Galectin-15 in ovine uteroplacental tissues

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Abstract

Galectin-15 is the newest member of a secreted β -galactoside-binding lectin family. The galectin-15 gene is expressed specifically by the endometrial luminal epithelium (LE) and superficial ductal glandular epithelium (sGE) of the ovine uterus. The proposed extracellular role of secreted galectin-15 is to regulate implantation and placentation by functioning as a heterophilic cell adhesion molecule between the conceptus trophoctoderm and endometrial LE, while that of intracellular galectin-15 is to regulate cell survival, differentiation and function. The present study determined galectin-15 expression in uteroplacental tissues during gestation and in the postpartum uterus. In the uterine lumen, secreted galectin-15 was found as multimers, particularly on days 14 and 16 of pregnancy. In the endometrial epithelium and conceptus trophoctoderm, intracellular galectin-15 protein was found associated with crystalline structures. Between days 20 and 120 of pregnancy, galectin-15 mRNA was expressed specifically by the LE and sGE of the intercaruncular endometrium of ewes. Immunoreactive galectin-15 protein was most abundant in the trophoctoderm with lower levels in the endometrial LE and sGE. Galectin-15 protein was detected in allantoic fluid, but not in amniotic fluid. After parturition, galectin-15 mRNA declined in the endometrium from postpartum day (PPD) 1 to 28 and exhibited a variegated expression pattern in the LE and sGE. These results indicate that galectin-15 is synthesized and secreted throughout gestation by the endometrial LE/sGE and is absorbed by the placenta and forms crystals within the trophoctoderm, whereas the remainder is cleared into the allantois after being transported into the fetal circulation via the placental areolae. Based on the biological properties of other galectin family members, galectin-15 is hypothesized to have biological roles in conceptus–endometrial interactions, uterine immune and inflammatory responses, and placental morphogenesis and function.

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Introduction

The endometrium of the uterus synthesizes and secretes or selectively transports a variety of substances, collectively termed the histotroph, necessary for conceptus (embryo/fetus and associated placental membranes) survival, growth and implantation in mammals (Bazer 1975, Roberts & Bazer 1988, Fazleabas *et al.* 1997, Kane *et al.* 1997, Carson *et al.* 2000). Histotroph is a rather undefined, complex mixture of adhesion proteins, transport proteins, ions, growth factors, hormones, proteases, protease inhibitors, amino acids and other molecules (Bazer 1975, Roberts & Bazer 1988). Uterine secretions are of particular importance for conceptus survival and growth in domestic animals due to their protracted peri-implantation period and non-invasive types of placentation (Bazer 1975, Roberts *et al.* 1987, Roberts & Bazer 1988). Indeed, histotroph is absorbed by the placenta, transported into the fetal circulation and cleared by the kidney into the allantois via the urachus (Bazer 1975, Roberts &

Bazer 1988). Although the allantois was traditionally considered to be a reservoir for fetal wastes (Alexander & Williams 1968), studies in the pig and sheep support the idea that components of allantoic fluid have an important role in fetal nutrition (Bazer 1989). Analysis of recurrent pregnancy loss in the uterine gland-knockout ewe model indicates that histotroph emanating from the endometrial luminal epithelium (LE) and glandular epithelium (GE) is clearly required for peri-implantation conceptus survival and growth in sheep (Gray *et al.* 2001, 2002). However, uterine histotroph is hypothesized to remain important for conceptus growth and development throughout gestation as a complement to hematrophic nutrition derived from increased uterine blood flow through placentomes.

Recently, a new galectin family member and component of uterine histotroph, galectin-15, was discovered in the endometrium of sheep (Gray *et al.* 2004). Galectin-15 was originally identified in ovine intestinal epithelium as being induced in response to infection by *Haemonchus contortus*, a nematode parasite (Dunphy *et al.* 2000). Expression

of the galectin-15 gene was detected only after day 10 of pregnancy in the endometrial LE and superficial ductal GE (sGE) and was induced by progesterone and further stimulated by interferon τ , the pregnancy-recognition signal produced by the conceptus. In the endometrium, galectin-15 protein had a nucleocytoplasmic distribution within the LE and sGE and was also concentrated near and on the apical surface. Further, secreted galectin-15 protein was abundant in the histotroph recovered from the uterine lumen and was immunologically similar to the previously unknown 14 kDa protein that is a component of crystals in the endometrial epithelium and conceptus trophoctoderm (Gray *et al.* 2004, Kazemi *et al.* 1990). Galectins bind β -galactosides and functionally crosslink glycoprotein and glycolipid receptors on the surface of cells, which initiates biological responses that include cell proliferation, differentiation, motility, adhesion and apoptosis (Cooper 2002, Yang & Liu 2003, Liu & Rabinovich 2005). The temporal and spatial alterations in galectin-15 mRNA and protein in the uterine endometrial epithelia and lumen during the peri-implantation period of early pregnancy, combined with the known biological activities of other galectins, make it a strong candidate mediator of conceptus–endometrial interactions during implantation (Gray *et al.* 2004). One proposed extracellular role of galectin-15 in the uterine lumen is to functionally bind and crosslink β -galactosides on glycoproteins and glycolipids, thereby allowing it to function as a heterophilic cell adhesion molecule bridging the conceptus trophoctoderm and the endometrial LE and stimulating biological responses within the trophoblast, such as migration, proliferation and differentiation, which are critical for successful conceptus implantation (Spencer *et al.* 2004).

Our working hypothesis is that galectin-15 is secreted as a component of histotroph that is secreted by the endometrial epithelia throughout pregnancy in sheep. Therefore, the present study investigated: (1) extracellular and intracellular galectin-15 protein in the uterus and conceptus on days 12 to 16 of pregnancy; (2) galectin-15 mRNA and protein in uteroplacental tissues between days 20 and 120 of pregnancy; and (3) galectin-15 mRNA in the uterus after parturition.

Materials and Methods

Animals and tissue collection

Experimental and surgical procedures on crossbred Suffolk sheep (*Ovis aries*) complied with the Guide for Care and Use of Agriculture Animals in Teaching and Research, and were approved by the Institutional Animal Care and Use Committees of Texas A&M University.

Study 1

Cyclic ewes were mated at estrus (day 0) to either a vasectomized or an intact ram and were hysterectomized

($n = 5$ sheep/day) on days 12, 14 or 16 of the estrous cycle or on days 12, 14 and 16 of pregnancy (the gestation period is 147 days). For all surgeries performed, ewes were administered isoflurane (5%) via an inhalation mask to induce anesthesia, which was maintained with isoflurane (1–5%). A midventral laparotomy was performed to expose the reproductive tract. The uterine lumen was flushed with saline and examined for the presence of a morphologically normal conceptus to confirm pregnancy. Uterine flushes were clarified by centrifugation (3000g for 30 min at 4°C), aliquoted and frozen at -80°C . Cross-sections of the uterine horn ipsilateral to the ovary bearing the corpus luteum and conceptuses from day 16 pregnant ewes were fixed for analysis by electron microscopy.

Study 2

As described previously (Kwon *et al.* 2004), ewes were mated to rams of proven fertility at estrus and 12 and 24 h later. Ewes were assigned randomly to be hysterectomized ($n = 4$ per day) on day 20, 40, 60, 80, 100 or 120 of gestation to allow collection of uteroplacental tissues and fetal fluids. At hysterectomy, samples of the amniotic and allantoic fluids were collected, snap-frozen in liquid nitrogen and stored at -80°C . Total endometrium was collected on days 16 and 20 of pregnancy, whereas caruncular and intercaruncular endometria were dissected from the myometrium collected between days 40 and 120 of gestation. Portions of these tissues were snap-frozen in liquid nitrogen and stored at -80°C . Sections of the uterine wall with the attached trophoctoderm were also fixed in freshly prepared 4% (w/v) paraformaldehyde in PBS (pH 7.2) or embedded in Optimal Cutting Temperature (OCT) compound (Miles, Oneota, NY, USA), snap-frozen in liquid nitrogen and stored at -80°C .

Study 3

As described previously (Gray *et al.* 2003), multiparous 5–8-year-old ewes were bred with rams of proven fertility, and pregnancy was determined by transabdominal ultrasonography. Ewes were assigned randomly to be ovariohysterectomized on postpartum days (PPD) 1, 7, 14 or 28 ($n = 4$ or 5 ewes/day). Ewes were checked twice daily for signs of labor or parturition. At hysterectomy, the residual placentomal or caruncular and intercaruncular areas of the uterine wall were dissected from the uteri of ewes on PPDs 1, 7 or 14, and sections (~ 1 cm) were fixed in 4% (w/v) paraformaldehyde. On PPD 28, sections from the mid-portion of each uterine horn were fixed in 4% (w/v) paraformaldehyde.

After 24 h, fixed tissues from all studies were dehydrated through a graded series of ethanol solutions and embedded in Paraplast Plus (Oxford Labware, St Louis, MO, USA) for histological analyses.

Western-blot analyses

Uterine flushes from study 1 were concentrated using Centricon-3 columns (Amicon, Beverly, MA, USA). Protein content of concentrated flushes (study 1) and fetal fluids (study 2) was determined using a Bradford protein assay (Bio-Rad, Hercules, CA, USA) with BSA as the standard. For study 1, uterine flush proteins (20 µg) were separated by non-denaturing/non-reducing 12% PAGE. For study 2, allantoic and amniotic fluid proteins (20 µg) were denatured and separated by 15% SDS/PAGE. Western-blot analyses of separated protein was conducted as described previously (Spencer *et al.* 1999a) using enhanced chemiluminescence detection. Immunoreactive galectin-15 was detected using primary rabbit anti-ovine galectin-15 serum (provided kindly by Dr Els N T Meeusen, Monash University, Melbourne, Australia; Dunphy *et al.* 2000) at a 1:2500 dilution. Negative control blots were performed in which primary antibody was replaced by rabbit serum at the same concentration.

Transmission electron microscopy (TEM)

Endometrium and conceptuses from day 16 of gestation (study 1) were fixed in a solution of 2% paraformaldehyde/3% glutaraldehyde in 0.08 M sodium cacodylate (pH 7.2). After fixation, tissues were dehydrated in a gradient of 10–100% ethanol and embedded in Unicryl resin (SPI Supplies, West Chester, PA, USA). Routine staining of semi-thick sections with 0.1% Toluidine Blue and 1% sodium borate (Borax; Na₂B₄O₇·10H₂O) was utilized to isolate areas of interest. Trimmed materials were then sectioned using an LKB ultramicrotome (type 4802A). The 70–90 nm-thick sections were stained with 10% uranyl acetate in 30% ethanol for 30 min followed by Reynolds' lead citrate for 10 min (Reynolds 1963). Sections were examined and photographed using a Zeiss 10CA transmission microscope at 80 kV on Kodak electron microscope film 4489.

Immunogold TEM

Endometrium and conceptuses from day 16 of gestation (study 1) were fixed in 3% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 5% (w/v) sucrose, dehydrated through ethanol, and embedded in Unicryl resin. Ultrathin sections were picked up on 300-hex nickel grids and floated on 10% heat-inactivated normal goat serum in 0.1% BSA/Tris for 30 min. Grids were transferred to drops of rabbit anti-ovine galectin-15 serum (1:2000) or rabbit serum for 1 h at room temperature. After washing three times with 50 mM Tris (pH 7.4) in 0.1% BSA for 1 min each, five times in 50 mM Tris (pH 7.4) for 1 min each and twice in PBS for 5 min each, the grids were post-fixed in 2% glutaraldehyde in PBS for 5 min and rinsed with water. Grids were then dried at room temperature and stained lightly

with uranyl acetate in 50% (v/v) alcohol. TEM images were photographed as described above.

RNA isolation and analyses

Total cellular RNA was isolated from frozen intercaruncular and caruncular endometria from study 2 and intercaruncular endometrium from study 3 using the Trizol reagent (Gibco-BRL, Grand Island, NY, USA). For each ewe, denatured total cellular RNA (20 µg) was analyzed by slot-blot hybridization using a radiolabeled antisense cRNA probe generated by *in vitro* transcription with [α -³²P]UTP (Amersham Pharmacia Biotech, Piscataway, NJ, USA) as described previously (Gray *et al.* 2004). Plasmid templates containing cDNAs for ovine galectin-15 (Gray *et al.* 2004) and 18 S rRNA (pT718S; Ambion, Austin, TX) were used to produce radiolabeled cRNA probes as described previously (Choi *et al.* 2001). Hybridization signals were detected by exposing washed slot blots to a phosphorimager screen and visualized and quantified using a Typhoon 8600 Variable Mode Imager (Molecular Dynamics, Sunnyvale, CA, USA).

In situ hybridization

Galectin-15 mRNA was localized in uterine tissue sections by *in situ* hybridization as described previously (Spencer *et al.* 1999b). Deparaffinized, rehydrated and deproteinized uterine sections (5 µm) were hybridized with radiolabeled antisense or sense cRNAs generated from linearized ovine galectin-15 (Gray *et al.* 2004) plasmid templates using *in vitro* transcription with [α -³⁵S]UTP. Autoradiographs of slides were prepared using Kodak NTB-2 liquid photographic emulsion. Slides were stored at 4 °C for 1 week as judged from autoradiographs, developed in Kodak D-19 developer, lightly counterstained with Gill's hematoxylin (StatLab, Lewisville, TX, USA), dehydrated through a graded series of alcohol to xylene, and coverslips affixed with Permount (Fisher Scientific, Atlanta, GA, USA).

Immunofluorescence analyses

Proteins were localized in frozen intercaruncular and caruncular endometrial tissue sections (8–10 µm) collected in study 2 by immunofluorescence staining as described previously (Johnson *et al.* 1999). Briefly, frozen tissues were sectioned (8 µm) with a Hacker-Bright OTF cryostat (Hacker Instruments, Fairfield, NJ, USA) and mounted on Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA, USA). Frozen sections were fixed in –20 °C methanol for 10 min, permeabilized with 0.3% Tween-20 (v/v) in 0.02 M PBS, and then blocked in antibody dilution buffer [two parts 0.02 M PBS/1.0% BSA (w/v)/0.3% Tween-20 (w/v; pH 8.0) and one part glycerol] containing 5% normal goat serum (v/v) for 1 h at room temperature, and incubated overnight at 4 °C with the rabbit anti-ovine galectin-15 antibody (1:1000) or rabbit serum (1:1000).

Immunoreactive protein was then detected using a fluorescein-conjugated secondary antibody for 1 h at room temperature. Slides were overlaid with Prolong antifade mounting reagent and then coverslips added (Molecular Probes, Eugene, OR, USA).

Photomicroscopy

Photomicrographs of *in situ* hybridization slides were taken using a Nikon Eclipse E1000 photomicroscope (Nikon Instruments, Melville, NY, USA). Digital images were captured using a Nikon DXM 1200 digital camera and assembled using Adobe Photoshop 7.0 (Adobe Systems, Seattle, WA, USA). For immunofluorescence analyses, representative fluorescence images were recorded using a Zeiss Axioplan microscope (Carl Zeiss, Thornwood, NY, USA) equipped with a Hamamatsu chilled 3CCD color camera (Hamamatsu Photonics, Bridgewater, NJ, USA) using Adobe Photoshop 7.0 image-capture software. TEM negatives were converted to digital images using an Epson Perfection 3200 photo scanner (Epson America, Long Beach, CA, USA) and assembled using Adobe Photoshop 7.0.

Statistical analyses

All quantitative data were subjected to least-squares analysis of variance using the General Linear Models procedures of the Statistical Analysis System (Cary, NC, USA). Analyses of steady-state levels of endometrial mRNA determined by slot-blot hybridization included the 18 S rRNA as a covariate to correct for differences in sample loading. Least-squares regression analyses were used to determine effects of gestational or postpartum day on endometrial galectin-15 mRNA levels. In all analyses, error terms used in tests of significance were identified according to the expectation of the mean squares for error. Data are presented as least-squares means with overall S.E. values.

Results

Galectin-15 in uterine flush and fetal fluids

Western-blot analyses of uterine flushes from cyclic and pregnant ewes (study 1) revealed multimeric forms of immunoreactive galectin-15 protein separated by non-denaturing/non-reducing PAGE (Fig. 1). Although the predicted size of galectin-15 from the inferred amino acid sequence was only 15.5 kDa (Gray *et al.* 2004), immunoreactive galectin-15 forms of ~60 and 80 kDa were observed in the uterine flush of day 14 and 16 cyclic ewes and day 14 pregnant ewes. Interestingly, immunoreactive galectin-15 forms of ~60, 80 and 100 kDa were observed in the uterine flush of day 16 pregnant ewes. Multimeric forms of galectin-15 protein were only minimally present, or were absent, in the uterine flush of day 12 cyclic or pregnant ewes.

Figure 1 Analysis of immunoreactive galectin-15 protein in the uterine lumen of early pregnant ewes. Uterine flush proteins were separated under native conditions. Western-blot analyses found that immunoreactive galectin-15 was present as multimers of ~60, 80 or 100 kDa. The predicted size of the galectin-15 monomer is 15.5 kDa. Positions of prestained molecular-mass standards ($\times 10^{-3}$) are indicated on the left.

TEM analysis of galectin-15 in the endometrium and conceptus trophoderm

Crystalline inclusion bodies were observed in both the endometrial LE (Fig. 2) and conceptus trophoderm (Fig. 2) from day 16 pregnant ewes. These crystal structures were localized in the cytoplasm near the nucleus in the LE cells and were more abundant in the conceptus trophodermal cells. The crystals appeared to be rhomboidal and were surrounded by a membrane. Immunogold labeling localized galectin-15 protein primarily to the crystalline inclusion bodies in both endometrial LE and conceptus trophoderm.

Galectin-15 mRNA and protein in uteroplacental tissues

In pregnant ewes (study 2), steady-state levels of galectin-15 mRNA were very low to undetectable in the carcuncular endometrium (Fig. 3). Galectin-15 mRNA expression in the intercaruncular endometrium was lowest during early pregnancy from days 16 to 40, increased 3.5-fold between days 40 and 60 of pregnancy, and remained elevated to day 120 (quadratic effect of day, $P < 0.001$).

Galectin-15 mRNA was observed specifically in the endometrial LE, if present, and sGE, but not middle to deep GE, stroma, myometrium or placenta (Fig. 4). Between days 20 and 40 of pregnancy, galectin-15 mRNA was detected only in the sGE of the endometrium, because the LE was absent. During ovine placentation, the LE fuses with the trophoblast giant binucleate cells (BNCs) to form a syncytia beginning on day 16 when the BNCs differentiate (Wimsatt 1951, Wooding 1984). The LE reappears in the intercaruncular endometrial areas between days 40 and 60. On day 60 and thereafter,

Figure 2 Structural analysis and galectin-15 localization in endometrial LE and conceptus trophoderm. Cross-sections of endometrium and filamentous conceptus collected on gestational day 16 were analyzed via TEM. Crystalline structures were present in both the endometrial LE and trophoderm. Galectin-15 protein in the cells was visualized by immunogold TEM (immunogold-EM) using a rabbit anti-ovine galectin-15 antibody. Immunoreactive galectin-15 was localized to these crystals as indicated by the gold labeling. Nu, nucleus; Cr, crystal protein; Li, lipid. Scale bar, 0.1 μ m.

galectin-15 mRNA was detected in both the LE and sGE of the intercaruncular endometrium. Galectin-15 mRNA was not detected in the caruncular or cotyledonary areas of the placentomes (data not shown).

Immunoreactive galectin-15 was detectable at the fetal–maternal interface throughout gestation (Fig. 5). On day 40 of pregnancy, galectin-15 was observed at the apical surface of the sGE. By day 60, galectin-15 protein was observed on the LE as well as within the overlying trophoderm of the chorion. This spatial pattern of expression was consistent throughout the latter portion of pregnancy.

Figure 3 Steady-state levels of galectin-15 mRNA in the intercaruncular (ICAR) and caruncular (CAR) endometrium during pregnancy as determined by slot-blot hybridization analysis. Data are presented as relative units (least-squares means $\pm$ s.e.). Galectin-15 mRNA levels increased after day 40 of pregnancy ($P < 0.001$, quadratic effect of day).

Galectin-15 protein was undetectable in the caruncular or cotyledonary areas of the placentomes (data not shown).

Immunoreactive galectin-15 was detectable in allantoic fluid throughout gestation. In the allantoic fluid, galectin-15 appeared as a single immunoreactive protein of approximately 15 kDa in size, as analyzed by SDS/PAGE under reducing and denaturing conditions (Fig. 6). Galectin-15 protein was not detectable in amniotic fluid on any day of gestation (data not shown).

Galectin-15 mRNA in the postpartum uterus

In postpartum ewes (study 3), steady-state levels of galectin-15 mRNA tended to be highest on PPD 1 and decreased through PPD 28 (linear effect of day, $P = 0.08$; Fig. 7A). In study 3, galectin-15 mRNA was localized primarily to the LE during the postpartum period, with low expression also detected in the sGE (Fig. 7). On PPD 1, galectin-15 mRNA expression was variable, ranging from high to moderate or low abundance depending on the ewe. The pattern of galectin-15 expression in the LE was variegated, particularly on PPDs 1 and 7. Expression of galectin-15 mRNA was still detected in some areas of the LE on PPD 28.

Discussion

Available results indicate that the galectin-15 gene is expressed in the endometrial LE and sGE of the intercaruncular endometrium of the pregnant ovine uterus from day 12 to term, encompassing the periods of implantation and placentation, as well as during uterine involution after parturition. In cyclic and early pregnant ewes, galectin-15

Figure 4 *In situ* hybridization analysis of galectin-15 mRNA expression in the intercaruncular areas of the endometrium from the uterus of pregnant ewes. Cross-sections of the uterine wall were hybridized with radiolabeled antisense or sense galectin-15 cRNA probes. Hybridized sections were digested with RNase A, and protected transcripts were visualized by liquid emulsion autoradiography. Developed slides were counterstained lightly with hematoxylin, and photomicrographs were taken under brightfield or darkfield illumination. Expression of galectin-15 mRNA was detected only in the endometrial LE and sGE. D, day; Tr, trophoblast. Scale bar, 100 μ m.

mRNA was also confined to the endometrial LE and sGE after day 10 (Gray *et al.* 2004). In that study, progesterone induced and interferon τ stimulated galectin-15 gene expression in the endometrium. In the present study, analysis of galectin-15 in uterine flushes indicated that secreted extracellular galectin-15 is in a multimeric form, particularly as the amount of galectin-15 mRNA increases in the endometrial LE and sGE and galectin-15 protein increases in the uterine lumen on days 14 and 16 of pregnancy (Gray *et al.* 2004). Other galectin family members are known to homodimerize and form multimers (Cho & Cummings 1996, Yang *et al.* 1998, Dunphy *et al.* 2002). The effects of galectin multimerization on biological function are not well understood, particularly in regard to ability of galectins to bind to glycoproteins and/or glycolipid receptors and initiate biological responses.

Immunogold electron microscopy revealed that within the endometrial LE and conceptus trophoblast, galectin-15 protein was localized to large, membrane-bound rhomboidal crystal structures. The working hypothesis is that galectin-15 protein forms crystals within the endometrial LE and is also secreted into the uterine lumen as a part of histotroph. The conceptus trophoblast then absorbs extracellular galectin-15 in the uterine histotroph, wherein it becomes a component of intracellular crystals as originally described for the 14 kDa protein, e.g. galectin-15, by Kazemi and coworkers (Kazemi *et al.* 1990). In the ovine conceptus trophoblast, crystals were found to increase in number and size between days 10 and 18 of pregnancy (Wintenberger-Torres & Flechon 1974). The presence of crystals in sheep uterine histotroph (or uterine

milk), present between maternal and fetal intercotyledonary membranes, is well documented (Wimsatt 1951, Hoffman & Olson 1984). Progesterone-induced crystal structures in the endometrium and/or conceptus trophoblast have been described in the sheep (Wintenberger-Torres & Flechon 1974, Hoffman & Olson 1984, Kazemi *et al.* 1990), mouse (Calarco & Szollosi 1973), rabbit (Nakoa *et al.* 1971, Daniel & Kennedy 1978) and human (Nakoa *et al.* 1971). Although the biological role(s) of galectin-15 crystals in the uterine epithelia is not known, the intracellular role of other galectins include modulation of cell growth, differentiation and apoptosis through functioning as pre-mRNA splicing factors and interacting with specific intracellular ligands such as Ras and Bcl-2 (Hernandez & Baum 2002, Liu *et al.* 2002).

During synepitheliochorial placentation in sheep, trophoblast giant BNCs begin to differentiate between days 14 and 16 and then fuse apically with the endometrial LE and form syncytia, thereby assimilating and replacing the endometrial LE (Wimsatt 1951, Wooding 1984). In the intercaruncular endometrium, the LE reappears between days 40 and 60 of pregnancy, and this was associated with an increase in galectin-15 mRNA in the intercaruncular endometrium in the present study. Galectin-15 mRNA and protein expression was not observed in the placentomes, which are comprised of placental cotyledons and endometrial caruncles devoid of LE. Although the placenta is expelled shortly after parturition, regrowth of the entire LE covering the endometrial caruncles takes at least 4 weeks during postpartum involution of the uterus (Gray *et al.* 2003). The expression of galectin-15 mRNA in

Figure 5 Immunofluorescence localization of galectin-15 protein in the intercaruncular areas of the endometrium from uteri of pregnant ewes on days (D) 40, 60, 80, 100 and 120. Galectin-15 protein was observed predominantly in the conceptus trophoblast and, to a lesser extent, in the endometrial LE and sGE. S, stroma; Tr, trophoblast. Scale bar, 50 μ m.

the epithelia is variegated during the postpartum period and steady-state levels declined slowly over the month following parturition. This unique expression pattern suggests that the phenotype of the endometrial epithelia is changing rapidly during uterine involution.

Although galectin-15 mRNA was found exclusively in the endometrial LE and sGE of the uterus, immunoreactive galectin-15 protein was detected predominantly in the chorion/trophoblast of the placenta as well as in the allantoic fluid. Galectin-15 protein is present in the uterine lumen of early pregnant ewes as well as in uterine milk recovered from the non-gravid horn of unilaterally pregnant ewes (Gray *et al.* 2004). Other galectin family members also exhibit dual localization, being found in both extracellular (cell-surface and medium) and intracellular (cytoplasm and, in some cases, the nucleus) compartments (Hughes 1997). Although galectins are often present on cell surfaces or in the extracellular matrix,

galectins lack a classical secretion signal sequence and are secreted by a novel apocrine mechanism that does not involve the endoplasmic reticulum–Golgi pathway (Hughes 1999, Boulianne *et al.* 2000). Similarly, ovine galectin-15 lacks a signal peptide (Dunphy *et al.* 2000, Gray *et al.* 2004). After implantation, the chorioallantois develops unique structures, termed areolae, that develop over the mouth of each uterine gland as specialized areas for absorption and transport of uterine histotroph into the conceptus (Bazer 1975). These results support the idea that galectin-15 protein is synthesized by the endometrial LE and sGE and then secreted into the uterine lumen, where it is absorbed by the placenta, transported into the fetal circulation, and cleared by the kidney into the allantois via the urachus (Bazer 1975, Roberts & Bazer 1988). Although the allantois was initially considered a reservoir for waste products of the fetus, it serves to store most secreted proteins from the endometrium (Bazer *et al.*

Figure 6 Analysis of immunoreactive galectin-15 protein in the allantoic fluid of pregnant ewes. Allantoic fluid proteins were separated under reducing conditions. Western-blot analysis found a single immunoreactive protein of ~15 kDa in all allantoic fluid samples. Positions of prestained molecular-mass standards ($\times 10^{-3}$) are indicated on the left.

1975, Moffatt *et al.* 1987). In contrast, amniotic fluid is not in the path for protein clearance by the fetal kidney and, therefore, does not function in this capacity. In the present study, galectin-15 protein was not detected in amniotic fluid, but was present in allantoic fluid where it may simply serve as a source of amino acids for fetal and placental growth and function or possess other biological functions. During human pregnancy, galectin-13 (or placental protein-13) plays a role in calcium-mediated depolarization, liberation of arachidonic acid and the formation of thromboxane in the placenta (Burger *et al.* 2004). Indeed, a reduction in galectin-13 is associated with intrauterine growth retardation (Burger *et al.* 2004).

Another potential biological function of endometrial galectin-15 throughout pregnancy and during involution may be to modulate inflammatory and immune responses. Under the influence of progesterone, uterine lymphocyte dynamics and functions are altered that inhibit maternal responses to the fetal-placental semi-allograft which is critical for pregnancy success (Clark *et al.* 1999). As in other mammals, the ovine uterus has pregnancy-associ-

Figure 7 Spatial and temporal patterns of galectin-15 mRNA expression during the postpartum period. (A) Steady-state levels of galectin-15 mRNA in the endometrium during the postpartum period as determined by slot-blot hybridization analysis. Data are presented as least-squares means $\pm$ s.e. Galectin-15 mRNA levels declined after parturition ($P = 0.08$, linear effect of day). (B) *In situ* hybridization analysis of galectin-15 mRNA expression in the intercaruncular areas of the endometrium of the postpartum ovine uterus. Cross-sections of the uterine wall were hybridized with radiolabeled antisense or sense cRNA probes generated from linearized plasmid cDNA clones. Hybridized sections were digested with RNase A, and protected transcripts were visualized by liquid emulsion autoradiography. Developed slides were counterstained lightly with hematoxylin, and representative photomicrographs were taken under brightfield or darkfield illumination. Expression of galectin-15 mRNA was detected only in the endometrial LE and sGE. S, stroma; V, blood vessel. Scale bar, 100 μ m.

ated changes in lymphocytes, macrophages and natural killer cells which are differentially regulated by conceptus and maternal factors (Gogolin-Ewens *et al.* 1989, Lee *et al.* 1992, Fox *et al.* 1998, Tekin & Hansen 2002, 2004). Evidence suggests that progesterone stimulates the endometrium to induce synthesis of regulatory molecules that affect lymphocyte dynamics (Majewski *et al.* 2001). A progesterone-stimulated immunoregulatory protein family is the uterine serpins or uterine milk proteins (Leslie & Hansen 1991, Hansen 1998). Similarly, galectin-15 is also induced by progesterone and a component of uterine milk (Gray *et al.* 2004). Interestingly, galectin-15 was originally found to be induced in gastrointestinal tissue and secreted into the intestinal lumen in response to inflammation and eosinophil infiltration after infection of sheep with the helminth, *Haemonchus contortus* (Dunphy *et al.* 2000). Given that several other galectins regulate innate and adaptive immune responses (Rabinovich *et al.* 2002, Young & Meeusen 2004), one may speculate that galectin-15 in the ovine endometrium is another progesterone-induced immunoregulatory factor that modulates the maternal intrauterine immune system, thereby protecting the conceptus and promoting placental growth, which is a process strikingly similar to host–parasite interactions (Guimond *et al.* 1999). Future experiments will assess the intracellular and extracellular role(s) of galectin-15 in regulation of uterine immune responses, placental morphogenesis and function, and fetal growth.

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