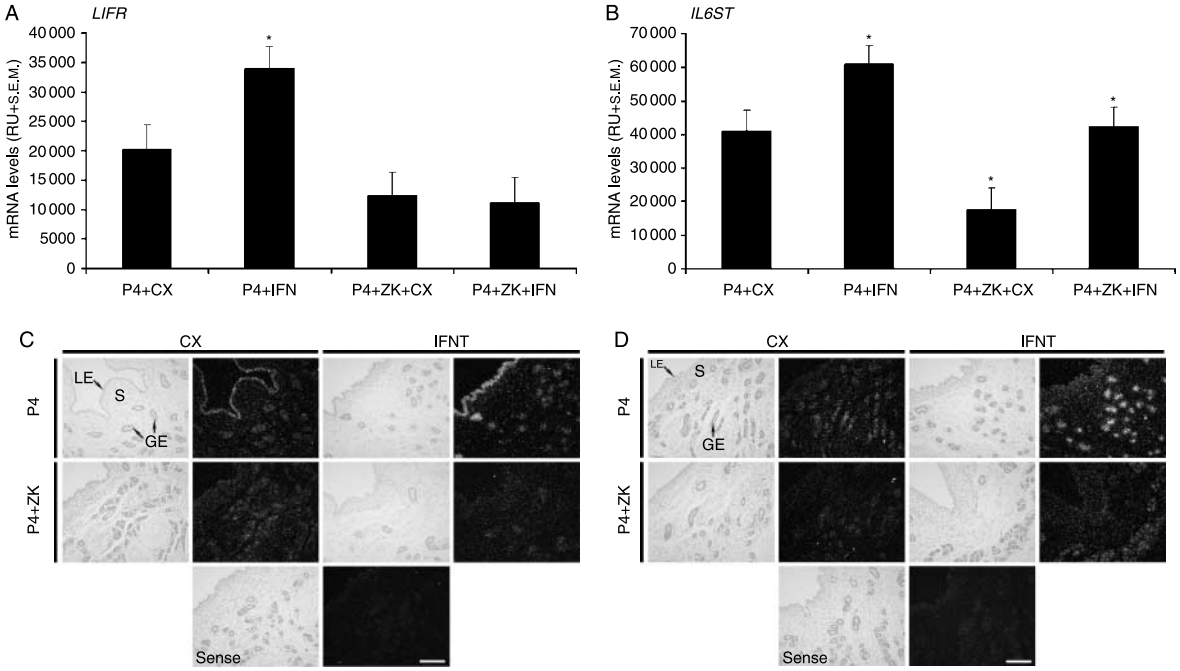


**Figure 5** Effects of progesterone (P4) and IFNT on *LIFR* mRNA in the ovine uterus. (A and B) Steady-state levels of *LIFR* and *IL6ST* mRNA in endometria as determined by slot-blot hybridization analysis. Endometrial *LIFR* mRNA was not affected by P4 (P4+CX versus P4+ZK+CX, $P>0.10$). However, roIFNT infusion increased *LIFR* mRNA levels by 1.7-fold (P4+CX versus P4+IFN, $P<0.04$), but not in P4+ZK-treated ewes. *IL6ST* mRNA levels were increased 2.3-fold by P4 (P4+CX versus P4+ZK+CX, $P<0.03$), and roIFNT increased *IL6ST* mRNA abundance 1.5-fold (P4+CX versus P4+roIFNT, $P<0.05$) and 1.4-fold (P4+roIFNT versus P4+ZK+roIFNT, $P<0.04$) respectively in both P4- and P4+ZK-treated ewes. Asterisk denotes an effect of treatment (*$P<0.05$). (C and D) *In situ* hybridization revealed that IFNT increased *LIFR* mRNA primarily in the LE and *IL6ST* mRNA in the GE and stroma of the endometrium respectively. In P4+ZK ewes, IFNT increased *IL6ST* mRNA in the deep GE of the endometrium. LE, luminal epithelium; GE, glandular epithelium; S, stroma. Scale bar, 10 μm.

of LIFR or IL6ST proteins (Fig. 9A and B). However, LIF increased the levels of p-STAT3 and p-MAPK3/1 MAPK proteins by 6.1-fold ($P<0.01$) and 3.6-fold ($P<0.01$) over basal levels respectively within 5 min; the effect was maintained to 90 min (Fig. 9C and D).

## Discussion

The present studies revealed that both *LIFR* and *IL6ST* expression in the ovine endometrium increases during the peri-implantation period of early pregnancy. Co-expression of *LIFR* and *IL6ST* mRNA and protein as well as phophorylated (activated) STAT3 protein was primarily in the superficial and upper glands of the endometrium, as well as in the trophectoderm of the conceptus. Vogiagis *et al.* (1997*a*) found that *LIF* mRNA and protein were present in relatively constant amounts throughout the estrous cycle and early pregnancy in sheep and localized to all uterine cell types with particular abundance in the LE, and that immunoreactive LIF was present in conceptus trophectoderm. Thus, the paracrine and perhaps autocrine actions of LIF within the uterus during early pregnancy in sheep are most likely manifest on the upper glands of the endometrium and conceptus trophectoderm. The results of the present studies indicate that the biological effects of LIF on those cells involve activation of STAT3 and MAPK3/1 MAPK signaling pathways to alter gene expression patterns and cell proliferation and differentiation.

The results of the present study found that *LIFR* and *IL6ST* in the endometrial LE/GE and GE respectively are stimulated by P4 and IFNT in a complex stage- and cell-specific manner. The induction of many genes by P4, such as galectin 15 (*LGALS15*; Gray *et al.* 2004), cathepsin L1 (*CTSL1*; Song *et al.* 2005), cystatin C (*CST3*; Song *et al.* 2006*a*), endothelial PAS domain protein 1 (*EPAS1*; Song *et al.* 2008*a*), gastrin-releasing peptide (*GRP*; Song *et al.* 2008*b*), and insulin-like binding proteins 1 and 3 (*IGFBP1* and *IGFBP3*; Satterfield *et al.* 2008), in the endometrial LE/sGE of the ovine uterus appears to be indirect and require P4-induced down-regulation of P4 receptors (PGR) in those epithelia (Spencer *et al.* 2004*b*, 2007), as well as perhaps factors from PGR-positive uterine stromal cells, such as fibroblast growth factor 10 (FGF10; Chen *et al.* 2000, Satterfield *et al.* 2008). Indeed, the increase in *LIFR* mRNA abundance in the LE/sGE and upper GE between days 10 and 14 post-estrus/mating in study 1 is coincident with the loss of *PGR* mRNA and protein in these epithelia (Wathes & Hamon 1993, Spencer *et al.* 1995). Furthermore, in studies 2 and 3, *LIFR* mRNA increased in endometrial LE/sGE and upper GE of

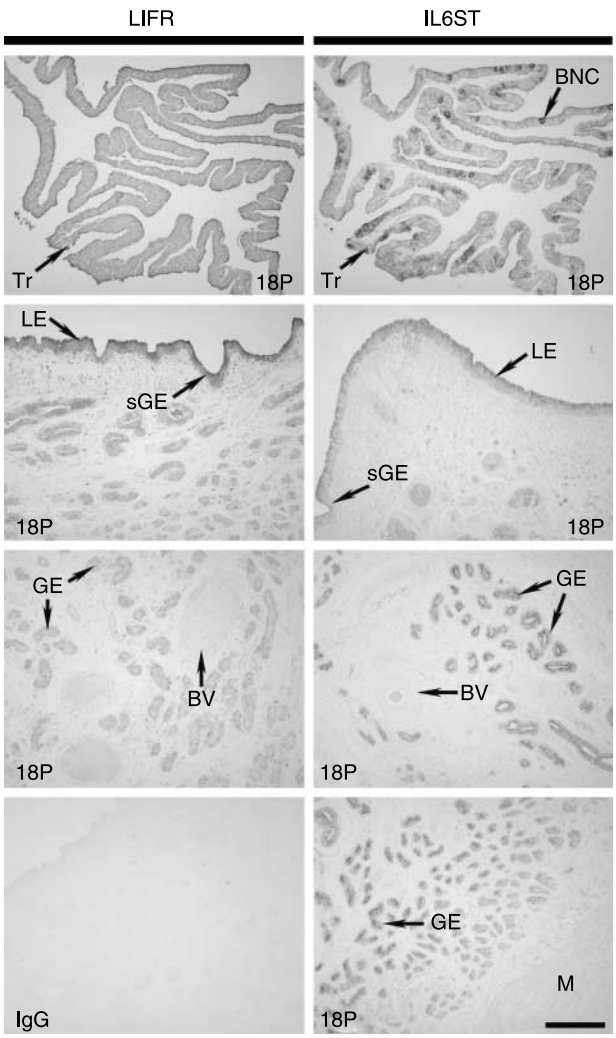


**Figure 6** Immunolocalization of LIFR and IL6ST proteins in the uteri from cyclic and pregnant ewes. Immunoreactive LIFR and IL6ST proteins were localized predominantly near the apical surface of endometrial LE/sGE and all GE respectively in uteri and in conceptus trophectoderm from day 18 pregnant ewes using a rabbit anti-human LIFR polyclonal antibody and a rabbit anti-human gp130 polyclonal antibody. For the IgG control, normal rabbit IgG was substituted for the primary antibody. Sections were not counterstained. LE, luminal epithelium; GE, glandular epithelium; Tr, trophectoderm; M, myometrium; BV, blood vessels; sGE, superficial GE; BNC, binucleate cells. Scale bar, 10 μm.

P4-treated ewes, but not in the ewes treated with P4 and a PGR antagonist, i.e., ZK 136 317 or RU486. Continuous exposure of the sheep uterus to P4 for 8–10 days down-regulates *PGR* mRNA and protein in endometrial LE/sGE, but not in stroma or myometrium (Johnson *et al.* 2000*b*); however, PGR are present in the endometrial epithelia of the ewes treated with P4 and a PGR antagonist (Johnson *et al.* 2000*b*), because PGR antagonists prevent P4 from down-regulating the expression of PGR. Consequently, the increase in *LIFR* mRNA may be due to P4-induced down-regulation of PGR in LE/sGE between days 10 and 12 of the estrous cycle and pregnancy (Spencer & Bazer 2004, Spencer *et al.* 2004*b*). The increase in endometrial *LIFR* expression by early treatment with P4 in study 2 also occurred coincident with early loss of PGR by endometrial LE/sGE (Satterfield *et al.* 2006). Similarly, *IL6ST* is expressed predominantly in endometrial GE as is the case for secreted phosphoprotein 1 (commonly referred to as osteopontin), serpin peptidase inhibitor (also known as uterine milk protein or *UTMP*), stanniocalcin 1 (*STC1*), and *GRP*, which encode proteins secreted into the uterine lumen (Moffatt *et al.* 1987, Ing & Roberts 1989, Johnson *et al.* 1999*b*, 2003, Song *et al.* 2006*b*, 2008*b*). All four of those genes are induced in endometrial GE by the long-term effects of continuous P4 that also requires loss of the PGR as a permissive event preceding IFNT effects to enhance gene expression (Spencer & Bazer 2002, Spencer *et al.* 2004*b*). Collectively, available evidence supports the idea that treatment of ewes with PGR antagonists prevents P4-induced loss of PGR in the LE/sGE and GE, as well as stromal-derived progestomedins, and then subsequent induction of gene expression in those epithelia, which, in turn, produces a uterus unsupportive of conceptus development and implantation. Indeed, no blastocysts were recovered from the P4+RU486-treated ewes in study 2 that had reduced the levels of endometrial *LIFR* and *IL6ST* expression (Satterfield *et al.* 2006). In addition to being an antiprogestin, RU486 or mifepristone is a high-affinity antagonist of the nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor; NR3C1, also known as GR; Baulieu 1989), suggesting that NR3C1 and glucocorticoids may also regulate uterine LE/sGE gene expression. However, little is known of *NR3C1* expression and glucocorticoid effects within the ovine uterus during early pregnancy.

Available results support our working hypothesis that uterine LIF regulates endometrial function and conceptus growth and development during the periimplantation period of pregnancy in sheep. In the present study, LIFR, IL6ST, and phosphorylated STAT3 were found in the upper GE of early pregnancy during the period of conceptus elongation and implantation. Similarly, *Lifr* and *Il6st* are co-expressed in the uterine LE of mice (Song & Lim 2006), which responds to LIF from the endometrial GE by phosphorylating STAT3 that is essential for implantation (Cheng *et al.* 2001, Song & Lim 2006). In the present studies, treatment of ovine endometrial GE cells with LIF increased the abundance of activated phosphorylated STAT3 and MAPK3/1 MAPK proteins. Future studies will determine which genes in the upper GE are the targets of LIF actions.

Novel results from the present studies implicate LIF, LIFR, IL6ST co-receptor, and STAT3 and MAPK3/1 MAPK in the growth and differentiation of conceptus trophectoderm and, in particular, trophoblast giant BNC. In particular, LIFR signaling via STAT3 regulates trophoblast giant cell differentiation in mice (Takahashi *et al.* 2008).

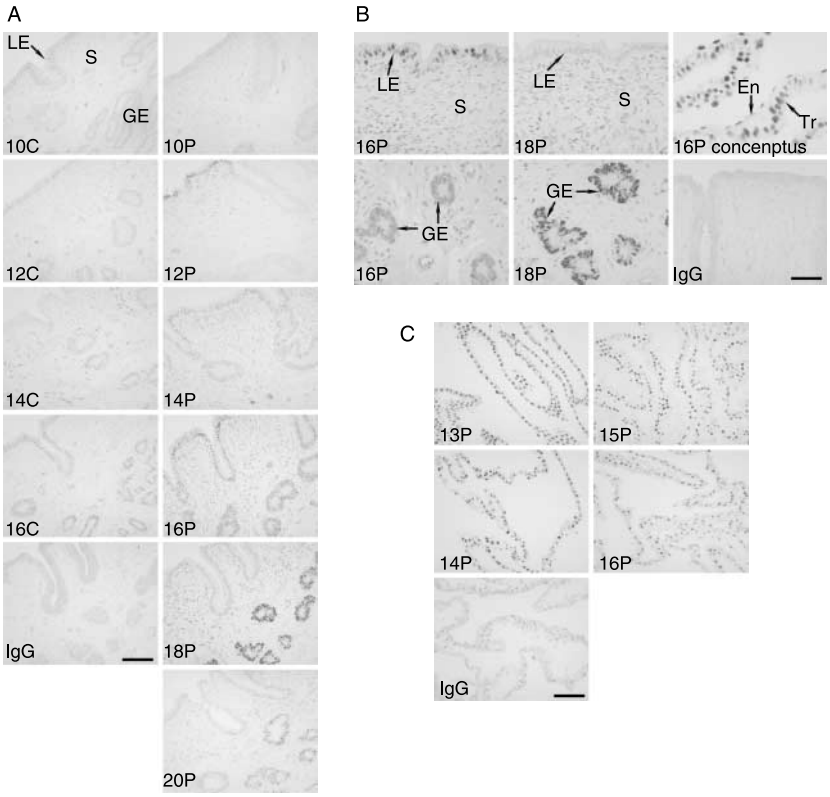


**Figure 7** Immunohistochemical localization of phosphorylated STAT3 protein in endometria from cyclic and pregnant ewes. Immunoreactive p-STAT3 protein was localized using a rabbit anti-mouse phospho-STAT3 (Tyr705) polyclonal antibody. Normal rabbit IgG was substituted for the primary antibody as a negative control. Sections were not counterstained. Immunoreactive p-STAT3 protein was present in the nuclei of endometrial LE, GE, and conceptus trophectoderm of pregnant ewes. Interestingly, after day 16 of pregnancy, p-STAT3 protein was predominantly localized to the endometrial upper glands while significantly decreased in the LE. The p-STAT3 protein was also particularly abundant in conceptus trophectoderm in pregnant ewes. Localization of immunoreactive p-STAT3 protein in conceptus trophectoderm, including binucleate giant cells, was based on cell morphology. LE, luminal epithelium; GE, glandular epithelium; S, stroma; En, endoderm; Tr, trophectoderm. (A and C) Scale bar, 5 μm; (B) 2.5 μm.

Indeed, the polyploid trophoblast giant cells of the mouse placenta are terminally differentiated and mediate trophoblast invasion of maternal decidua (Simmons & Cross 2005). Similarly, human extravillous trophoblast invasiveness can be regulated by LIF and STAT3 (Poehlmann *et al.* 2005). The trophoblast giant BNC of the ovine placenta are similar to the trophoblast giant cells of the mouse placenta, in that they are polyploid, invasive, and express unique genes such as *CSH1* (Hoffman & Wooding 1993). In addition to STAT3, the MAPK3/1 MAPK pathway also plays important roles in differentiation, including embryonic and placental development (Mudgett *et al.* 2000, Wang *et al.* 2004, Daoud *et al.* 2005). Given that little is known about the cellular and molecular mechanisms governing trophoblast growth and differentiation in ruminants, the potential roles of LIF, its receptor complex, STAT3, and MAPK3/1 MAPK in development and differentiation within the sheep conceptus need to be discerned.

## Materials and Methods

### *Animals*

Mature crossbred Suffolk sheep (*Ovis aries*) were observed daily for estrus in the presence of vasectomized rams and used in experiments after they had exhibited at least two estrous cycles of normal duration (16–18 days). At estrus, the ewes were assigned randomly to cyclic or pregnant status. All experimental and surgical procedures were in compliance with the Guide for the Care and Use of Agriculture Animals in Teaching and Research and were approved by the Institutional Animal Care and Use Committee of Texas A&M University.

### *Experimental design*

#### *Study 1*

At estrus (day 0), the ewes were mated to either an intact or vasectomized ram and then hysterectomized ($n=5$ ewes/day) on either day 10, 12, 14, or 16 of the estrous cycle or day 10, 12, 14, 16, 18, or 20 of pregnancy as described previously (Spencer *et al.* 1999*a*). At hysterectomy, the uterus was flushed with 20 ml sterile saline. Pregnancy was confirmed on days 10–16 post-mating by the presence of a morphologically normal conceptus(es) in the uterine flushing. It was not possible to obtain uterine flushings on either day 18 or 20 of pregnancy, because the conceptus had firmly adhered to the endometrial LE and basal lamina. At hysterectomy, several sections (~0.5 cm) from the mid-portion of each uterine horn ipsilateral to the CL were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2) (v/v). After 24 h, the fixed tissues were changed to 70% ethanol (v/v) for 24 h, dehydrated through a graded series of alcohol to xylene, and then embedded in Paraplast Plus (Oxford Labware, St Louis, MO, USA). The remaining endometrium was physically dissected from myometrium, frozen in liquid nitrogen, and stored at −80 °C for subsequent RNA extraction. In monovulatory pregnant ewes, uterine tissue samples were marked as either contralateral or ipsilateral to the ovary bearing the CL and only tissues from the ipsilateral

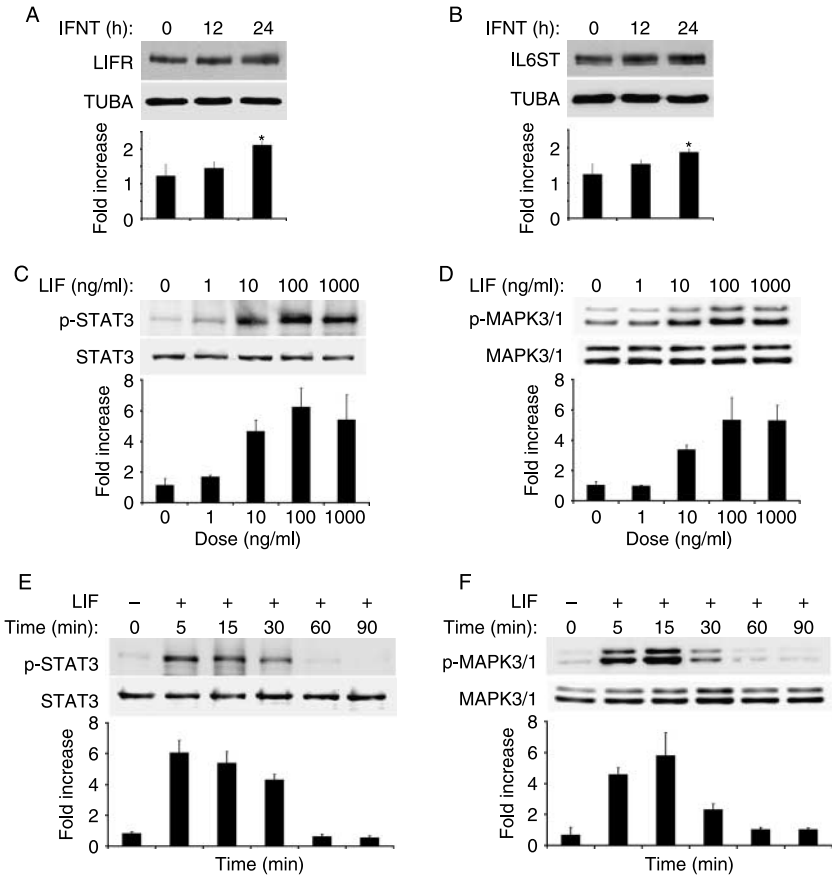


**Figure 8** Effects of IFNT on LIFR and IL6ST proteins and dose- and time-dependent effects of human LIF on the phosphorylation of STAT3 and MAPK3/1 MAPK in ovine trophectoderm (oTr1) cells. (A and B) The oTr1 cells were starved for 24 h in serum-free medium and then treated with IFNT ($10^4$ AVU/ml) for the indicated periods of time. Blots were imaged to calculate the normalized values presented in graphs (bottom) by measurements of the levels of mouse α-tubulin (TUBA) protein relative to total proteins. This time-course design was replicated in four independent experiments. (C–F) oTr1 cells were starved for 24 h in serum-free medium and then treated with LIF by indicated concentration (C and D) and time (100 ng/ml LIF) (E and F). Blots were imaged to calculate the normalized values presented in graphs by measurements of the levels of phosphorylated protein relative to total proteins. All quantitative data are presented as least-squares means (LSM) with overall S.E.M. This time-course design was replicated in three independent experiments.

uterine horn were used in subsequent analyses. In addition, conceptuses ($n>5$ per day) were collected on days 13, 14, 15, and 16 of pregnancy by uterine flush and then fixed and embedded in paraffin as described above.

*Study 2*

As described previously (Satterfield *et al.* 2006), the ewes were mated at estrus (day 0) to intact rams and then assigned randomly to receive daily i.m. injections from days 1.5 to 9 of either corn oil vehicle (CO; $n=6$) or 25 mg P4 ($n=6$). All ewes were hysterectomized on day 9, and uteri processed as described for experiment 1. In a complementary study, the ewes were mated and assigned randomly to receive daily i.m. injections of either (a) CO ($n=8$), (b) 25 mg P4 (Sigma Chemical Co.) from days 1.5 to 12 ($n=7$), or (c) 25 mg P4 (from days 1.5 to 8, $n=5$) and 75 mg of RU486 (mifepristone; Sigma-Aldrich Inc.), a P4 receptor (PGR) antagonist, from days 8 to 12 (P4+RU486). All ewes were hysterectomized on day 12, and uteri processed as described for study 1. Pregnancy was confirmed by the recovery of a morphologically normal blastocyst or conceptus in the uterine flushing.

*Study 3*

Cyclic ewes ($n=20$) were checked daily for estrus and then ovariectomized and fitted with indwelling uterine catheters on day 5 as described previously (Johnson *et al.* 2000*a*). The ewes were then assigned randomly ($n=5$ per treatment) to receive daily i.m. injections of P4 and/or a PGR antagonist (ZK 136,317; Schering AG, Berlin, Germany) and intrauterine (i.u.) infusions of control serum proteins and/or recombinant ovine IFNT protein (roIFNT) as follows: 1) 50 mg P4 (days 5–16) and 200 μg control serum (CX) proteins (days 11–16) (P4+CX), 2) P4 plus 75 mg ZK 136,317 (days 11–16) and CX proteins (P4+ZK+CX), 3) P4 and IFNT ($2 \times 10^7$ antiviral units, days 11–16) (P4+IFN), or 4) P4 plus ZK and IFNT (P4+ZK+IFN). Steroids were administered i.m. daily in corn oil vehicle. Both uterine horns of each ewe received twice daily injections of either CX proteins (50 μg/horn/injection) or roIFNT ($5\times10^6$ antiviral units/horn/injection). The roIFNT was produced in *Pichia pastoris* and purified as described previously (Van Heeke *et al.* 1996). CX proteins were prepared for intrauterine injection as described previously (Spencer *et al.* 1995). This regimen of P4 and roIFNT mimics the effects of P4 from the CL and IFNT from the conceptus on endometrial expression of hormone receptors and ISGs during early pregnancy in sheep (Song *et al.* 2005, 2006*a*, 2007, 2008*a*, 2008*b*). All ewes were hysterectomized on day 17, and uteri processed as described for study 1.

*Study 4*

A mononuclear ovine trophectoderm cell line (oTr1), derived from a day 15 conceptus (Farmer *et al.* 2008, Kim *et al.* 2008),

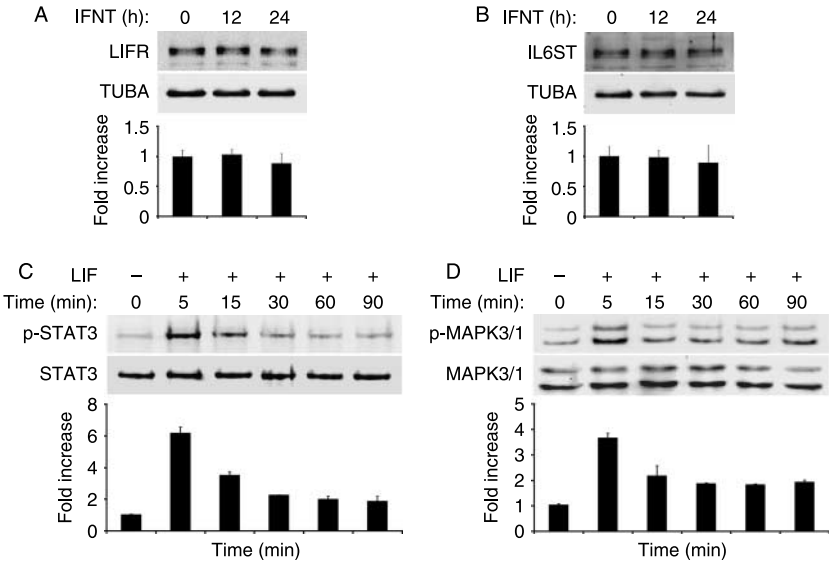


**Figure 9** Dose- and time-dependent effects of human LIF on phosphorylation of STAT3 and MAPK3/1 MAPK in an immortalized ovine uterine glandular epithelial (oGE) cell line. (A and B) The oGE cells were starved for 24 h in serum-free medium and then treated with IFNT ($10^4$ AVU/ml) for the indicated periods of time. Blots were imaged to calculate the normalized values presented in graphs (bottom) by measurements of the levels of mouse α-tubulin (TUBA) protein relative to total proteins. This time-course design was replicated in four independent experiments. (C and D) The oGE cells were starved for 24 h in serum-free medium and then treated with LIF by indicated time (100 ng/ml LIF). Blots were imaged to calculate the normalized values presented in graphs by measurements of the levels of phosphorylated - protein relative to total proteins. All quantitative data are presented as least-squares means (LSM) with overall S.E.M. This time-course design was replicated in three independent experiments.

was cultured as described previously in DMEM–F12 (Sigma-Aldrich Corp.) that included 10% FBS (v/v), 50 U penicillin, 50 μg streptomycin, 0.1 mM each non-essential amino acids, 1 mM sodium pyruvate, 2 mM glutamine, and 0.7 μM insulin. When cell density in dishes reached about 80% confluence, they were passaged at a ratio of 1:3 and frozen stocks of cells were prepared at each passage. For experiments, monolayer cultures of oTr1 cells (between passages 9 and 13) were grown in culture medium to 80% confluence on 100 mm tissue culture plates. Immortalized ovine endometrial GE (oGE) cells were cultured as described previously (Johnson *et al.* 1999*a*). Ovine GE cells were maintained in DMEM–F12 supplemented with 10% FBS (v/v) and antibiotics and then both cell monolayer cultures were grown to 90% confluence on 100 mm tissue culture plates. All cell lines were incubated in serum-free medium for 24 h and then left untreated as a control or treated with roIFNT ($10^4$ AVU/ml) for the indicated time. In other experiments, oTr1 and oGE cells were serum starved for 24 h and then treated with recombinant human LIF (rhLIF, catalog no. L-5283; Sigma-Aldrich Inc.) for 0, 5, 15, 30, 60, or 90 min. Based on dose–response experiments (tested concentrations of 1, 10, 100, and 1000 ng/ml LIF) with doses based on bovine embryo culture conditions (Fukui & Matsuyama 1994, Han *et al.* 1995, Funston *et al.* 1997, Sirisathien *et al.* 2003), the 100 ng/ml dose of LIF was selected and used for study. All studies were replicated in three independent experiments.

### *RNA isolation*

Total cellular RNA was isolated from the frozen endometria or cultured cells using the TRIzol reagent (Gibco-BRL), according to the manufacturer's recommendations. The quantity and quality of total RNA were determined by spectrometry and denaturing agarose gel electrophoresis respectively.

### *Cloning of partial cDNAs for ovine LIFR and IL6ST*

Partial cDNAs for ovine *LIFR* and *IL6ST* mRNAs were amplified by RT-PCR using total RNA from day 18 pregnant endometrium using specific primers based on bovine *LIFR* mRNA (GenBank accession no. XM_587754; forward, 5′-AGC CAC TGA CCG AGT TTC C-3′; reverse, 5′-ATG GGA AGA AAT TCC TGT GG-3′) and bovine *IL6ST* mRNA (GenBank accession no. XM-600430; forward, 5′-TCC TGG AAG GGA AAC ATA CC-3′; reverse, 5′-GCT TCT TCA CTC CAG TCA CTC C-3′). Reverse transcription of total RNA into cDNA was performed as described previously (Taylor *et al.* 2001). PCR amplification was conducted as follows for *LIFR* and *IL6ST*: 1) 95 °C for 5 min; 2) 95 °C for 30 s, 56.5 °C for 40 s, and 72 °C for 1 min for 35 cycles; and 3) 72 °C for 10 min. The partial cDNAs for ovine *LIFR* and *IL6ST* PCR products were cloned into pCRII using a T/A Cloning Kit (Invitrogen) and their sequences verified using an ABI PRISM Dye Terminator Cycle Sequencing Kit and ABI PRISM automated DNA sequencer (Applied Biosystems, Foster City, CA, USA).

### *Slot-blot hybridization analyses*

The steady-state levels of mRNA in ovine endometria were assessed by radioactive slot-blot hybridization as described previously (Spencer *et al.* 1999*b*). Denatured total endometrial RNA (20 μg) from each ewe was hybridized with radiolabeled antisense cRNA probes. To correct for variation in total RNA loading, a duplicate RNA slot-blot membrane was hybridized with radiolabeled antisense 18S cRNA (pT718S; Ambion, Austin, TX, USA), because 18S RNA content of the endometrium was not affected by day, pregnancy status, or hormonal treatment in any of the studies (data not shown). Following washing, the blots were digested with RNase A and radioactivity associated with slots quantified using a Typhoon 8600 MultiImager (Molecular Dynamics, Piscataway, NJ, USA).

### In situ *hybridization analyses*

Location of mRNA in uterine sections (5 μm) was determined by radioactive *in situ* hybridization analysis as described previously (Spencer *et al*. 1999*b*). After hybridization, washing and RNase A digestion, slides were dipped in NTB-2 liquid photographic emulsion (Kodak), and exposed at 4 °C for 10 days. After development and counterstaining, images of the representative fields were recorded under bright- or dark-field illumination.

### *Immunohistochemical analyses*

Immunohistochemical localization of LIFR and IL6ST proteins in ovine uteri was performed as described previously (Spencer *et al*. 1999*c*) using rabbit anti-human LIFR polyclonal IgG (catalog no. sc-659; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 1:500 dilution (0.4 μg/ml), rabbit anti-human IL6ST polyclonal IgG (catalog no. 06-261; Upstate, Lake Placid, NY, USA) at 1:1000 dilution (1.0 μg/ml), and rabbit anti-mouse phospho-STAT3 (Tyr705) polyclonal IgG (catalog no. 9131; Cell Signaling Technology, Danvers, MA, USA) at 1:100 dilution. Antigen retrieval was performed using the boiling citrate method. Negative controls included substitution of the primary antibody with purified non-immune rabbit IgG at the same final concentration. Sections were not counterstained prior to affixing coverslips.

### *Western blot analyses*

Whole cell extracts and immunoblot assays were prepared and performed as described previously (Stewart *et al*. 2001). Proteins in cell lysates were denatured, separated using SDS-PAGE, transferred to nitrocellulose, and western blot analyses performed as described previously (Spencer *et al*. 1999*a*) using ECL detection (SuperSignal West Pico, Pierce, Rockford, IL, USA) and X-OMAT AR X-ray film (Kodak), according to the manufacturer's recommendations. Immunoreactive proteins were detected using anti-human LIFR polyclonal IgG (catalog no. sc-659; Santa Cruz Biotechnology Inc.) at 1:1000 dilution (0.2 μg/ml), anti-human IL6ST polyclonal IgG (catalog no. 06-261; Upstate) at 1:2000 dilution (0.5 μg/ml), anti-mouse phospho-STAT3 (Tyr705) polyclonal IgG (catalog no. 9131; Cell Signaling Technology) at 1:1000 dilution (1.0 μg/ml), anti-human phospho-MAPK3/1 polyclonal IgG (catalog no. 9101; Cell Signaling Technology) at 1:2000 dilution (0.5 μg/ml), anti-rat STAT3 polyclonal IgG (catalog no. S21320; Transduction Laboratories Inc., Lexington, KY, USA) at 1:2500 dilution (1.0 μg/ml), and anti-human MAPK3/1 monoclonal IgG (catalog no. 4695; Cell Signaling Technology) at 1:5000 dilution. Multiple exposures of each western blot were performed to ensure linearity of chemiluminescent signals. Western blots were quantified using a ChemiDoc EQ system and Quantity One software (Bio-Rad).

### *Statistical analyses*

All quantitative data were subjected to least-squares ANOVA using the Statistical Analysis System (SAS Institute, Cary, NC, USA). Slot-blot hybridization data were corrected for differences in sample loading using the 18S rRNA data as a covariate. Data from ewes between days 10 and 16 (study 1) were analyzed for the effects of day, pregnancy status (cyclic or pregnant), and their interaction. Data from studies 2 and 3 were analyzed using orthogonal contrasts (day 9: CO versus P4, day 12: CO versus P4, and day 12: P4 versus P4+RU486 for study 2; P4+CX versus P4+IFN, P4+ZK+CX versus P4+ZK+IFN, P4+CX versus P4+ZK+CX for study 3) to determine the effects of the treatment. All tests of significance were performed using the appropriate error terms according to the expectation of the mean squares for error. $P \leq 0.05$ was considered significant. Data are presented as least-squares means with (S.E.M).

## Declaration of interest

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

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