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Progesterone and interferon tau-regulated genes in the ovine uterine endometrium: identification of periostin as a potential mediator of conceptus elongation

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Abstract

During early pregnancy in ruminants, progesterone (P_4) and interferon tau (IFNT) act on the endometrium to regulate genes hypothesized to be important for conceptus development and implantation. The present study was conducted to verify several candidate genes (actin α-2, smooth muscle, aorta (*ACTA2*), collagen, type III, α-1 (*COL3A1*), periostin (*POSTN*), secreted protein acidic cysteine-rich (*SPARC*), S100 calcium-binding protein A2 (*S100A2*), *STAT5A* and transgelin (*TAGLN*)) regulated by pregnancy, P_4, and/or IFNT in the endometrium determined using a custom ovine cDNA array. *S100A2* mRNA was detected primarily in endometrial epithelia and conceptuses. *S100A2* mRNA increased in endometrial epithelia from days 10 to 16 in cyclic ewes and from days 10 to 14 in pregnant ewes and declined thereafter. The abundance of *S100A2* mRNA was less in endometrial luminal epithelium of IFNT-infused ewes receiving P_4. Expression of *COL3A1, SPARC, ACTA2,* and *TAGLN* was independent of pregnancy, P_4, or IFNT. *POSTN* mRNA was detected primarily in compact stroma of intercaruncular and caruncular endometria, but not in the conceptus. Endometrial *POSTN* mRNA increased between days 12 and 14 in pregnant but not cyclic ewes, and *POSTN* mRNA was more abundant in uterine stroma of ewes treated with P_4. POSTN protein was detected in uterine flushings of pregnant ewes and found to mediate attachment and stimulate migration of ovine trophectoderm cells *in vitro*. These results support the ideas that POSTN and S100A2 are regulated by P_4 and IFNT respectively, and that POSTN is involved in conceptus elongation during early pregnancy.
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Introduction

Maternal recognition of pregnancy and implantation of conceptuses (embryo/fetus and its extraembryonic/fetal membranes) in early pregnancy are crucial for successful establishment and maintenance of pregnancy. The elongating ruminant conceptus secretes the pregnancy recognition signal, interferon tau (IFNT), which prevents luteolysis. The corpus luteum produces progesterone (P_4), which is required for successful pregnancy by regulating uterine functions that are permissive to implantation, placentation, and successful growth and development of the conceptus to term (Spencer & Bazer 2004). In most mammalian uteri, progesterone receptors (PGR) are expressed in the endometrial epithelia and stroma during the early to mid-luteal phase, allowing direct regulation of a number of genes by P_4 via activation of PGR. The loss of PGR in the endometrial luminal epithelia (LE) and then glandular epithelia (GE) is associated with reprograming of gene expression in the endometrium (Spencer *et al*. 2004, 2008). In addition to antiluteolytic effects, IFNT acts on the endometrium in a cell-specific manner to regulate expression of IFN-stimulated genes (ISGs) that are hypothesized to play roles in uterine receptivity to implantation as well as conceptus elongation, development, and implantation (Spencer & Bazer 2004).

During early pregnancy, gene expression by cells of the uterine endometrium in sheep is regulated by independent and combined actions of P_4 and IFNT (Spencer *et al*. 2007). We previously developed a custom endometrial cDNA array from day 14 pregnant ewes in order to identify effects of pregnancy, P_4, and IFNT on gene expression in ovine endometria (Gray *et al*. 2006). A subset of genes listed in Table 1 was found to be either increased or decreased in response to pregnancy, P_4, and/or IFNT in that study. Periostin (POSTN) was originally isolated as an

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Table 1 Candidate pregnancy, progesterone (P_4) and interferon tau (IFNT)-regulated genes in the ovine uterus[a].

Symbol	Name	Day 14 pregnant/cyclic	Effect of P_4	Effect of IFNT
ACTA2	Actin, α-2, smooth muscle, aorta	↓	–	–
COL3A1	Collagen, type III, α-1	↓	–	↓
SPARC	Secreted protein, acidic, cysteine-rich (osteonectin)	↓	–	↓
POSTN	Periostin, osteoblast specific factor	↑	↑	↓
S100A2	S100 calcium-binding protein A2	–	–	↓
STAT5A	STAT5A	↑	↑	↑
TAGLN	Transgelin	–	–	↑

↑, increase; ↓, decrease; –, no effect.
[a]From Gray *et al.* (2006).

osteoblast-specific factor that functions as a cell adhesion molecule for preosteoblasts and is thought to be involved in osteoblast recruitment, attachment, and spreading (Gillan *et al.* 2002). S100 calcium-binding protein A2 (S100A2) is a member of the S100 family of proteins containing two EF-hand calcium-binding motifs. S100 proteins are localized in the cytoplasm and/or nucleus of a wide range of cells, and involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation and may act as a modulator against excess calcium accumulation (Wicki *et al.* 1997). Collagen, type III, α-1 (COL3A1) and secreted protein, acidic, cysteine-rich (SPARC) are extracellular matrix (ECM) components that are regulated by interleukin 11 (IL11) during decidualization in mice (White *et al.* 2004). Actin, α-2, smooth muscle, aorta (ACTA2) belongs to the actin family of proteins, which are highly conserved proteins that play a role in cell motility, structure, and integrity. ACTA2 is expressed in uterine myometrium during pregnancy to prepare the myometrium for optimal contractions during labor (Shynlova *et al.* 2005). Transgelin (TAGLN) is an actin-binding protein involved in actin organization by causing gelling of

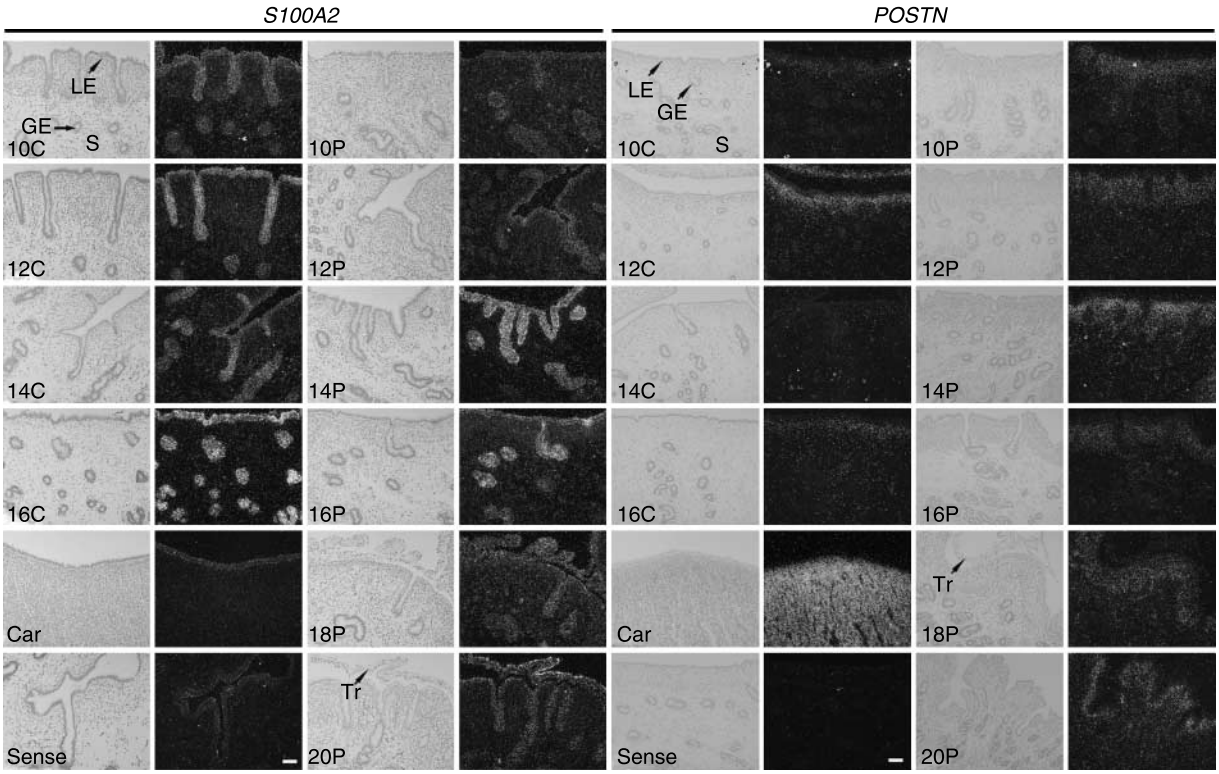

Figure 1 *In situ* localization of *S100A2* and *POSTN* mRNAs in the cyclic (C) and pregnant (P) ovine endometria. Cross sections of uteri were hybridized with radiolabeled antisense or sense ovine *S100A2* and *POSTN* cRNA probes. *S100A2* mRNA is localized in LE, GE, and conceptus on days 18 and 20. *POSTN* mRNA is localized in stroma and caruncle. Bright-field photomicrographs (left) and dark-field photomicrographs (right) were taken for each gene. LE, luminal epithelium; GE, glandular epithelium; S, stroma; Car, caruncle; Tr, trophectoderm. Scale bar represents 10 μm.

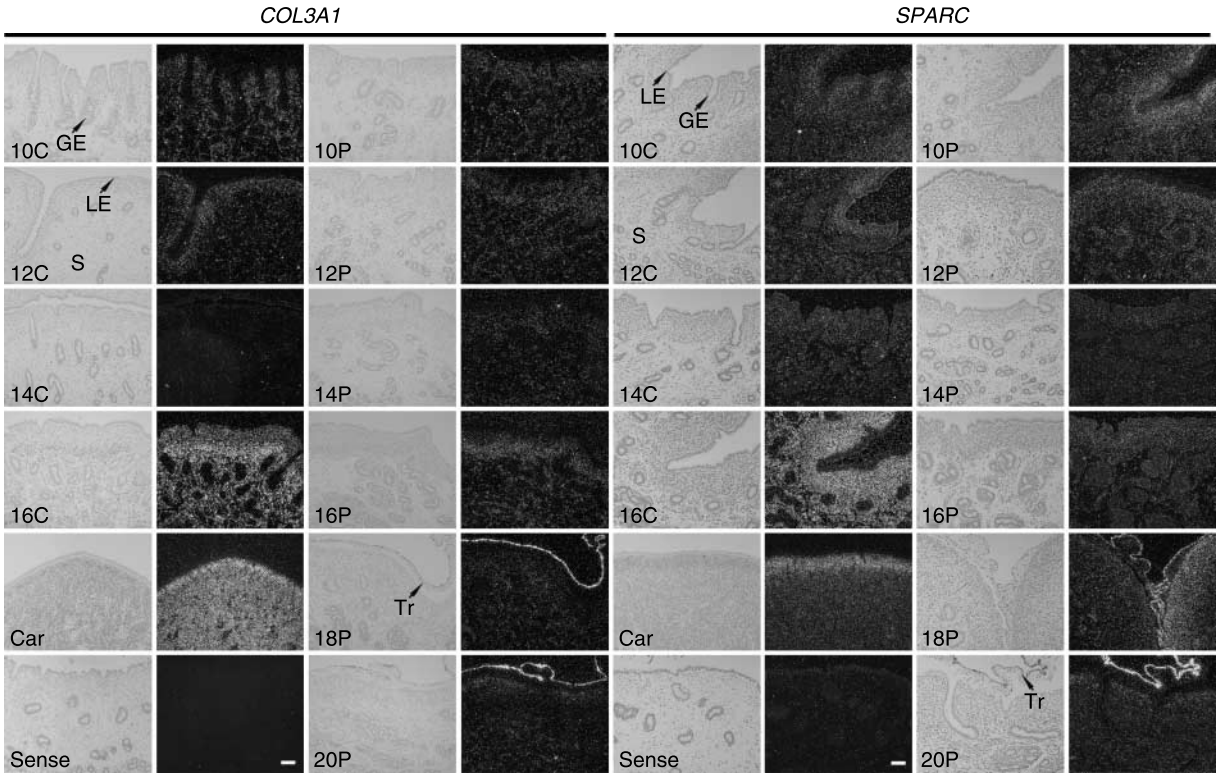

Figure 2 *In situ* localization of *COL3A1* and *SPARC* mRNAs in the cyclic (C) and pregnant (P) ovine endometria. Cross sections of uteri were hybridized with radiolabeled antisense or sense ovine *COL3A1* and *SPARC* cRNA probes. *COL3A1* and *SPARC* mRNAs are localized in stroma, caruncle, and conceptus on days 18 and 20. Bright-field photomicrographs (left) and dark-field photomicrographs (right) were taken for each gene. LE, luminal epithelium; GE, glandular epithelium; S, stroma; Car, caruncle; Tr, trophectoderm. Scale bar represents 10 μm.

actin (Shapland *et al*. 1993). STAT5A is one member of the JAK–STAT-signaling pathway that mediates actions of lactogenic hormones including prolactin and placental lactogen (Darnell 1997).

Our working hypothesis is that many of the genes identified by custom microarray analysis of ovine endometria have physiological roles in establishment of pregnancy and conceptus elongation and implantation. Although the genes listed in Table 1 have been identified as candidate pregnancy, P_4, and/or IFNT-regulated genes in the ovine uterine endometrium, confirmation of cell-specific and temporal changes in their expression in the ovine endometrium during early pregnancy and in response to P_4 and IFNT has not been reported. Therefore, these studies were conducted to validate regulation of the candidate genes by pregnancy, P_4, and/or IFNT and, if feasible, ascertain their biological function using ovine trophectoderm (oTr) cells.

Results

In situ *hybridization analysis of uteri from cyclic and pregnant ewes (experiment 1)*

In situ hybridization detected *S100A2* mRNA predominantly in uterine LE and GE as well as the conceptus. *S100A2* mRNA was present in both conceptus Tr and endoderm (Fig. 1). In cyclic ewes, *S100A2* mRNA increased between days 10 and 16. In pregnant ewes, *S100A2* mRNA was low on days 10 and 12, increased on days 14 and 16, and decreased on days 18 and 20.

POSTN mRNA was expressed predominantly in the stratum compactum stroma of intercaruncular and caruncular endometrium, but not in LE or GE, and not in conceptuses on days 18 and 20 (Fig. 1). In cyclic ewes, *POSTN* mRNA was low on day 10, increased on day 12, and was undetectable by day 14. In pregnant ewes, *POSTN* mRNA was detected constitutively in the stroma and was particularly abundant in caruncular stroma.

COL3A1 mRNA was detected in the intercaruncular stroma and caruncles of the ovine endometrium and endoderm of conceptuses (Fig. 2). In cyclic ewes, *COL3A1* mRNA decreased from days 10 to 14 and then increased substantially by day 16. In pregnant ewes, *COL3A1* mRNA was low in uterine stroma between days 10 and 20. However, abundant *COL3A1* mRNA was detected in caruncular stroma and endoderm of conceptuses on days 18 and 20 of pregnancy. Similar temporal and spatial alterations in *SPARC* mRNA in uteri were observed during the estrous cycle and early pregnancy of ewes (Fig. 2). *SPARC* mRNA was also abundant in the endoderm of conceptuses.

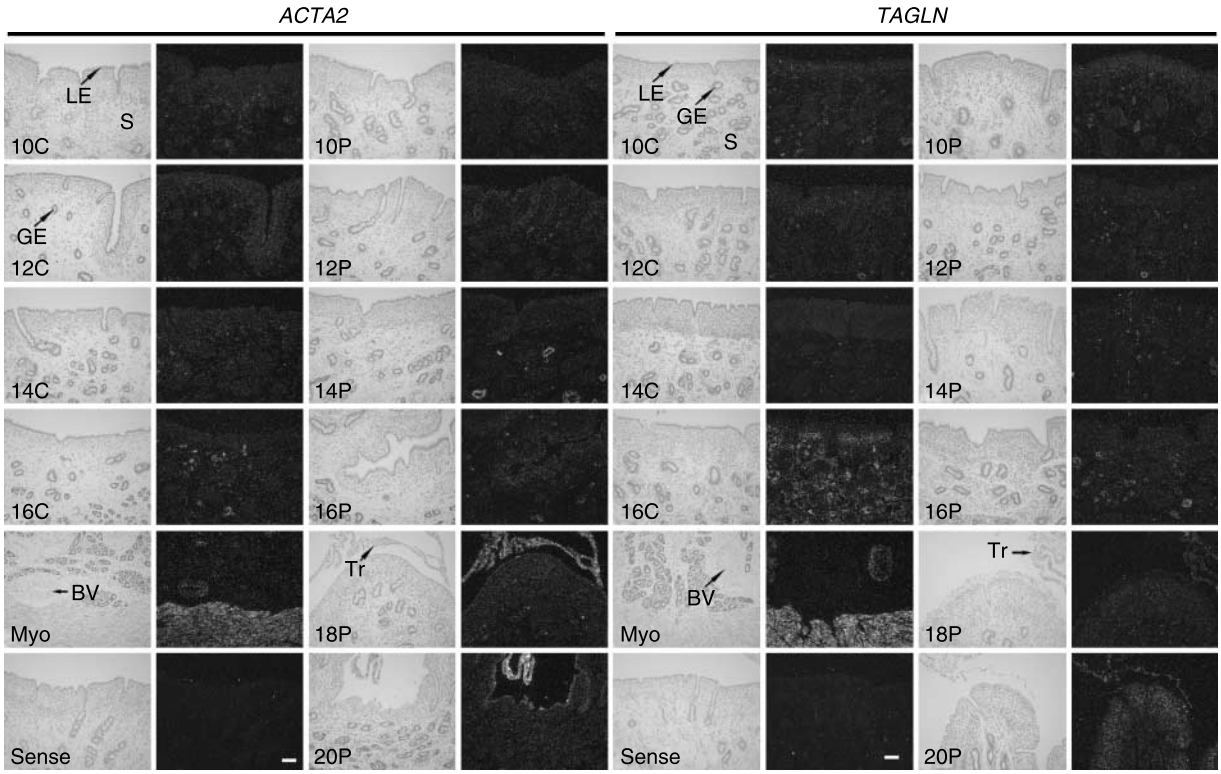

Figure 3 *In situ* localization of *ACTA2* and *TAGLN* mRNAs in the cyclic (C) and pregnant (P) ovine endometria. Cross sections of uteri were hybridized with radiolabeled antisense or sense ovine *ACTA2* and *TAGLN* cRNA probes. *ACTA2* mRNA was localized in stroma, myometrium, and conceptus on days 18 and 20. *TAGLN* mRNA is localized in stroma and myometrium. Bright-field photomicrographs (left) and dark-field photomicrographs (right) were taken for each gene. LE, luminal epithelium; GE, glandular epithelium; S, stroma; M, myometrium; BV, blood vessel; Tr, trophectoderm. Scale bar represents 10 μm.

ACTA2 and *TAGLN* mRNAs were low to undetectable in most cells of the endometrium, except for blood vessels in both cyclic and pregnant ewes (Fig. 3). However, *TAGLN* mRNA increased in the stroma of day 16 cyclic ewes. Abundant *ACTA2* and *TAGLN* mRNAs were detected in the myometrium of both cyclic and pregnant ovine uteri. *ACTA2*, but not *TAGLN* mRNA was observed in conceptus Tr on days 18 and 20 of pregnancy.

STAT5A mRNA was not detectable in uteri of either cyclic or pregnant ewes (data not shown).

Steady-state levels on S100A2 and POSTN mRNAs in endometria from cyclic and pregnant ewes (experiment 1)

The temporal and cell-specific changes in mRNA localization in uteri of cyclic and pregnant ewes, as well as the abundance of their mRNAs, were such that *S100A2* and *POSTN* were subjected to further study with respect to regulation by pregnancy, P_4, and/or IFNT. Steady-state levels of *S100A2* and *POSTN* mRNAs in endometria from cyclic and pregnant ewes are illustrated in Fig. 4. The abundance of *S100A2* mRNA in the endometrium was affected by day ($P<0.05$) and pregnancy status ($P<0.05$). In cyclic ewes, *S100A2* mRNA increased nearly fivefold between days 12 and 16 (linear effect of day, $P<0.01$). In pregnant ewes, *S100A2* mRNA increased between days 12 and 14 and then decreased to day 20 (quadratic effect of day, $P<0.05$).

As illustrated in Fig. 4, endometrial *POSTN* mRNA levels were affected by day ($P<0.01$) and day ×pregnancy status interaction ($P<0.05$). In cyclic ewes, *POSTN* mRNA was high on days 10 and 12 and then decreased to day 16 (linear effect of day, $P<0.05$). In pregnant ewes, *POSTN* mRNA abundance increased from days 10 to 12 and remained elevated to day 20 (quadratic effect of day, $P<0.05$).

Effects of P_4 and IFNT on S100A2 and POSTN mRNA expression (experiment 2)

The effects of P_4 and IFNT on expression of *S100A2* and *POSTN* were determined in experiment 2. As shown in Fig. 5A, intrauterine injections of IFNT decreased steady-state levels of *S100A2* mRNA about 2.5-fold in ewes treated with P_4 (P_4+CX versus P_4+IFN, $P<0.05$); however, there was no effect of the antiprogestin ZK 136317 in ewes receiving CX proteins (P_4+CX versus

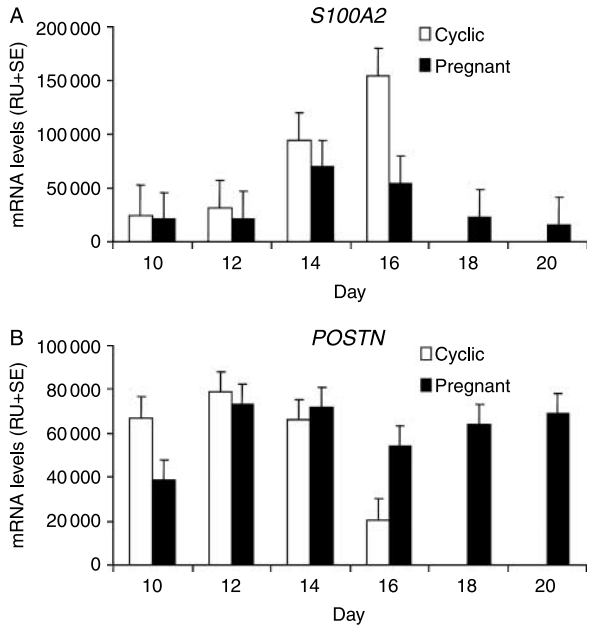

Figure 4 Steady-state levels of *S100A2* (A) and *POSTN* (B) mRNAs in endometria from cyclic and early pregnant ewes as determined by slot blot hybridization analysis. (A) *S100A2* mRNA increased from day 12 to day 16 ($P<0.01$) in cyclic ewes. In pregnant ewes, *S100A2* mRNA increased from day 12 to day 14 ($P<0.01$) and decreased to day 20 ($P<0.05$). (B) In cyclic ewes, *POSTN* mRNA was high in day 12, then decreased till day 16 ($P<0.05$). In pregnant ewes, *POSTN* mRNA was increased on day 12, decreased till day 16 ($P<0.05$), and maintained thereafter. Data are presented as least-square mean-relative units (RU) with S.E.M.

P_4+ZK+CX, $P>0.10$). Interestingly, treatment of ewes with the ZK 136317 prevented inhibitory effects of IFNT (P_4+ZK+CX versus P_4+ZK+IFN, $P>0.10$). *In situ* hybridization results (Fig. 5B) confirmed that *S100A2* mRNA was reduced in LE and superficial GE of P_4 +IFNT-treated ewes.

Treatment of ewes with P_4 increased *POSTN* mRNA levels by about 4.8-fold in the endometrium (P_4+CX versus P_4+IFN, $P=0.001$), particularly in the stratum compactum stroma (Fig. 5A and B). Intrauterine infusion of IFNT did not affect ($P>0.10$) endometrial *POSTN* mRNA levels in either P_4 or P_4+ZK-treated ewes.

Immunoreactive POSTN protein

POSTN protein was observed in the stratum compactum stroma adjacent to GE and in caruncles of ovine endometria (Fig. 6A). In the stratum compactum stroma, POSTN protein was localized to either subepithelial area adjacent to LE or areas proximal to the stratum spongiosum stroma. Changes in abundance of POSTN protein during the estrous cycle and early pregnancy are summarized in Table 2. In cyclic ewes, POSTN protein increased on day 12 and was maintained to day 16. In pregnant ewes, POSTN protein increased on day 12, decreased to day 16, and then increased again to day 20. Less POSTN protein was detected in pregnant than cyclic ewes on day 16. On days 16–20 of pregnancy in intercaruncular areas and along the sides of concave caruncles, abundant POSTN protein was observed just below the LE (Fig. 6B). In the caruncles, focal accumulation of POSTN protein on days 18 and 20 was observed. A 90 kDa form of immunoreactive POSTN protein was detected in uterine flushings of pregnant ewes (Fig. 6C). In fact, POSTN protein was consistently observed in the uterine flush of pregnant but not cyclic ewes, and the amount of POSTN protein was most abundant on days 14 and 16 of pregnancy.

Effect of POSTN on attachment and migration of trophectoderm cells

POSTN protein promotes cell adhesion and motility via integrin αvβ3 and αvβ5 subunits (Gillan *et al.* 2002). Therefore, attachment and migration analyses were conducted to test the hypothesis that POSTN has attachment and migration functions on oTr cells (Fig. 7). In these assays, recombinant human (rh) POSTN mediated the attachment of oTr cells similar to that observed for bovine fibronectin (FN), which was used as a positive control (Fig. 7A). Moreover, the attachment function mediated by POSTN was greater ($P<0.01$) when culture wells were coated with 100 ng as compared with 10 ng POSTN protein.

As shown in Fig. 7B, POSTN also stimulated ($P<0.01$) the migration of oTr cells, although lower migration of oTr cells was observed when cells were treated with 100 ng POSTN as compared with 10 ng POSTN.

Discussion

The endometrium undergoes dynamic changes with autocrine and paracrine factors acting in a cell-specific and temporal manner in response to complex regulation by hormones and their relevant receptors signaling via various pathways during the estrous cycle and early pregnancy. Therefore, it is necessary to corroborate microarray data through studies of gene expression by cells of the endometrium during various physiological states, e.g. day of pregnancy or the oestrous cycle. In complex tissues like uterine endometria, localization of mRNA by *in situ* hybridization is essential to appreciate temporal and cell-specific changes in gene expression that provide insight into mechanisms regulating functions of gene products within complex epitheliomesenchymal tissues (White & Salamonsen 2005). In addition, laser capture microdissection enables isolation of specific cells of the endometrium, which have remarkably different phenotypes, such as LE, GE, and stromal cells, to identify genes uniquely expressed

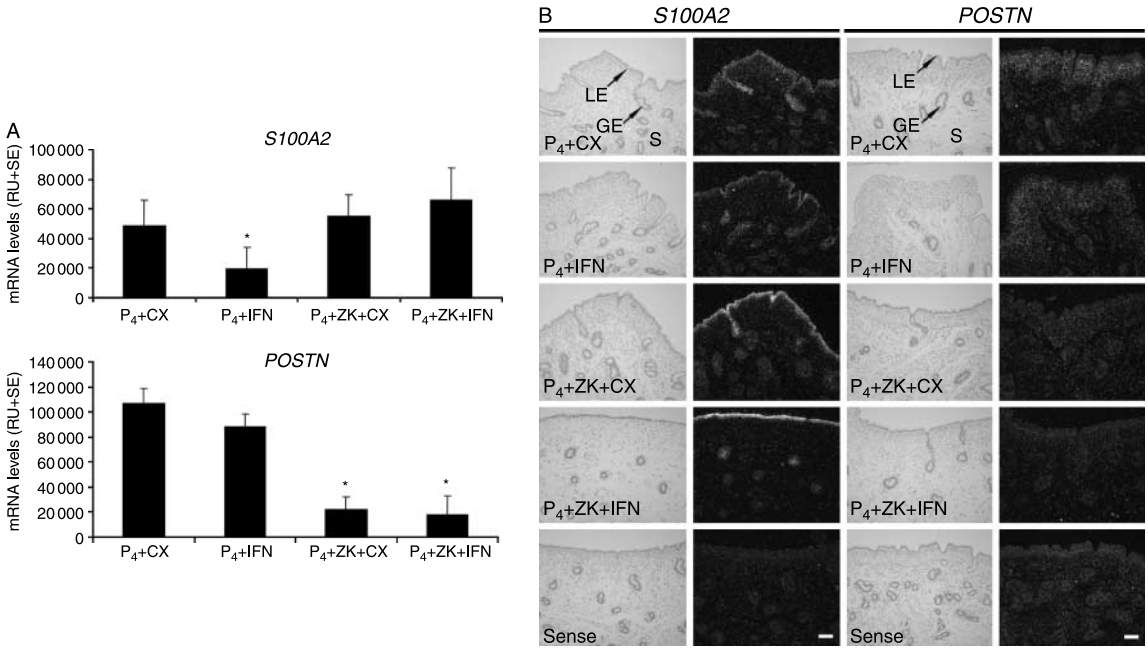

Figure 5 Steady-state levels of *S100A2* and *POSTN* mRNAs in endometria. Intrauterine infusion of IFNT decreased *S100A2* mRNA in the presence of P_4 (P_4+CX versus P_4+IFN, $P<0.05$) but not in P_4+ZK-treated ewes (P_4+ZK+CX versus P_4+ZK+IFN, $P>0.10$). The PGR antagonist ZK reduced *POSTN* mRNA (P_4+CX versus P_4+ZK+CX, $P>0.05$), whereas IFNT did not affect *POSTN* mRNA levels (P_4+CX versus P_4+IFN, $P>0.10$). (B) *In situ* localization of *S100A2* and *POSTN* mRNAs in pregnant endometria from pregnant ewes in study 2. *S100A2* mRNA tended to be less abundant in P_4+IFN-treated ewes compared with ewes from other treatment groups. P_4 increased *POSTN* mRNA in the stroma (primarily stratum compactum stroma) of P_4-treated ewes (P_4+CX and P_4+IFN). The asterisk (*) denotes an effect of treatment. Data are presented as least-square mean-relative units (RU) with S.E.M. LE, luminal epithelium; GE, glandular epithelium; S, stroma. Scale bar represents 10 μm.

by each during the estrous cycle and peri-implantation period as reported for studies using mice (Niklaus & Pollard 2006). Indeed, gene profiling using microarray analyses must extend to proteomic and functional genomics to understand the ultimate roles of genes in organ systems (Horcajadas *et al*. 2007).

Based on microarray analysis, *ACTA2* was predicted to be down-regulated by pregnancy and *TAGLN* was expected to be up-regulated by IFNT (Gray *et al*. 2006). *In situ* hybridization performed in human and monkey myometrium revealed that *ACTA2* is the primary type of actin in the myometrium (Cavaille 1985, Cavaille *et al*. 1986). These reports as well as Shynlova *et al*. (2005) found expression of *ACTA2* in uterine myometrium. Results of the present study indicated that *ACTA2* and *TAGLN* mRNAs were not detected in uterine LE, GE, or stromal cells of the ovine endometrium; however, both *ACTA2* and *TAGLN* mRNAs were observed in the conceptus Tr and blood vessels of the endometrium. The absence of changes in these mRNAs in either the myometrium or blood vessels of ovine uterine endometrium was unexpected as they are increased during early pregnancy in primates (Christensen *et al*. 1995). Studies performed in the rat also show an alteration in γ-actin mRNA expression in the myometrium of rat uterus (Shynlova *et al*. 2005). Results of the present study did not confirm effects of P_4 and/or IFNT on expression of *ACTA2* or *TAGLN* in ovine endometria during early pregnancy. ACTA2 is a marker of decidualization-like changes in stromal cells, as are secreted phosphoprotein 1 (SPP1, also known as osteopontin) and desmin (Johnson *et al*. 2003*b*). In the ovine uterus, *ACTA2* mRNA is abundant in stromal cells of intercaruncular and caruncular endometria of ewes from days 25 to 120 of pregnancy, but not during the estrous cycle or earlier stages of pregnancy (Johnson *et al*. 2003*b*), which is consistent with results of the present study.

TAGLN is a marker for smooth muscle cells and smooth muscle-containing tissues including the uterus (Lees-Miller *et al*. 1987, Gimona *et al*. 1992, Duband *et al*. 1993, Shanahan *et al*. 1993). In the present study, *TAGLN* mRNA was expressed in a similar manner as *ACTA2* mRNA in myometrium, stroma, and blood vessels, as well as conceptus Tr and endometrial stroma on day 20 of pregnancy. *TAGLN* is a repressor of proteolytic activity of matrix metallopeptidase 9 (MMP9; Nair *et al*. 2006), which plays a role in remodeling of uterine endometrial tissue. In uteri of mice, *Mmp9* is regulated by estrogen (Zhang *et al*. 2007) and its expression may be regulated by estrogen in ewes, as *TAGLN* was most abundant on day 16 of the estrous cycle when concentrations of estrogen and endometrial

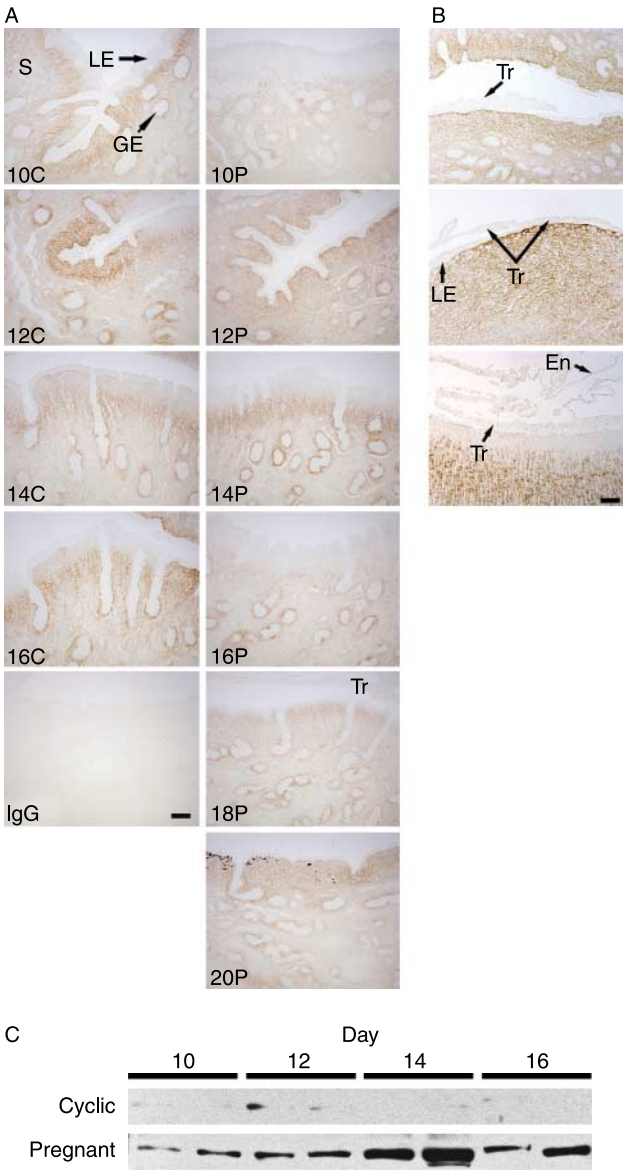

Figure 6 POSTN protein in the ovine uterus. (A) Immunolocalization of POSTN protein in cyclic (C) and pregnant (P) ovine endometria. Immunoreactive proteins were detected using specific rabbit polyclonal antibody against POSTN protein. For the IgG control, normal rabbit IgG was substituted for the primary antibody. POSTN protein was localized in the stroma, area adjacent to GE, and caruncle. POSTN protein increased on day 12 in both cyclic and pregnant ewes and maintained in cyclic ewes, but decreased in pregnant ewes. LE, luminal epithelium; GE, glandular epithelium; S, stroma; Tr, trophectoderm. Scale bar represents 10 μm. (B) Representative photomicrographs of immunoreactive POSTN protein in the pregnant endometria and caruncles from days 16 to 20. LE, luminal epithelium; Tr, trophectoderm; En, endoderm. Scale bar represents 20 μm. (C) Representative western blot analysis of POSTN protein in uterine flushings from cyclic and pregnant ewes. Proteins in uterine flushings were separated by 10% SDS-PAGE (40 μg/lane), and immunoreactive POSTN protein was detected using specific rabbit polyclonal antibody. An immunoreactive protein of ~90 kDa POSTN was detected primarily in uterine flush of pregnant ewes.

expression of ESR1 are increasing (see Fig. 3). ProMMPs-1, -2, and -3 are secreted by cultured ovine endometrial stromal but not epithelial cells, and expression of proMMP-1 and proMMP-3 is inhibited by IFNT, and TIMP metallopeptidase inhibitor 1 (TIMP1) and TIMP2 are most abundant in ovine endometrium between days 12 and 20 (Salamonsen *et al.* 1995). Those authors suggested that the TIMPs play a role in preventing trophoblast invasion as well as modulating endometrial remodeling associated with early placentation. Other potential proteases and protease inhibitors having potential roles in implantation and regulated by P_4 or IFNT include cathepsin L (CTSL1) and cystatin C (CST3; Song *et al.* 2006, 2007). The inability of the present studies to confirm our previous microarray analysis identifying *ACTA2* and *TAGLN* as pregnancy and/or IFNT-regulated genes could be due to the abundance of these mRNAs in the myometrium and potential contamination of endometrium with myometrium due to the physical method of isolating the endometrium at surgery (Gray *et al.* 2006). One solution to this problem would be isolation of endometrial cell types by laser capture microdissection.

Our previous microarray analysis indicated that *COL3A1* and *SPARC* were down-regulated by IFNT and pregnancy (Gray *et al.* 2006). Results of the present study confirmed that *COL3A1* mRNA was down-regulated during pregnancy, but not affected by P_4 or IFNT. *COL3A1* mRNA was abundant during proestrus (day 16 cycle), suggesting that estrogen up-regulates *COL3A1* expression in ovine uterine stromal cells. This is consistent with estrogen stimulation of expression of collagen types I, III, and V in rat uteri (Frankel *et al.* 1988) and collagen types I, III, and VI in uterine stromal cells of postmenopausal women (Iwahashi *et al.* 1997). *COL3A1* is the collagen type that increases tensile strength (van der Rest & Garrone 1991, Burgeson & Nimni 1992, Aumailley & Gayraud 1998). A reduction in collagen fibrils surrounding endometrial stromal cells during early pregnancy in mice (Finn 1971) may be related to decreases in junctional complexes to enhance paracellular transport of molecules to and from the conceptus. Junctional proteins in the LE decrease from day 12 in uteri of both cyclic and pregnant ewes, making a potentially 'leaky' epithelium to facilitate transport of molecules from the uterine stroma and/or selective movement of serum transudate directly to the uterine lumen and conceptus (Satterfield *et al.* 2007).

SPARC, a matricellular protein, is not a component of the ECM, but it modulates cell–matrix interactions and cell function (Bornstein & Sage 2002) as an upstream regulator of several collagens including COL3A1 (Zhou *et al.* 2006). In the present study, temporal and cell-specific changes in expression of *SPARC* and *COL3A1* were similar (see Fig. 2), and both *COL3A1* and *SPARC* were particularly abundant in conceptus Tr. In mice, *Col3a1* and *Sparc* are

Table 2 Distribution and relative abundance of immunoreactive POSTN protein in the cyclic and pregnant ewes in study 1[a].

		Endometrium							
		Intercaruncular					Caruncular		
PND	**Status**	LE	GE, shallow	GE, deep	Stroma, compactum	Stroma, spongiosum	LE	Stroma	**Myometrium**
10	C	−	−	−	++/+++	+	−	+/++	−
12	C	−	−	−	+++	+	−	+	−
14	C	−	−	−	++	+	−	++	−
16	C	−	−	−	++/+++	+	−	ND	−
10	P	−	−	−	−/+	+	−	+	−
12	P	−	−	−	++/+++	+	−	++	−
14	P	−	−	−	++/+++	+	−	−	−
16	P	−	−	−	−/+	+	−	++	−
18	P	−	−	−	++	+	−	++/+++	−
20	P	−	−	−	++/+++	+	−	++/+++	−

ND, not determined.
[a]Protein staining intensity was evaluated visually as absent (−), weak (+), moderate (++), or strong (+++).

co-ordinately down-regulated in IL11 receptor alpha (*Il11ra*) null mice, which are unable to undergo normal uterine decidualization (White *et al.* 2004). Collectively, *SPARC* and *COL3A1* may have co-operative roles during early pregnancy through effects on uterine stromal cells and conceptus Tr in sheep, which, based on the literature, might influence stromal remodeling (White *et al.* 2004) and participation of endothelial cells involved in angiogenesis (Germain 2002).

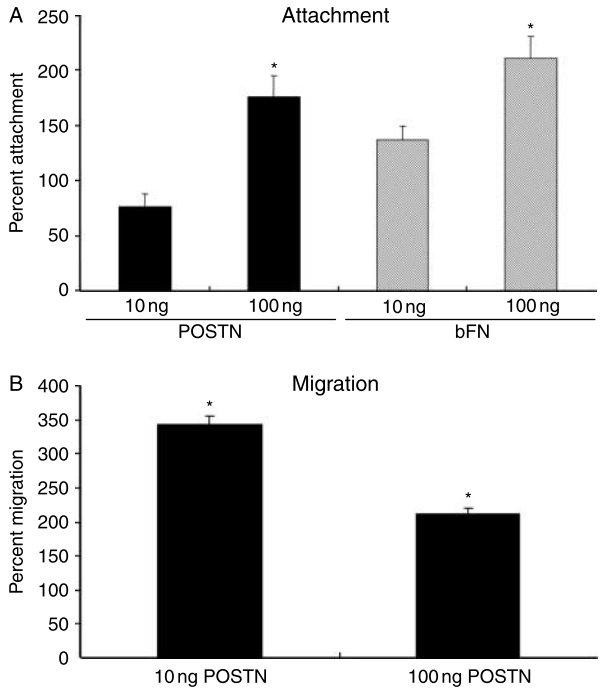

Figure 7 Effects of POSTN on attachment and migration of ovine trophectoderm cells. For attachment (A), wells of suspension culture plates were precoated overnight with either BSA (negative control), bovine FN (positive control), or recombinant human POSTN. Equal numbers of cells were added to each well, and the number of attached cells was determined after 1.5 h of incubation. For migration (B), cells were seeded in a transwell plate in serum- and insulin-free medium and treated with recombinant human POSTN or BSA as a control. Data are presented as percent increase in attachment or migration relative to negative control BSA treatment. POSTN protein mediated attachment and stimulated migration of oTrF cells.

S100A2 was predicted to be down-regulated by IFNT (Gray *et al.* 2006), and results of the present study confirmed that *S100A2* mRNA is down-regulated by pregnancy (Figs 3 and 4A) and IFNT (Fig. 5A). However, in the absence of functional PGR, IFNT did not decrease *S100A2* mRNA, suggesting that IFNT regulation of *S100A2* is P_4 dependent. It has been reported that the *S100A2* gene promoter lacks IFN-responsive elements; however, co-stimulation of human melanoma cells with IFNA and transforming growth factor-β1 (TGFB1) induced *S100A2* expression in human melanoma cells (Foser *et al.* 2006). S100A2 may act as a modulator against excess calcium accumulation in endometrial epithelial cells. *S100A2* mRNA was also observed in the conceptus, particularly in the endoderm. In the conceptus and perhaps endometrial epithelia, S100A2 may be involved in cell cycle progression and differentiation (Wicki *et al.* 1997).

POSTN, a secreted ECM protein in osteoblasts (Takeshita *et al.* 1993), shares homology with fasciclin I, which is a cell adhesion protein that guides development of the growth cone in insects (Elkins *et al.* 1990). *POSTN* also increases cell migration, invasion, and adhesion in tumorigenic 293T cells (Yan & Shao 2006) and mediates ovarian epithelial cell adhesion and motility via αvβ3 and αvβ5 integrins (Gillan *et al.* 2002). During the peri-implantation period of pregnancy in sheep, integrin subunits α (v,4,5) and β (1,3,5) are constitutively expressed on apical surfaces of both conceptus Tr and endometrial LE (Johnson *et al.* 2001). Thus, functional activation of integrins on both Tr and LE must be mediated by specific integrin-binding proteins (Burghardt *et al.* 2002). *POSTN* is expressed in uteri, placentae, and mammary glands of humans (Hiroi *et al.* 2008), and *Postn* null mice exhibit postnatal lethality and severe growth retardation (Rios *et al.* 2005). *POSTN* was predicted to be up-regulated by P_4 and pregnancy, but

down-regulated by IFNT (Gray *et al*. 2006). Results of the present study confirmed that *POSTN* expression is regulated by P_4 and pregnancy, but *POSTN* was not regulated by IFNT. As an ECM component, *POSTN* mRNA was localized in the intercaruncular and caruncular stratum compactum stroma, and POSTN protein was localized to the apical side of stratum compactum stroma and site of conceptus attachment on days 18 and 20 of pregnancy, but absent in the conceptus. Functional studies demonstrated that POSTN protein mediated attachment of oTr cells *in vitro*, which is undoubtedly important for the rapid elongation of the conceptus Tr observed from days 12 to 19 of early pregnancy in sheep (Wales & Cuneo 1989).

In human endometrium, the abundance of POSTN protein in both stromal and epithelial cells is affected by stage of the menstrual cycle and pregnancy (Hiroi *et al*. 2008). POSTN contains a FAS-binding domain, which enables it to interact with integrins (Kim *et al*. 2000), enhance effects of growth factors like fibroblast growth factor 2 (FGF2), and stimulate vascular smooth muscle cells. Interestingly, FGF2 is expressed in the endometrium of the ovine uterus (Ocón-Grove *et al*. 2008) and stimulates IFNT production by bovine Tr cells (Cooke *et al*. 2009). The effects of *POSTN* can be inhibited by silencing the phosphatidylinositol-3-kinase (PI3K)/AKT cell-signaling pathway (Li *et al*. 2006), which is present in oTr (Kim *et al*. 2008). Thus, POSTN may induce PI3K/AKT signaling to stimulate attachment and/or migration of oTr cells as POSTN is present in the uterine lumen, where it theoretically binds ECM and/or integrin subunits on conceptus Tr and influences elongation. POSTN was abundant in ovine caruncles on days 18 and 20 of pregnancy and it is expressed by human and mouse placentae (Gillan *et al*. 2002, Rios *et al*. 2005). Therefore, POSTN may influence placentation and/or placental functions throughout pregnancy.

Collectively, results from the present study confirm that expression of *S100A2* and *POSTN* is regulated by P_4 and IFNT in the ovine uterine endometrium. Of particular note, *POSTN* was found to be expressed by stromal cells and stimulated attachment of oTr cells similar to galectin 15 (LGALS15) and SPP1 (Johnson *et al*. 2003*a*, Farmer *et al*. 2008). Future studies will be directed towards identification of biological effects and signaling pathways involved in conceptus Tr response to endometrial-derived POSTN.

Materials and Methods

Animals

Mature crossbred Suffolk ewes (*Ovis aries*) were observed daily for oestrus 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, ewes were assigned randomly to cyclic or pregnant status. All experimental and surgical procedures were in agreement with the Guide for the Care and Use of Agriculture Animals in Research and Teaching and approved by the Institutional Animal Care and Use Committee of Texas A&M University, USA.

Experimental design

Experiment 1

At estrus (day 0), ewes were mated to an intact 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. On days 10–16 of pregnancy, the uterine lumen was flushed with 20 ml sterile 10 mM Tris–HCl (pH 8.0), and pregnancy was confirmed by recovery of one or more morphologically normal conceptus(es). Uterine flushings were not obtained from either days 18 or 20 of pregnancy, because the conceptuses are firmly adhered to the endometrial LE and basal lamina (Guillomot *et al*. 1981). Uterine flushings were clarified by centrifugation (3000 ***g*** for 30 min at 4 °C) and frozen at −80 °C until analysis. At hysterectomy, several sections (~0.5 cm) from the midportion of each uterine horn ipsilateral to the corpus luteum were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2). After 24 h, fixed tissues were changed to 70% ethanol for 24 h and then embedded in Paraplast Plus (Oxford Labware, St Louis, MO, USA) for *in situ* hybridization and immunohistochemical analyses. 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 corpus luteum. No tissues from the contralateral uterine horn were used for study.

Experiment 2

As described previously (Gray *et al*. 2006), cyclic crossbred ewes were checked daily for oestrus and then ovariectomized and fitted with indwelling uterine catheters on day 5. Ewes were assigned randomly ($n=5$ per treatment) to daily treatments as follows: 1) 50 mg P_4 from days 5 to 16 and intrauterine serum proteins (200 μg) as a control (CX) from days 11 to 16 (P_4+CX); 2) P_4 and 75 mg of the antiprogestin ZK 137316 (Schering AG, Berlin, Germany; Klein-Hitpass *et al*. 1991) from days 11 to 16 and intrauterine control proteins (P_4+ZK+CX); 3) P_4 and intrauterine IFNT (2×10^7 antiviral units) from days 11 to 16 (P_4+IFN); or 4) P_4 and ZK and IFNT (P_4+ZK+IFN). The P_4 and ZK 137316 were dissolved in corn oil vehicle and administered to ewes by i.m. injection. Recombinant ovine IFNT was produced in *Pichia pastoris* and purified (Van Heeke *et al*. 1996) and prepared for intrauterine injection as described previously (Spencer *et al*. 1999*b*). Each uterine horn of each ewe received twice-daily intrauterine injections of either control serum proteins (50 μg/horn per injection) or recombinant ovine IFNT (5×10^6 antiviral units/horn per injection). This regimen mimics the effect of P_4 from CL and IFNT from the conceptus on endometrial expression of hormone receptors, as well as P_4-induced and ISGs during

Table 3 Sequences of primers used for RT-PCR and cloning.

Gene	Sequence (5′–3′): forward and reverse	GenBank	Annealing temperature (°C)	Product size (bp)
ACTA2	GTGTGTGACAATGGCTCTGG TCTCAAAGTCCAGGGCTACG	BC102699	55.1	649
COL3A1	GAAAGCCTTGAAGCTGATGG TGCTCTGAAAATGGGCTAGG	L47641	54.9	552
POSTN	CCATCTGTGGACAGAAAACG CACCATTTGTTGCAATCTGG	D13666	55	465
S100A2	AGAGGGCGACAAGTTCAAGC ATGAGGGCCAGGAAGACG	BC102570	56.1	192
SPARC	GGCCTGGATCTTCTTTCTCC AGTCCAGGTGGAGTTTGTGG	J03233	55	428
STAT5A	TGACCTGCTCATCAACAAGC AATAGGACCCTGCACACTGG	NM_001012673	54.9	634
TAGLN	GTCCTTCCTATGGCATGAGC CTGTAGGCCGATGACATGC	NM_001046149	54.6	521

early pregnancy in ewes (Song *et al.* 2006). All ewes were hysterectomized on day 17. Uteri were processed for histology and the endometria obtained for RNA extraction as described for experiment 1.

RNA isolation

Total cellular RNA was isolated from frozen samples of endometrium using Trizol reagent (Life Technologies, Inc.) 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 cDNA

Partial cDNAs for ovine *ACTA2*, *COL3A1*, *POSTN*, *S100A2*, *SPARC*, *STAT5A*, and *TAGLN* were generated by RT-PCR using total RNA from day 16 or 18 pregnant ovine endometrium-specific primers and annealing temperatures summarized in Table 3 and methods described previously (Hayashi *et al.* 2007). PCR products of the predicted size were cloned into pCRII using a T/A cloning kit (Invitrogen), and their sequences verified by sequencing using an ABI PRISM Dye Terminator Cycle Sequencing Kit and ABI PRISM automated DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA).

Slot blot hybridization analyses

Steady-state levels of mRNA in ovine endometria were quantified using slot blot hybridization as described previously (Song *et al.* 2007). Briefly, radiolabeled antisense and sense cRNA probes were generated by *in vitro* transcription using [α-^{32}P] UTP. Denatured total endometrial RNA (20 μg) from each ewe was hybridized with radiolabeled cRNA probes. To correct for variation in total RNA loading, a duplicate membrane was hybridized with radiolabeled antisense 18S cRNA (pT718S; Ambion, Austin, TX, USA). After washing, blots were digested with RNase A and radioactivity associated with slots quantified using a Typhoon 8600 MultiImager (Molecular Dynamics, Piscataway, NJ, USA). Data are expressed as relative units.

In situ *hybridization analyses*

Cell-specific localization of mRNAs in sections (5 μm) of the ovine uterus was determined by radioactive *in situ* hybridization analysis as described previously (Spencer *et al.* 1999*b*, Choi *et al.* 2001). Briefly, radiolabeled antisense and sense cRNA probes were generated by *in vitro* transcription using linearized plasmid cDNA templates, RNA polymerases, and [α-^{35}S] UTP. Deparaffinized, rehydrated, and deproteinated uterine tissue sections were hybridized with radiolabeled antisense or sense cRNA probes. After hybridization, washing, and RNase A digestion, slides were dipped in NTB-2 liquid photographic emulsion (Kodak) and exposed at 4 °C for 3 days to 4 weeks. Slides were developed in Kodak D-19 developer (Kodak), counterstained with Gill's hematoxylin (Fisher Scientific, Pittsburgh, PA, USA), and then dehydrated through a graded series of alcohol to xylene. Coverslips were then affixed with Permount (Fisher Scientific).

Immunohistochemistry

Immunolocalization of POSTN was performed using sections (5 μm) of the ovine uteri and methods described previously (Spencer *et al.* 1999*a*). Vectastain Elite ABC rabbit IgG Kit (PK-6101; Vector Laboratories, Burlingame, CA, USA) and rabbit polyclonal antibody specific to POSTN protein (ab14041, Abcam Inc., Cambridge, MA, USA) at a final concentration of 0.5 μg/ml were used. Negative controls were performed in which the primary antibody was substituted with the same concentration of normal rabbit IgG (Sigma). Multiple tissue sections from each ewe were processed as sets within an experiment. Immunoreactive proteins were visualized using diaminobenzidine tetrahydrochloride (Sigma). Sections were subsequently dehydrated and coverslips were affixed with Permount (Fisher Scientific).

Western blot analyses

Concentrations of protein in uterine flushings were determined using the Bradford protein assay (Bio-Rad Laboratories) with BSA as the standard. Proteins were denatured and separated by

10% SDS-PAGE and western blot analysis conducted as described previously (Spencer *et al.* 1999*a*). Rabbit polyclonal POSTN antibody (cat no. ab14041, Abcam) was used at 1 μg/ml final concentration. Blots were washed, and placed in affinity-purified antibody peroxidase-labeled goat anti-rabbit IgG (H+L) (1:10 000 dilution of 1 mg/ml stock; cat no. 474-1506, KPL Inc., Gaithersburg, MD, USA). Immunoreactive POSTN protein was detected using ECL (SuperSignal West Pico, Pierce, Rockford, IL, USA) and X-OMAT AR film (Kodak).

Attachment assay

oTr cells were isolated from day 15 ovine conceptuses and cultured *in vitro* as described previously (Farmer *et al.* 2008). Attachment assays were conducted using oTrF cells as described previously (Farmer *et al.* 2008). Cell suspension plates with 24 wells (Greiner Multiwell Tissue Culture Plates, PGC Scientific Co., Monroe, NC, USA) were coated with either BSA (BSA Fraction V, Pierce) as a negative control, bovine FN (0.1% (w/v) solution from bovine plasma, Sigma) as a positive control as described previously (Farmer *et al.* 2008), or rh POSTN protein (R&D Systems, Minneapolis, MN, USA) in triplicate and allowed to dry overnight in a sterile hood at room temperature. Wells were then blocked with 1 ml BSA (10 mg/ml) in PBS for 1 h, and each was rinsed three times with 1 ml serum and insulin-free DMEM. Equal numbers of freshly trypsinized oTrF cells were seeded into each well and plates incubated for 1.5 h. Wells were washed three times with 1 ml serum- and insulin-free medium to remove unattached cells. Cell numbers were determined using a Janus Green assay as described previously (Farmer *et al.* 2008). The entire experiment was repeated at least three times with different passages of oTrF cells.

Trophectoderm cell migration assay

Migration assays were conducted with oTrF cells as described previously (Farmer *et al.* 2008). Briefly, oTrF cells (50 000 cells per 100 μl serum and insulin-free DMEM) were seeded on 8 μm pore Transwell inserts (Corning Costar #3422, Corning, Lowell, MA, USA). Treatments were then added to each well ($n=3$ wells per treatment) that included combinations of: a) serum and insulin-free DMEM (600 μl); b) rh POSTN protein (R&D Systems) at 10 or 100 ng/ml; c) BSA (BSA Fraction V, Pierce) at 10 or 100 ng/ml; and d) trophoblast growth medium including serum and insulin as a positive control. After 12 h, cells on the upper side of the inserts were removed with a cotton swab. For evaluation of cells that migrated onto the lower surface, inserts were fixed in 50% ethanol for 5 min. The transwell membranes were then removed, placed on a glass slide with the side containing cells facing up, overlaid with Prolong antifade mounting reagent with DAPI (Invitrogen–Molecular Probes, Eugene, OR, USA), and overlaid with a coverslip. The migrated cells were systematically counted using a Zeiss Axioplan 2 fluorescence microscope with Axiocam HR digital camera and Axiovision 4.3 software (Carl Zeiss Microimaging, Thornwood, NY, USA). The entire experiment was repeated at least three times with different passages of oTrF cells.

Statistical analyses

Data from all quantitative studies were subjected to least-squares ANOVA using the general linear models' procedures of the Statistical Analysis System (SAS Institute, Cary, NC, USA). Slot blot hybridization data were corrected for differences in sample loading by using the 18S rRNA data as a covariate. Data are presented as least-squares means with overall S.E.M. A P value of 0.05 or less was considered significant.

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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