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The IGF system in the neonatal ovine uterus

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Abstract

Postnatal development of the ovine uterus primarily involves uterine gland morphogenesis or adenogenesis. Adenogenesis involves the budding differentiation of the glandular epithelium (GE) from the luminal epithelium (LE) and then GE proliferation and coiling/branching morphogenetic development within the stroma between birth (postnatal day or PND 0) and PND 56. Insulin-like growth factor (IGF)-I and IGF-II mRNAs were previously found to be expressed only in the endometrial stroma, whereas the IGF receptor (IGF-1R) mRNA was most abundant in epithelia and in stroma, suggesting that an intrinsic IGF system regulates postnatal development of the uterus. Given that the biological activities of IGFs are modulated by a family of six IGF binding proteins (IGFBPs) and specific proteases, the objective was to determine the effects of age and estrogen disruption on expression of IGFs, IGFBPs and pregnancy-associated plasma protein A (PAPP-A or IGFBP-4 protease) in the ovine uterus. In Study One, circulating levels of IGF-I and IGF-II in the serum of neonatal ewes did not change between PND 0 and PND 56. Levels of immunoreactive IGF-I, IGF-II and IGF-1R protein were most abundant on the apical surface of the endometrial LE and GE. RT-PCR analyses detected expression of IGFBPs (3, 4, 5 and 6) as well as PAPP-A mRNAs in the uterus, but not IGFBP-1 and IGFBP-2 mRNAs. IGFBP-3 and IGFBP-4 mRNAs were expressed specifically in the endometrial stroma and myometrium and increased after birth. PAPP-A mRNA was expressed specifically in the endometrial stroma and increased after birth. In Study Two, ewes were treated from birth with estradiol-17β valerate (EV), which reduces uterine growth and inhibits endometrial adenogenesis. On PNDs 14 and 56, IGFBP-3 mRNA was decreased in the uterus of EV-treated ewes, but IGF-1R and IGFBP-4 mRNAs were not affected. PAPP-A mRNA was increased by EV treatment on PND 14, but decreased on PND 56. These results support the hypothesis that an intrinsic IGF system in the uterus regulates epithelial–stromal interactions important for postnatal uterine growth and endometrial gland morphogenesis in the sheep.
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Introduction

Postnatal development of the ovine uterus involves the emergence, proliferation and differentiation of endometrial glands, specification of intercaruncular stroma, development of endometrial folds and, to a lesser extent, growth of endometrial caruncular areas and the myometrium (Taylor *et al.* 2000, Gray *et al.* 2001*a*). Uterine gland development or adenogenesis is initiated between postnatal days (PNDs) 1 and 7 when shallow epithelial invaginations appear along the luminal epithelium (LE) in presumptive intercaruncular areas. Between PNDs 7 and 14, the nascent glandular epithelia (GE) bud and invaginate into the stroma and then proliferate to form tubules or ducts that begin to coil and branch at the tips by PND 21. After PND 14, endometrial adenogenesis primarily involves coiling and slight branching morphogenetic growth of tubular endometrial glands from the upper stroma (e.g. stratum compactum) underneath the LE into the lower stroma (e.g. stratum spongiosum) adjacent to the inner circular layer of the myometrium. By PND 56, uterine morphogenesis is essentially complete, as the aglandular caruncular and glandular intercaruncular endometrial areas appear histoarchitecturally similar to that of the adult uterus (Taylor *et al.* 2000). The success of uterine development in the neonate directly impacts on the embryotrophic capacity of the adult uterus. Inappropriate exposure to progestins from birth to PND 56 permanently ablates endometrial gland development and results in a uterine gland knockout (UGKO) phenotype in the adult (Gray *et al.* 2001*b*). Adult UGKO ewes are infertile and exhibit a defect in peri-implantation conceptus survival

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and growth (Gray *et al.* 2001*a*, 2002). Therefore, it is important to understand the hormonal, cellular and molecular mechanisms governing postnatal uterine development (Bartol *et al.* 1999).

Insulin-like growth factors (IGF-I and IGF-II) function as endocrine and paracrine/autocrine hormones that stimulate proliferation and differentiation of many diverse cell types (Jones & Clemmons 1995, Baxter 2000). Established components of the IGF system include IGF-I and IGF-II, type I and type II IGF receptors (IGF-1R and IGF-2R), six high affinity IGF binding proteins (IGFBP-1 to -6), and IGFBP proteases (Clemmons 1997, Hwa *et al.* 1999). In the neonatal ovine uterus, IGF-I and IGF-II mRNAs are expressed only in the endometrial stroma, whereas IGF-1R mRNA is expressed in all uterine cell types with the highest levels in the GE (Taylor *et al.* 2001). IGF-2R was not detected in the neonatal ovine uterus (Taylor *et al.* 2001). Interestingly, IGF-I and IGF-II mRNAs were most abundant in the stroma surrounding the developing glands in the intercaruncular areas of the endometrium (Taylor *et al.* 2001). Recently, estrogen disruption of uterine growth and endometrial adenogenesis was correlated with alterations in normal patterns of IGF-I, IGF-II and IGF-1R expression in the neonatal ewe (Carpenter *et al.* 2003*a*, Hayashi *et al.* 2004), supporting the idea that this intrinsic growth factor system regulates uterine development after birth. The IGF system is also implicated in postnatal rodent uterine development (Adesanya *et al.* 1999, Gu *et al.* 1999), and adult IGF-I null female mice have severe reductions in uterine weight and size (Baker *et al.* 1996). In mice, locally produced IGF-I can maintain normal growth and development in the absence of circulating IGF-I (Yakar *et al.* 1999). However, estrogen-induced uterine growth appears to require systemic IGF-I in mice (Sato *et al.* 2002). Collectively, available studies support the hypothesis that IGFs of local stromal origin are important mitogens that act in a classical epithelial–stromal manner on epithelial IGF-1R to regulate uterine growth and endometrial adenogenesis in the neonatal ewe. The systemic levels of IGFs in the neonatal ewe have not been reported in detail.

Six distinct yet structurally homologous IGF binding proteins, designated IGFBP-1 through IGFBP-6, function as regulators of the biological activities of IGF-I and IGF-II (Jones & Clemmons 1995, Firth & Baxter 2002). IGFBPs can either inhibit or enhance IGF activity and can also act independently of the IGFs. One IGFBP-4 protease is pregnancy-associated plasma protein A (PAPP-A) (Jones & Clemmons 1995), which is a metalloproteinase in the metzincin superfamily that includes astacins, serralysins, adamalysins, and matrix metalloproteinases (MMPs) (Overgaard *et al.* 2000). Although IGFBP mRNA distribution has been reported in the adult ovine uterus during gestation (Wathes *et al.* 1998*b*, Nayak & Giudice 2003), the IGFBP system has not been investigated in the developing neonatal ovine uterus. Our working hypothesis is that an intrinsic IGF system regulates postnatal uterine development and, in particular, endometrial adenogenesis in the neonatal ewe. Therefore, one objective of the present studies was to determine the effects of postnatal age on (1) systemic levels of IGF-I and IGF-II, (2) expression of IGF-I, IGF-II and IGF-1R protein in the uterus, and (3) expression of IGFBPs and PAPP-A in the uterus. The second objective was to determine the effects of estrogen-induced developmental disruption on expression of IGFBPs and PAPP-A in the neonatal ovine uterus.

Materials and methods

Experimental design and tissue collection

All experimental and surgical procedures were approved by the University Laboratory Animal Care and Use Committee of Texas A&M University.

Study One

Crossbred Suffolk ewes were mated to Suffolk rams between November and December 2002. Ewes included in the following experiment were born between February and March 2003. All lambs were suckling their dams throughout the experiment. Crossbred, spring-born Suffolk ewes ($n = 45$) were assigned randomly at birth (PND 0) to be hysterectomized on PND 0 ($n = 6$), 7 ($n = 4$), 14 ($n = 5$), 21 ($n = 5$), 28 ($n = 5$), 35 ($n = 5$), 42 ($n = 5$), 49 ($n = 5$), or 56 ($n = 5$). Before surgery, blood samples were collected by jugular venipuncture, and serum was obtained by centrifugation after coagulation. At hysterectomy, the entire reproductive tract was excised, and the uterus was trimmed free of the broad ligament, oviduct, and cervix. Each uterine horn and ovary was fixed in 4% (w/v) paraformaldehyde in PBS (pH 7.2). After 24 h, fixed tissues were changed to 70% ethanol and then embedded in Paraplast Plus (Oxford Labware, St Louis, MO, USA). Several sections from the middle of each uterine horn were snap-frozen in Tissue-Tek OCT compound (SAKURA Finetek USA Inc., Torrance, CA, USA). The remainder of the uterus was frozen in liquid nitrogen and stored at −80 °C.

Study Two

Crossbred Suffolk ewes were mated to Suffolk rams between July and October 2003. Ewes included in the following experiment were born between October 2003 and January 2004, and lambs were suckling their dams throughout the experiment. As described previously (Carpenter *et al.* 2003*a*), ewes ($n = 14$) were assigned randomly at birth (PND 0) to receive daily i.m. injections from PND 0 to PND 55 of: (1) corn oil vehicle as a control (CX) or (2) estradiol-17β valerate (EV; Sigma) in corn oil at a dose of 50 μg per kg body weight. Ewes were weighed and the EV dose adjusted every 7 days. On PND 14, the right ovarian pedicle was ligated with a suture, and the ovary and oviduct were removed. The right uter-

ine horn was ligated with a suture above the intercornual ligament, and the anterior portion of the right uterine horn above the ligature was removed, fixed in 4% (w/v) paraformaldehyde in PBS (pH 7.2), and processed for histology. On PND 56, all ewes were killed and necropsied. The entire left uterine horn was dissected from the remaining portion of the right uterine horn, fixed in 4% (w/v) paraformaldehyde in PBS (pH 7.2), and processed for histology. The remainder of the uterus was frozen in liquid nitrogen and stored at −80 °C.

IGF-I radioimmunoassay (RIA)

Blood samples for serum were allowed to clot for 1 h at room temperature. Serum was then collected by centrifugation (3000 × *g* for 30 min at 4 °C), removed, and stored at −20 °C. Serum concentrations of IGF-I were determined by a double-antibody RIA procedure (Bilby *et al.* 1999) with all the samples processed in a single assay. The primary antibody was obtained from Dr A F Parlow, National Hormone and Pituitary Program (UCLA Medical Center, Torrance, CA, USA). The standard curve, ranging from 2 pg to 1000 pg/tube, was constructed using recombinant IGF-I from Peninsula Labs (Belmont, CA, USA). Iodinated IGF-I (Cat# H-5406) tracer was from Peninsula Labs. Normal rabbit serum and goat anti-rabbit antibody were from Antibodies, Inc. (Davis, CA, USA). Briefly, serum samples (10 μl) were incubated with 500 μl assay buffer (0.02% protamine sulfate, 30 mM sodium phosphate, 0.05% Tween 20, 0.02% sodium azide, and 0.01 M EDTA, pH 7.5) and 400 μl 1 M glycine (pH 3.2) for 48 h at 37 °C. After incubation, the acidified samples were neutralized with 90 μl 0.05 M NaOH. Duplicate 50 μl aliquots of the acidified samples were assayed. The RIA tubes (polypropylene, 12 × 75 mm) were incubated at 4 °C in the presence of primary antibody for a 24-h period, after which iodinated IGF-I (20 000 c.p.m./100 μl) was added for an additional incubation period of 18 h at 4 °C. The assay was terminated by addition of 50 μl normal rabbit serum (1:100 dilution), 50 μl goat anti-rabbit antibody (1:60) and 300 μl of a polyethylene glycol solution (12.5% w/v; 8000 Da molecular mass; Sigma). The RIA tubes were incubated for 30 min before centrifugation at 4 °C. The supernatant was discarded and radioactivity associated with the pellet (bound fraction) was determined by counting for 2 min on a gamma counter. Acidified serum samples representing high, medium and low concentrations of IGF-I were assayed to represent internal quality control pools. The mean intra-assay coefficient of variation was 4.6%, and the assay sensitivity was 3.6 pg/ml.

IGF-II RIA

Concentrations of IGF-II in serum were determined by double antibody RIA in one assay as previously described (Spicer *et al.* 1995) with the following modifications. The tracer and standard were recombinant human IGF-II, and the extraction was performed using acid ethanol (16 h at 4 °C) as previously described (Echternkamp *et al.* 1990). The assay sensitivity, defined as 95% of total binding, was 14 ng/ml and the intra-assay CV was 2.8%.

RT-PCR analyses

Partial cDNAs for IGF-1R, IGFBP-1 through IGFBP-6, and PAPP-A mRNAs were generated by RT-PCR using total RNA, isolated from the neonatal ovary, neonatal uterus or endometrium from gestational day (GD) 16, as described previously (Taylor *et al.* 2000, 2001, Hayashi *et al.* 2003). Primer and annealing temperatures used for PCR are summarized in Table 1. The amplified PCR products were subcloned into the pCRII cloning vector using a T/A Cloning Kit (Invitrogen Life Technologies, Carlsbad, CA, USA) and sequenced in both directions using an ABI PRISM Dye Terminator Cycle Sequencing Kit and ABI PRISM automated DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) to confirm identity.

Table 1 Summary of PCR primer sequences and expected cDNA sizes.

Primer	**Sequences of forward and reverse primers** (5′-3′)	**GenBank** (species)	**Annealing temperature** (°C)	**cDNA size** (bp)
IGF-IR	GAGATTGCAGATGGCATGG GCTGATGATCTCCAGGAACG	X54980 (bovine)	56	441
IGFBP-1	GATGACCGAGTCCAGTGAGG CCATTCTTGTTGCAGTTTGG	AF327650 (ovine)	54	210
IGFBP-2	AGCAGGTTGCAGACAATGG ACAGTTGGGGATGTGTAGGG	S44612 (ovine)	54	353
IGFBP-3	CAGAACTTCTCCTCCGAGTCC CCACACACCAGCAGAAACC	AF327651 (ovine)	54	205
IGFBP-4	CTTGGCCAAAATTCGAGACC AAGCTTCACTCCCGTCTTCC	S77394 (ovine)	54	286
IGFBP-5	GCGGCGTCTACACTGAGC GAAGATCTTGGGCGAGTAGG	S52657 (bovine)	54	212
IGFBP-6	AAGGAGAGTAAGCCCCAAGC GGCACGTAGAGTGTATGAGACC	AF327653 (ovine)	54	212
PAPP-A	ACACATGTGGCTTCAACAGC AGACAAAGGTCACCCAGACG	AF421140 (ovine)	54	552

In-situ hybridization

Expression of mRNAs in uterine tissues was determined by *in-situ* hybridization using methods described previously (Spencer *et al.* 1999). Briefly, deparaffinized, rehydrated, and deproteinated cross-sections (5 μm) of the uterus and ovary from each ewe were hybridized with radiolabeled sense or antisense cRNA probes generated from linearized plasmid templates using *in vitro* transcription with [α-^{35}S]UTP. After hybridization, washing and ribonuclease A digestion, slides were dipped in NTB-2 liquid photographic emulsion (Kodak, Rochester, NY, USA), stored at 4 °C for 7 to 28 days, and developed in Kodak D-19 developer. Slides were then counterstained with Gill's modified hematoxylin (Stat Lab, Lewisville, TX, USA), dehydrated through a graded series of alcohol to xylene, and protected with a coverslip. Images of representative fields were recorded using a Nikon Eclipse 1000 photomicroscope (Nikon Instruments Inc., Lewisville, TX, USA) fitted with a Nikon DXM1200 digital camera.

Immunofluorescence analyses

Rabbit anti-human IGF-I (H-70), IGF-II (H-103) and IGF-1R (H-78) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Normal rabbit IgG was from Santa Cruz, and fluorescein-conjugated goat antibody against rabbit IgG was from Chemicon International (Temecula, CA, USA). Proteins were localized in frozen uterine cross-sections (8 μm) using methods previously described (Johnson *et al.* 1999). Briefly, tissues were fixed in −20 °C methanol, permeabilized with 0.3% Tween 20 in PBS, blocked in 10% normal goat serum, incubated overnight at 4 °C with 2 to 10 μg/ml primary antibody, and detected with fluorescein-conjugated secondary antibodies. Slides were then overlaid with a coverglass. Photomicrographs of representative fields of immunofluorescence were evaluated using a Zeiss Axioplan2 microscope interfaced with a AxioCam high resolution digital camera and AxioVision 3.1 software (AxioVision, Thornwool, NY, USA). Representative fluorescence images of cross-section for each antibody and for each ewe were recorded digitally.

As described previously (Taylor *et al.* 2001, Hayashi *et al.* 2003), relative hybridization signal intensity for staining intensity for immunoreactive protein expression was assessed visually in uterine sections from each ewe by two independent observers and scored as follows: absent i.e. no staining above IgG control (−), weak (+), moderate (++), or strong (+++). Intercaruncular endometrial tissues, including LE, stroma, GE, and myometrium were scored. The GE was separated into stratum compactum and stratum spongiosum.

Statistical analyses

All quantitative data were subjected to least-squares ANOVA using General Linear Models procedures of the Statistical Analysis System (Cary, NC, USA). Data are presented as least-square means with overall standard errors (S.E.).

Results

Systemic levels of IGF-I and IGF-II

Circulating levels of IGF-I and IGF-II in the serum of neonatal ewes did not change ($P > 0.10$) between birth and PND 56 (Fig. 1).

Expression of IGF-I, IGF-II and IGF-1R protein in the neonatal uterus

Immunofluorescence analyses were performed to localize immunoreactive IGF-I, IGF-II and IGF-1R protein in the neonatal uterus (Fig. 2). Results of immunofluorescence analyses were quantified and are summarized in Table 2. Low levels of immunoreactive IGF-I protein were observed on the apical surface of the endometrial LE and GE between birth and PND 56. Immunoreactive IGF-II protein was detected in all endometrial cell types; however, abundant IGF-II protein was detected on the apical surface of the endometrial LE and GE. Similarly, immunoreactive IGF-1R protein was detected predominantly on the apical surface of the endometrial LE and GE and at much lower abundance in the stroma and myometrium. The abundance of immunoreactive IGF-1R protein increased in the endometrial GE after PND 21.

Expression of IGFBPs and PAPP-A in the neonatal uterus

In order to determine which of the six IGFBP family members was expressed in the neonatal ovine uterus, RT-PCR analysis was performed using total RNA isolated from the neonatal uterus. Total RNA isolated from ovine endometrium from day 16 of gestation (GD 16) and a PND 42 ovary were used as positive controls (data not shown).

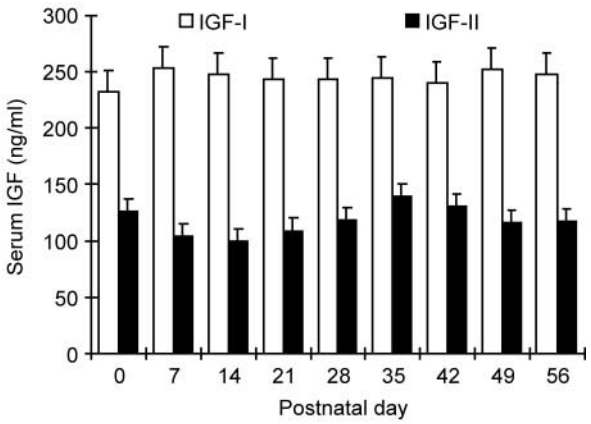

Figure 1 Systemic levels of IGF-I and IGF-II in the serum of neonatal ewes between postnatal day (PND) 0 and PND 56 ($n = 4–6$ ewes per day). Levels of both IGFs were not affected by postnatal age ($P > 0.10$).

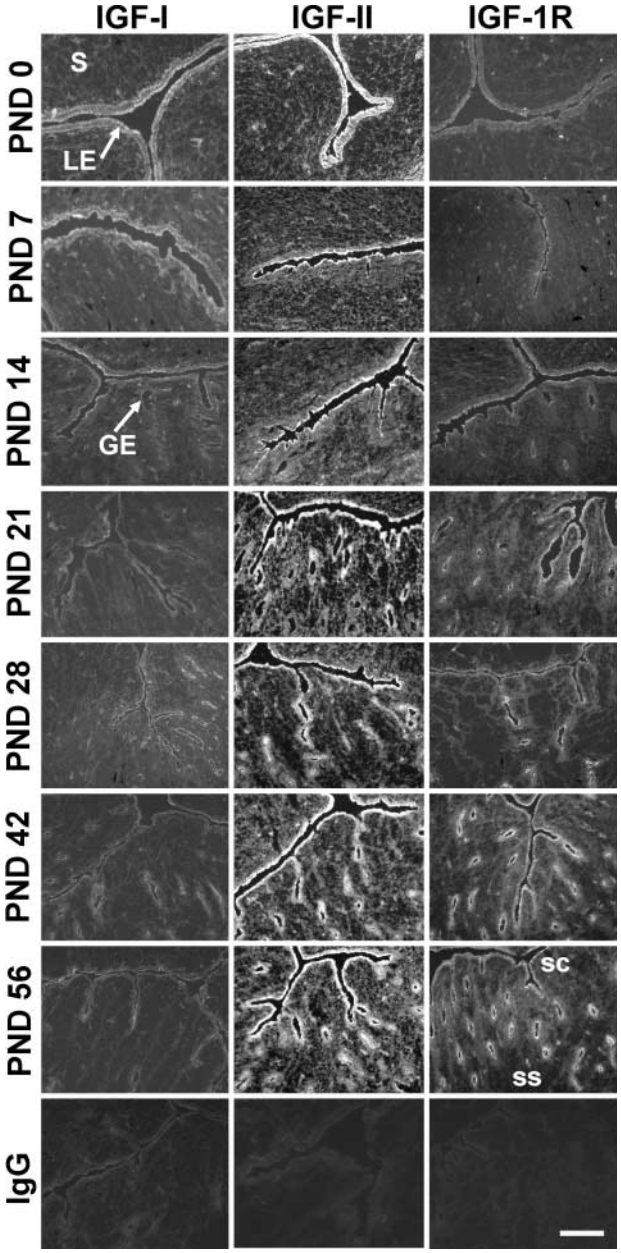

Figure 2 Distribution of immunoreactive IGF-I, IGF-II and IGF-1R in the neonatal ovine uterus on postnatal days (PND) 0, 7, 14, 21, 28, 42 and 56. GE, glandular epithelium; LE, luminal epithelium; S, stroma; sc, stratum compactum; ss, stratum spongiosum. Scale bar, 100 μm.

IGFBP-1 mRNA was not detected in either the neonatal ovine uterus or ovary, but was detected in the endometrium from GD 16. IGFBP-2 mRNA was detected in GD 16 endometrium and PND 42 ovary, but not in the neonatal uterus. IGFBP-3, -4, -5 and -6 mRNAs were detected in the neonatal ovine uterus and ovary as well as GD 16 endometrium. PAPP-A mRNA was detected in the uterus on all PNDs (data not shown).

In-situ hybridization analyses were conducted to localize expression of IGFBP mRNAs in the neonatal ovine uterus (Fig. 3). Consistent with the results of RT-PCR analyses, expression of IGFBP-1 and IGFBP-2 mRNA was not detected in the neonatal ovine uterus (data not shown). IGFBP-3 and IGFBP-4 mRNAs were detected only in the endometrial stroma and myometrium. The overall abundance of both IGFBP-3 and IGFBP-4 mRNAs was low or undetectable in the endometrial stroma and myometrium on PND 0 and increased thereafter. Although IGFBP-5 and IGFBP-6 mRNAs were detected in the neonatal ovine uterus by RT-PCR, the overall abundance was below the detectable limits of *in-situ* hybridization analysis (data not shown). PAPP-A mRNA was detected specifically in the stratum compactum stroma in both caruncular and intercaruncular endometrial areas, and increased between PND 0 and PND 56 (Fig. 4). PAPP-A mRNA was not observed in the endometrial LE or GE nor myometrium. Groups of pigmented melanocytes also produce a prominent signal in darkfield microscopy even though they do not express IGFBP or PAPP-A mRNAs.

Table 2 Distribution and relative abundance of immunoreactive IGF-I, IGF-II and IGF-1R protein in the neonatal uterus.

		GE			
PND	**LE**	**Str. comp.**	**St. spons.**	**Stroma**	**Myometrium**
IGF-I					
0	+++	NP	NP	+/−	+/−
7	+++	++	NP	+/−	+/−
14	++	+	NP	+/−	+/−
21	+	+	+	−	+/−
28	+/−	+/−	+/−	−	+/−
42	+/−	+/−	+/−	−	+/−
56	+/−	+/−	+/−	−	+/−
IGF-II					
0	+++	NP	NP	+	++
7	+++	++	NP	+	++
14	+++	++	NP	+	++
21	+++	++	+	+/++	++
28	+++	++	+	+/++	++
42	+++	++	+	+/++	++
56	+++	++	+	+/++	++
IGF-1R					
0	+	NP	NP	−/+	−
7	+	+	NP	−/+	−
14	+	+	NP	−/+	−
21	++	++	+	+	−
28	++	+++	+++	+	−
42	++	+++	+++	+	−
56	++	+++	+++	+	−

PND, postnatal day; LE, luminal epithelium; GE, glandular epithelium (shallow or stratum compactum and deep or stratum spongiosum); NP, not present.
− , no staining above IgG control; +, weak staining; ++, moderate staining; +++, strong staining.

Expression of IGF-IR, IGFBPs and PAPP-A in the EV-treated neonatal uterus

In Study Two, treatment of ewes from birth with EV affected uterine development and endometrial adenogenesis (Fig. 5). The endometrium from uteri of EV-treated ewes on both PND 14 and PND 56 did not contain any histologically

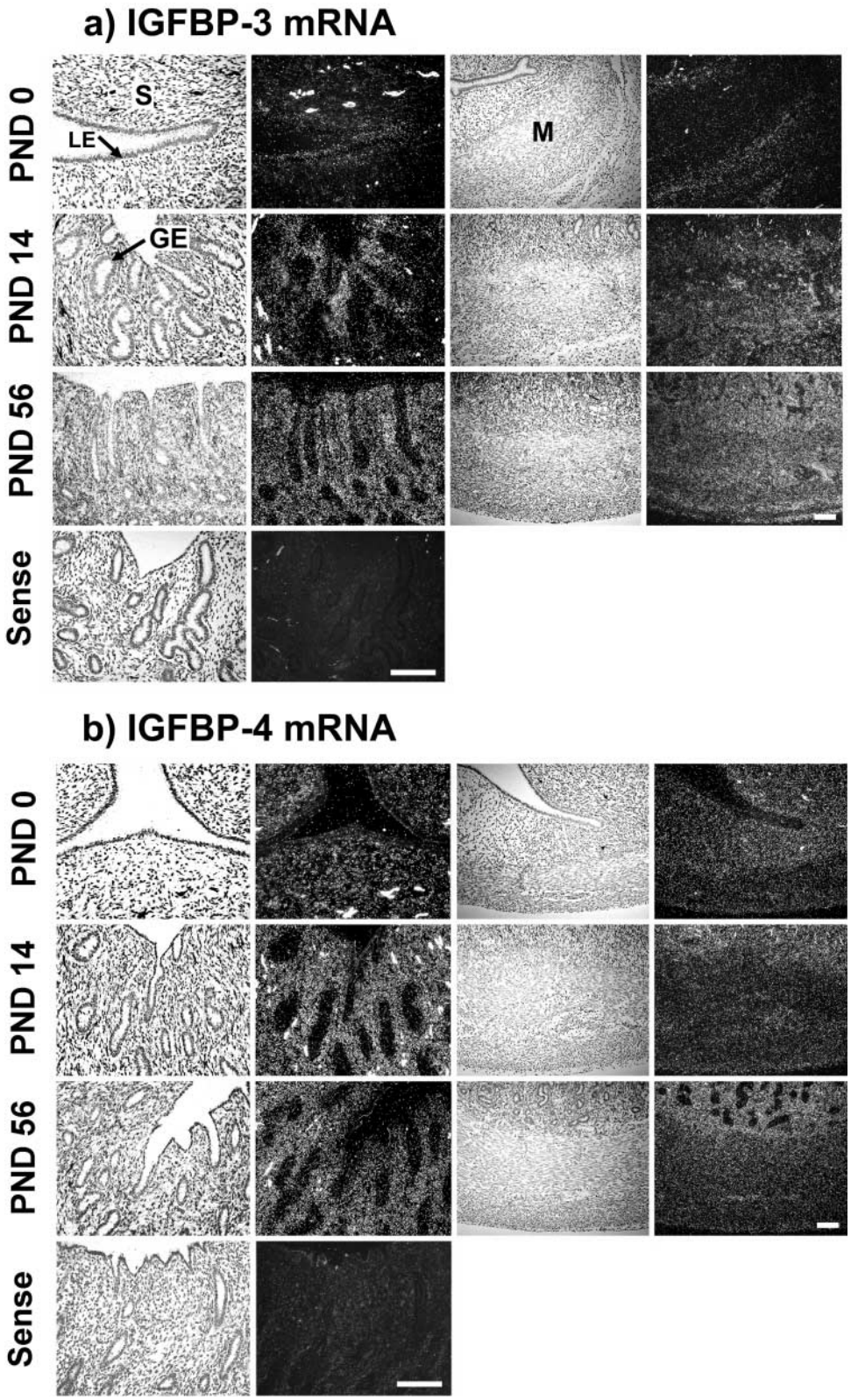

Figure 3 *In-situ* localization of (a) IGFBP-3 and (b) IGFBP-4 mRNA in the neonatal ovine uterus on postnatal days (PND) 0, 14 and 56. The photomicrographs are presented in brightfield (left) and darkfield (right) illumination. GE, glandular epithelium; LE, luminal epithelium; M, myometrium; S, stroma. Scale bar, 100 μm.

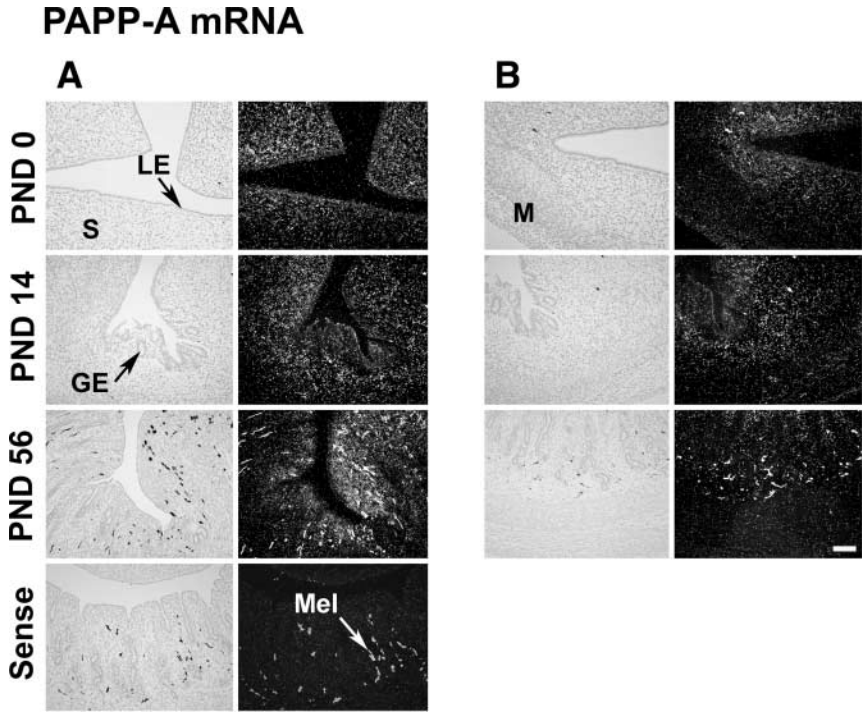

Figure 4 *In-situ* localization of PAPP-A mRNA in the neonatal ovine uterus. Left two columns (A) focus on endometrium, right (B) focus on myometrium. The photomicrographs are presented in brightfield (left) and darkfield (right) illumination. GE, glandular epithelium; LE, luminal epithelium; Mel, melanocyte; M, myometrium; S, stroma. Scale bar, 100 μm.

discernable endometrial glands. Expression of IGF-1R mRNA was observed abundantly in the endometrial LE and GE with lower levels in the stroma in CX ewes on PNDs 14 and 56 and was not affected by exposure to EV (Fig. 5a). IGFBP-3 mRNA was detected in the stroma and myometrium on PND 14, and expression was markedly increased on PND 56 in CX ewes (Fig. 5b). Treatment with EV decreased IGFBP-3 mRNA in the myometrium on PND 14 and predominantly in the stroma on PND 56 (Fig. 5b). In contrast, EV did not affect expression of IGFBP-4 mRNA in the stroma on PND 14 or PND 56 (Fig. 5c). On the other hand, PAPP-A mRNA was increased in the stroma of EV-treated ewes on PND 14, but was reduced on PND 56 as compared with CX ewes (Fig. 5d).

Discussion

In the present study, circulating levels of IGF-I and IGF-II were not different between birth and PND 56. The levels of circulating IGF-I and IGF-II observed in the present study were within the range of values previously reported for neonatal lambs (Mesiano *et al.* 1987, 1989). Plasma IGF-I increased in ram lambs between birth and PNDs 4 to 9 and was dependent on suckling (Greenwood *et al.* 2002). Furthermore, IGF-I and IGF-II increased between PNDs 0 and 20 in suckling ram and ewe lambs, and the increase between PNDs 1 and 16 was greater in ram lambs than in ewe lambs (Gatford *et al.* 1997). Overall, circulating IGF levels in ewes in the present study were not substantially different from previous reports. Therefore, systemic IGFs could play a regulatory role in postnatal uterine development. The importance of local and systemic IGFs in normal growth and development of tissues remains a subject of some debate. In mice, locally produced IGF-I can maintain normal growth and development in the absence of circulating IGF-I (Yakar *et al.* 1999), whereas estrogen-induced uterine growth appears to require systemic IGF-I (Sato *et al.* 2002). In the neonatal ewe, circulating levels of estradiol-17β are below detectable limits after birth to at least PND 56 (Carpenter *et al.* 2003*a*,*c*), suggesting that circulating IGF-I and IGF-II are not regulated by ovarian steroids in the neonatal ewe.

Previous studies found that IGF-I and IGF-II mRNAs were expressed in the endometrial stroma of the neonatal ovine uterus surrounding the developing glands (Taylor *et al.* 2001, Carpenter *et al.* 2003*a*). IGF-1R mRNA was expressed by all uterine cell types, but was particularly abundant in the endometrial epithelia. The same pattern of IGF-I, IGF-II and IGF-1R mRNA expression was observed in the adult ovine uterus (Stevenson *et al.* 1994). The present study is the first to localize the expression of immunoreactive IGF-I, IGF-II and IGF-1R protein in the ovine uterus. Immunoreactive IGF-I protein was detected at fairly low levels on the endometrial LE and, to some extent, GE. In contrast, immunoreactive IGF-II protein was localized in relative abundance on the apical surface of the endometrial LE and GE. The biological effects of IGF-I and IGF-II are mediated by the IGF-1R in most tissue and

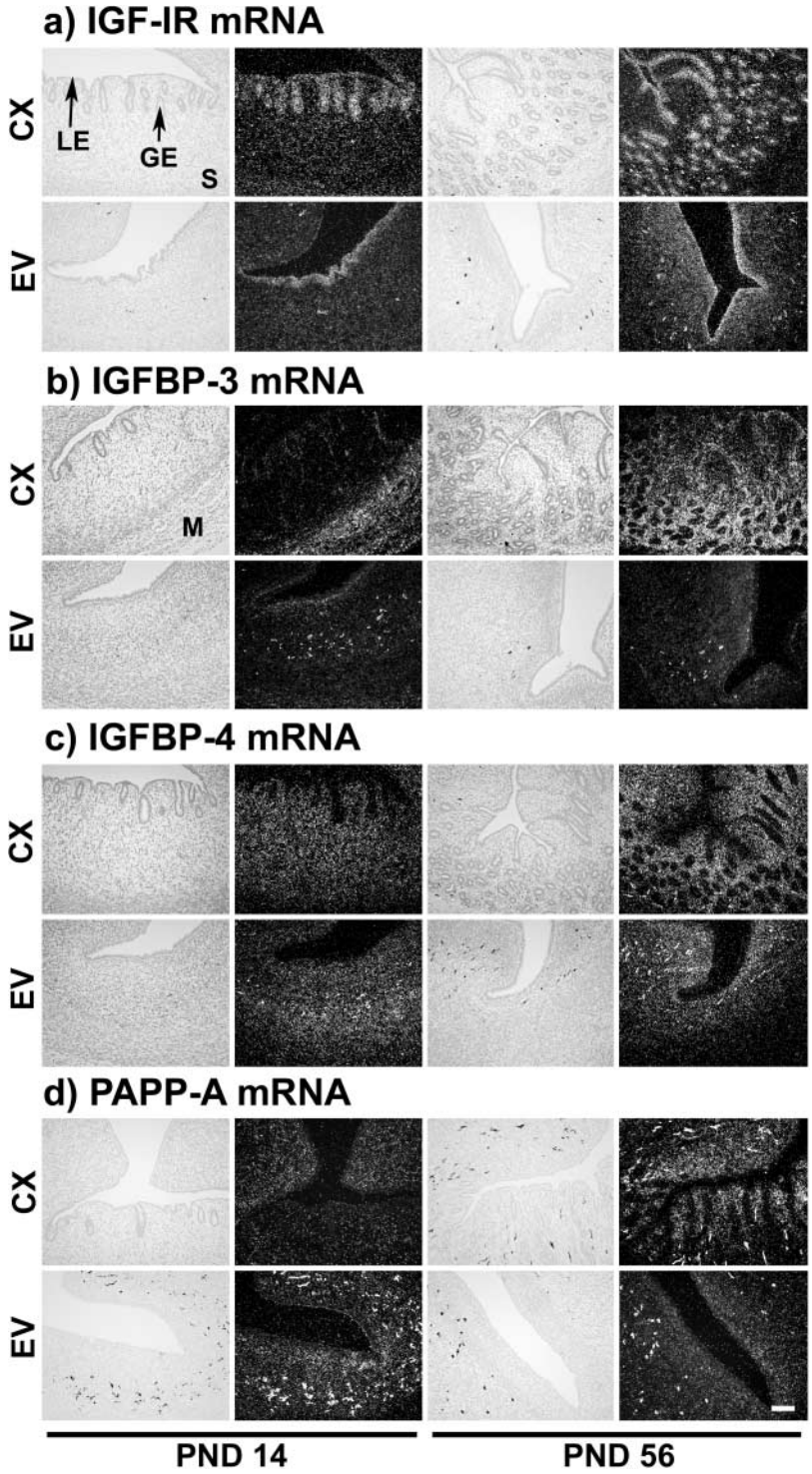

Figure 5 Expression of (a) IGF-1R, (b) IGFBP-3, (c) IGFBP-4 and (d) PAPP-A mRNAs in uteri from control (CX) and estradiol valerate (EV)-treated ewes on PNDs 14 and 56. The photomicrographs are presented in brightfield (left) and darkfield (right) illumination. GE, glandular epithelium; LE, luminal epithelium; M, myometrium; S, stroma. Scale bar, 100 μm.

cell systems (Jones & Clemmons 1995). Although immunoreactive IGF-1R protein was observed in all uterine cell types, the most abundant expression was detected on the apical surfaces of endometrial LE and GE during postnatal development. Collectively, available results strongly support the idea that local IGF-I and IGF-II of stromal origin act on IGF-1R in the epithelium and stroma to regulate uterine growth and endometrial adenogenesis in the neonatal ewe (Taylor *et al.* 2001, Carpenter *et al.* 2003*a*, Hayashi *et al.* 2004). Although IGF-II plays a

fundamental role in embryonic and fetal development, its role during the postnatal period is generally thought to be less important since it is substituted by IGF-I (DeChiara *et al.* 1990). Interestingly, IGF-II gene expression was not observed in the neonatal mouse uterus (Gu *et al.* 1999), but was detected in the neonatal ovine uterus in the present study, and in the adult ovine and human uterus (Wathes *et al.* 1998*a*, Nayak & Giudice 2003). Given the lack of IGF-II in the neonatal mouse uterus, the precise role of IGF-II in uterine development and function remains to be determined using other models such as domestic ruminants.

The activities of both IGF-I and IGF-II are modulated by their association with six IGFBPs (Jones & Clemmons 1995). By binding IGF-I and IGF-II, IGFBPs have growth-inhibitory effects by restricting the availability of these ligands for binding to IGF-1R (Rechler & Brown 1992, Jones & Clemmons 1995). On the contrary, the IGFBPs also regulate IGF bioavailability by maintaining a circulating reservoir of IGFs and prolonging their half-life (Ferry *et al.* 1999). At the tissue level, IGFBPs interact with either extracellular matrix constituents (IGFBP-2 and IGFBP-5) (Arai *et al.* 1996) or directly with cell membranes (IGFBP-1 and IGFBP-3) (Delbe *et al.* 1991). In the present study, RT-PCR analyses of the neonatal ovine uterus detected expression of only IGFBP-3, -4, -5 and -6 mRNAs. *In-situ* hybridization localized expression of IGFBP-3 and IGFBP-4 mRNAs only in the endometrial stroma and myometrium of the neonatal ovine uterus, whereas IGFBP-5 and IGFBP-6 mRNAs were below detectable limits of the *in-situ* hybridization procedure. Overall, expression of IGFBP-3 and IGFBP-4 mRNAs increased after birth and was highest in the stroma during the period of endometrial gland morphogenesis. Potentiation and inhibition of IGF actions by IGFBP-3 has been demonstrated in many cell culture systems (Firth & Baxter 2002). Interestingly, IGFBP-3 has IGF-1R-independent actions to inhibit cell growth (Valentinis *et al.* 1995). Moreover, IGFBP-3 interacts with and modifies the biological activities of a number of other growth factors, including transforming growth factor beta and retinoids (Firth & Baxter 2002).

The action of IGFBP-4 appears to be purely inhibitory with respect to both IGF-I and IGF-II (Jones & Clemmons 1995). IGFBP-4 availability in the microenvironment is determined not only by specific gene expression, but also through limited proteolysis by specific proteases such as PAPP-A (Fowlkes 1997). Enzymatic cleavage of IGFBP-4 by PAPP-A markedly reduces its binding of IGFs and potentiates the effectiveness of exogenous IGF-I in stimulating receptor-mediated growth responses in cultured cells (Qin *et al.* 1999). In the present study, PAPP-A mRNA was expressed specifically in the compact stroma of the endometrium in the neonatal ovine uterus. Expression of PAPP-A increased from birth to PND 56 in association with uterine growth and endometrial gland morphogenesis. Thus, bioavailability of local IGF-I and IGF-II in the uterine microenvironment may be regulated through controlled IGFBP-4 proteolysis by PAPP-A. Similarly, PAPP-A, IGFBP-4, and IGF appear to function together in the ovary and uterus in other species (Conover *et al.* 2001, Giudice *et al.* 2002, Mazerbourg *et al.* 2004).

In the present study, expression of IGFBP-1 and IGFBP-2 was not detected in the neonatal uterus by RT-PCR. Furthermore, expression of IGFBP-5 and IGFBP-6 mRNA was only detectable using very high cycles of amplification (30–35 cycles) in the RT-PCR analyses (K Hayashi and T E Spencer, unpublished results), and expression of these IGFBPs was below detectable limits of the *in-situ* hybridization analysis. In adult ewes, these IGFBPs are expressed in the uterus during the estrous cycle and pregnancy (Reynolds *et al.* 1997, Osgerby *et al.* 1999, Gadd *et al.* 2000). IGFBP-1 mRNA is expressed by the LE during early pregnancy and then declines to undetectable levels during later stages of gestation (Osgerby *et al.* 1999). IGFBP-2 mRNA is undetectable in the uterus during the estrous cycle, but is abundant in the dense stroma of the placentomal capsule after GD 29, which also expresses IGFBP-4 (Reynolds *et al.* 1997). In cyclic ewes, IGFBP-5 mRNA is present in the caruncular endometrium and in the intercaruncular endometrial LE (Gadd *et al.* 2000). During pregnancy, IGFBP-5 is also present in the endometrial LE and GE as well as in caruncular stroma surrounding the chorionic villi. Available studies indicate that the expression of the genes encoding IGFBP-1 through IGFBP-6 are regulated by steroid hormones during the estrous cycle and potential conceptus factors during pregnancy (Wathes *et al.* 1998*a*, Nayak & Giudice 2003). In the present study, IGFBP-3 and IGFBP-4 mRNAs were observed only in the endometrial stroma and not in the endometrial LE as in the adult ovine uterus. Therefore, factors from the ovary, e.g. estrogen and progesterone, and from the conceptus appear to act on the ovine endometrium to regulate IGFBP gene expression after puberty in a tissue- and cell type-specific manner.

Available results strongly support the hypothesis that the intrinsic IGF system (IGF-I, IGF-II, IGFBP-3, IGFBP-4, and PAPP-A) is an important regulator of endometrial gland morphogenesis and uterine growth in the developing neonatal ewe. In Study Two, exposure of neonatal ewes to EV from birth did not affect epithelial IGF-1R or stromal IGFBP-4 mRNA, but did decrease IGFBP-3 mRNA in the stroma. In the EV-treated ewes, PAPP-A mRNA in the stroma was increased initially on PND 14, but was decreased by PND 56. The negative effects of EV exposure to decrease IGFBP-3 and increase PAPP-A would be predicted to modify biological activities of IGFs in the endometrium. However, stromal IGF-I and IGF-II expression is substantially decreased in the endometrium of EV-treated ewes (Carpenter *et al.* 2003*a*). Similarly, disruption of uterine growth and endometrial adenogenesis by inappropriate exposure of the neonatal ewe to estradiol-17β benzoate from PNDs 14 to 28 or PNDs 42 to 56 negatively affected expression of IGF-I, IGF-II and IGF-1R in the endometrium (Hayashi *et al.* 2004). Collectively, these results support the

hypothesis that IGFs of local and perhaps systemic origin regulate critical interactions between the stroma and epithelium that are crucial for development of the uterus and other female reproductive tract organs (Cunha & Lung 1979, Cunha *et al.* 1989). Studies to determine the effects of modulating systemic and intrinsic IGF-I and IGF-II levels on postnatal ovine uterine development need to be conducted, but could be an effective tool for optimizing postnatal uterine development and perhaps adult uterine capacity and fertility in domestic ruminants as well as in humans (Carpenter *et al.* 2003*b*).

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