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Research

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## Immunohistochemical localization of integrin alpha V beta 3 and osteopontin suggests that they do not interact during embryo implantation in ruminants

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### Abstract

**Background:** It has been suggested that trophoblast attachment requires co-expression of integrin alpha V beta 3 and its ligand osteopontin at the fetal-maternal interface. Until now the expression patterns of integrin alpha V beta 3 and osteopontin in the pregnant bovine uterus were unknown. The objectives of this study were to localize integrin alpha V beta 3 and osteopontin in bovine and sheep endometrium during the periimplantation period and to compare the distribution patterns using antibodies that had not yet been tested in sheep.

**Methods:** Cell compartments within endometrial tissue sections were scored for immunohistochemical staining intensity and data were analyzed to determine the effects of day of pregnancy or cycle.

**Results:** In pregnant bovine endometrium, integrin alpha V beta 3 was detected in luminal epithelium, stroma, myometrium and smooth muscle. A strong band of immunoreactivity was observed in the subepithelial stroma of intercaruncular regions, but there was reduced reactivity in the caruncles and glands. Bovine trophoblast did not express integrin alpha V beta 3 at any stage of pregnancy. In ovine endometrium a different pattern of staining for integrin alpha V beta 3 was observed. Reactivity was not present in the luminal epithelium or trophoblast. There was strong staining of the deep glands and no reactivity in the superficial glands. Osteopontin distribution was similar for sheep and cattle. For both species, apical staining was present on the luminal epithelium and glands and on embryonic tissues.

**Conclusion:** In ruminants, integrin alpha V beta 3 and osteopontin do not co-localize at the fetal-maternal interface indicating that these proteins could not interact to facilitate embryo attachment as has been proposed in other species.

### Introduction

Comparative studies of placentation in domestic animals show that implantation is similar among these species [1-4]. However differences in the molecular properties of

receptive endometrium are just now being explored. Placentation in ruminants is categorized as non-invasive as chorionic cell migration into maternal tissue is restricted to the luminal epithelium [5,6]. The preimplantation

period in cattle and sheep is longer than in rodents, carnivores and primates. This period coincides with secretion of pregnancy supporting proteins from the glandular epithelium and trophoblast production of the pregnancy recognition factor, interferon-tau (IFN- $\tau$ ) [7]. Unique to ruminants are caruncles. These dense aglandular connective tissue regions are covered by columnar epithelium and their location defines where the finite placentae will form [1,8]. The molecular determinants which prevent villus development in the intercaruncular endometrial tissue, or promote development in the caruncles, are not yet identified.

Integrins are heterodimeric glycoproteins that facilitate cell-cell and cell-extracellular matrix attachment and are key facilitators of cellular processes involved in tissue remodeling such as cell migration and de-adhesion [9-11]. In many species endometrial expression of integrins and their extracellular matrix (ECM) ligands is correlated with embryo attachment, and uterine differentiation during the receptive phase and pregnancy [12-17]. The most promiscuous integrin in terms of ligand interactions is integrin  $\alpha_v\beta_3$ , which binds Arg-Gly-Asp (RGD) motifs in fibronectin, osteopontin and laminin, among others [18,19]. The affinity of integrins for their ligands is dependent on cell type, and a change in the activation state that is mediated by phosphorylation of the cytoplasmic domain (inside out signaling), and ligand availability (outside in signaling) [20,21]. Integrin activity is augmented in the presence of growth factors such as the increased binding of integrin  $\alpha_v\beta_3$  observed in smooth muscle cells in the presence of insulin-like growth factor I (IGF-1) [22].

Osteopontin was first described as a secreted 60-kDa phosphoprotein associated with bone ECM and as a lymphokine expressed by activated lymphocytes and macrophages [23]. Expression has since been identified on the epithelium of many tissues such as kidney, breast, and the reproductive tract [24]. Osteopontin expression and distribution differs between cycling and pregnant sheep; little secretion occurs in the glands of cycling animals while secretion increases from glandular epithelium during the periimplantation period [25]. As a secreted protein of the ruminant uterus regulated by progesterone, osteopontin has been proposed to support conceptus growth and act as an adhesive between trophoblast and luminal epithelium via integrin  $\alpha_v\beta_3$  [25,26].

In mice, humans, baboons and sheep, it has been postulated that co-expression of integrin  $\alpha_v\beta_3$  and its ligand osteopontin on uterine epithelium is required for trophoblast attachment and/or invasion; blocking integrin  $\alpha_v\beta_3$  in mice prevents embryo implantation [15,16,25,27]. Potential osteopontin receptors include

CD44 and integrins  $\alpha_v\beta_3$ ,  $\alpha_v\beta_1$ ,  $\alpha_9\beta_1$ ,  $\alpha_v\beta_5$  and  $\alpha_5\beta_1$  [28]. In sheep, several of these integrin subunits are expressed on the apical surface of uterine epithelium and on conceptus trophoblast, although no change in their expression patterns has been observed between cyclic and pregnant ewes [16,29]. Despite the similarity in placental anatomy of sheep and cattle this widespread distribution pattern of  $\alpha_v\beta_3$  is not observed in cycling cattle. In cycling heifers, integrin  $\alpha_v\beta_3$  is most strongly expressed in intercaruncular subepithelial stroma, and its expression is temporally regulated with the estrous cycle at least partially under the influence of the reproductive steroids [14,30].

Until now the distributions of integrin  $\alpha_v\beta_3$  and its ligand osteopontin have not been studied in the pregnant cow. The distribution of these proteins has been studied in cycling and pregnant sheep, however, the integrin  $\alpha_v\beta_3$  results were unlike what we observed in cycling cattle. This is surprising since histologically placentation in sheep and cows is similar. The objectives of the present study were to localize the expression of integrin  $\alpha_v\beta_3$  in bovine and sheep endometrium during the periimplantation period and to compare the distribution patterns using two monoclonal antibodies that had not been tested in sheep. In addition, the expression of osteopontin during the estrous cycle and periimplantation period in bovine and ovine endometrium was examined using an antibody to bovine osteopontin.

## Materials and methods

### Animals

All procedures performed were in accordance with the guidelines of the Canadian Council on Animal Care and were reviewed and approved by the Nova Scotia Agricultural College Animal Care and Use Committee. Sexually mature heifers of mixed beef breeds were synchronized to estrus using Estrumate® (500  $\mu$ g cloprostenol, Schering Canada Inc.; QUE, Canada). Animals were observed for estrous behavior three times a day and had cycle lengths of 19 to 20 days. Uteri were collected at slaughter on days 0 (estrus), 1, 3, 6, 10, 14, 15, 16, 17, 18, 19 and 20 of the estrous cycle, or days 16, 18, 21, 24 or 30 of pregnancy with at least three animals at each day. To confirm estrous cycle stage, blood samples taken at heat and slaughter were assayed for progesterone by radioimmunoassay (RIA) using a Coat-A-Count kit (Diagnostic Products Corporation, Los Angeles CA).

Mature Rideau Arcott ewes were synchronized to estrus using intravaginal progesterone pessaries for 14 days followed by observation for estrous behavior in the presence of an intact ram. Ewes were randomly assigned to either cyclic or pregnant status, and mating to rams occurred over a 2-day period. Reproductive tracts were collected on

days 13 and 17 of the cycle, and on day 13 of pregnancy ( $n = 5$ ).

A single sample of mammary gland parenchyma was excised from a randomly selected lactating cow at a provincial abattoir to serve as a positive control tissue for osteopontin antibody reactivity.

### **Tissue collection**

Ovarian dating was performed at collection according to the criteria of Ireland [31] and pregnancy was established by the presence of an embryo or trophoblast in the uterine lumen. The ipsilateral horn was dissected into 1 cm<sup>3</sup> cross-sectioned blocks and frozen in liquid nitrogen (-196°C) at the collection site before transfer to an ultra low temperature freezer (-80°C) for storage.

### **Antibodies**

Two murine monoclonal antibodies specific to human integrin  $\alpha_v\beta_3$  were used for immunohistochemical localization. Clone LM609 (Chemicon, Temecula, CA) targeted the functional binding region of the integrin  $\alpha_v\beta_3$  complex of several species, including cattle [32], and clone BV4 (Abcam product number ab7167, Novus Biologicals, Littleton, CO) was directed against the full length integrin  $\alpha_v\beta_3$  of endothelial cells. Osteopontin was localized using an anti-bovine milk osteopontin polyclonal antibody [33] kindly provided by Dr. G. Killian (Pennsylvania State University, Almqvist Research Centre, University Park, PA). Horseradish peroxidase (HRP) conjugated donkey-anti-rabbit IgG (Pierce, Rockford, IL) was used for detection of osteopontin. Negative controls were rabbit IgG (ICN Biomedical Inc., Aurora, ON, Canada) or purified mouse IgG (Chemicon) and were used at the same concentration as primary antibodies. Optimal dilutions of primary antibodies were empirically determined, and fixation and detection methods were selected based on the conditions that allowed for maximum specific immunostaining.

### **Western blotting**

To determine reactivity of bovine anti-milk osteopontin antibodies with bovine and ovine endometrium, 0.3 g samples of endometrial and mammary gland tissues were thawed and homogenized in 2 ml extraction buffer (50 mM Tris, pH 8.0, 300 mM NaCl, 20 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1 mM EDTA, 1 mM EGTA, 10% v/v glycerol, 1% v/v NP-40, 0.5 mM PMSF and 10 µg/mL aprotinin). Concentrations of protein were determined using a Bradford protein assay (Bio-Rad Laboratories, Hercules, CA) with BSA as the standard. Following denaturation in Laemmli buffer, proteins (200 µg) were separated using 10% SDS-PAGE and blotted to PVDF membrane. Blots were blocked in PBS containing 0.05% (v/v) Tween-20 (PBS-T) and 2% (w/v) BSA for 60 min at

RT. Blots were washed 3 times for 5 min each in PBS-T, then incubated for 2 h in PBS-T containing 1% BSA and 2.5 µg/ml anti-bovine osteopontin or rabbit IgG (negative control). Blots were washed 3 times for 5 min each, then incubated for 1 h in PBS-T containing 1% BSA and donkey anti-rabbit IgG conjugated to horseradish peroxidase. Following 3 washes for 5 min each in PBS-T, and one wash in PBS, immunoreactive proteins were detected using a chemiluminescent substrate (ECL<sup>®</sup>, Amersham Biosciences Inc., Baie d'Urfe, Quebec, Canada).

### **Immunohistochemistry**

Immunoperoxidase staining for integrins was performed on 5 to 8 µm cryostat cross-sections of endometrium. Serial sections were mounted on 3-aminopropyl-triethoxysilane-coated-Superfrost Plus<sup>®</sup> slides (Fisher Scientific, Whitby, ON, Canada), then fixed in acetone for 10 min and air dried overnight. Cut sections were stored at -80°C until use. Sections were blocked with 2% w/v BSA in PBS (pH 7.2 to 7.4) for 30 min. Primary antibodies were diluted in 2% w/v BSA in PBS and applied at concentrations of 10 µg/ml for anti-osteopontin, 0.6 µg/ml for anti- $\alpha_v\beta_3$  clone LM609 (Chemicon), and 0.2 µg/ml for anti- $\alpha_v\beta_3$  clone BV4 (Novus Biologicals). All primary antibodies were allowed to bind for 2 h, then the slides were washed in PBS 3 times for 3 min each, and secondary antibody was applied and allowed to react for 45 min followed by washing in PBS. For amplification of integrin  $\alpha_v\beta_3$  signal, the Vectastain Elite ABC kit<sup>®</sup> (Vector Laboratories, Burlingame, CA) was used. To prevent non-specific staining, avidin (reagent A) was used at half the recommended concentration. Immunostaining was visualized using the chromogen metal enhanced diaminobenzidine (integrin  $\alpha_v\beta_3$ ; Pierce) or 3-amino-9-ethylcarbazole (osteopontin; Sigma, Saint Louis, MO). Slides were counterstained with Mayers Hematoxylin (Electron Microscopy Sciences, Fort Washington, PA) and coated with Aquaperm<sup>®</sup> mounting media (Fisher). Slides were viewed on a Leica DMLB microscope (Leica Microsystems, Wetzlar, Germany) and photographed on Ektachrome 160T tungsten film (Eastman Kodak, Rochester, NY). Slides were scanned and images assembled in Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA). All experiments were replicated at least three times for each animal on different days.

### **Statistical analysis**

Cell compartments within sections were scored by two independent observers on a six point scale (0-negative, 1-very weak, 2-weak but clearly positive, 3-intermediate, 4-high, and 5-very high) for staining intensity. Data were checked for normalcy using the univariate procedure in SAS<sup>™</sup> (Statistical Analysis Software Version 8, SAS Institute Inc. Cary NC) and log transformed if transformation corrected to a normal distribution. Normal data were

analyzed using a general linear models procedure to examine the effect of day on staining intensity, and the Duncan's multiple range test was used to compare population means. Nonparametric statistical comparisons between day of cycle, day of pregnancy or cycle stage (stage 1-metestrus, stage 2-early diestrus; stage 3-late diestrus; stage 4-proestrus and estrus) were made by the Kruskal-Wallis test of population medians [34]. When significant effects were observed, Mann-Whitney's two sample rank test [34] was used to test the equality of population medians. The selected alpha level was 0.05.

## Results

### **Immunohistochemical localization of integrin $\alpha_v\beta_3$ in early pregnant bovine endometrium**

At all stages examined integrin  $\alpha_v\beta_3$  was detected in luminal epithelium, endometrial stroma, myometrium and arteriolar smooth muscle (Fig. 1). There was no difference in staining intensity or the distribution pattern in tissue sections treated with antibody to integrin  $\alpha_v\beta_3$  from either Chemicon or Novus Biologicals. As previously observed [14], sections prepared from heifers at day 18, but not day 16, of the estrous cycle showed a band of strong immunoreactivity in the stromal cells underlying the intercaruncular luminal epithelium and presumably in contact with basement membrane matrix, with reduced staining evident in the same region of the caruncles (Fig. 1B,1C). A diffuse weak reactivity was present throughout the stroma. Samples from days 16 and 18 of pregnancy showed a similar pattern of expression to the day 18 cycling heifers (Fig. 1C,1D,1E). Trophoblast did not express the antigen at any of the stages examined. Expression of integrin  $\alpha_v\beta_3$  in luminal epithelium was evident from days 18 to 24 of pregnancy (Fig. 1D,1E,1F,1G,1H). As binucleate cell migration proceeded to modify the luminal epithelium, reactivity to integrin  $\alpha_v\beta_3$  decreased in the hybrid luminal epithelium and increased in the underlying stroma (Fig. 1E,1F,1G,1H and Fig. 2). Reactivity scores were always highest in intercaruncular tissues and weak, if any, staining was detected in the caruncles (Fig. 1).

### **Immunohistochemical localization of integrin $\alpha_v\beta_3$ in ovine endometrium**

Both monoclonal antibodies to integrin  $\alpha_v\beta_3$  showed similar distribution patterns and reacted strongly with glandular epithelium and blood vessels in ovine endometrium, but did not react with luminal epithelium or trophoblast (Fig. 3). For all ewes examined, reactivity was extremely high in the deep glandular epithelium but absent from the most superficial glands ( $P < 0.05$ ; Fig. 3A,3B). Although there was some staining of the compact stroma in intercaruncular regions, reactivity was diffuse and not concentrated in the region of the basal lamina as seen in sections from cattle. Staining of the caruncular subepithelial stroma was consistently very weak or absent,

indicating differential staining between caruncular and intercaruncular regions (Fig. 3A,3C,3E). The deep caruncular stroma reacted positively with the anti-integrin  $\alpha_v\beta_3$  antibodies.

### **Osteopontin distribution in cyclic bovine endometrium**

Western blotting of total proteins extracted from cyclic bovine and ovine endometrium indicated that the antibody to bovine milk osteopontin specifically recognized the reported 70, 45, 34 and 24 kDa forms of the protein in both species (data not shown). Osteopontin localized to the blood vessel walls and to the uterine epithelium of bovine endometrium throughout the estrous cycle (Fig. 4A,4B,4C). The pattern of staining suggested intracellular accumulation as well as apical secretion of the phosphoprotein and reactivity was strongest in glandular epithelium in comparison to the luminal epithelium ( $P < 0.05$ ; Fig. 4 and Fig. 5A). An effect of cycle stage was observed on luminal epithelium, where reactivity ranged from weak to moderate and was least evident in sections collected from late luteal phase animals ( $P < 0.05$ ; Figs. 4B and 5A). There was no influence of cycle stage on osteopontin scores in blood vessels or glands ( $P < 0.05$ ).

### **Osteopontin distribution in early pregnant bovine endometrium**

The distribution of osteopontin was similar in cryosections prepared from pregnant animals to those prepared from cyclic animals, although reactivity scores for the apical surface of the luminal and glandular epithelium decreased with advancing pregnancy (Fig. 4D,4E,4F,4G). Staining was lowest in glandular epithelium in samples from day 30 of pregnancy in comparison to other pregnancy stages examined ( $P < 0.05$ ; Fig. 4G and Fig. 5B). Trophoblast was weakly reactive, and in many sections from day 24 and day 30 pregnant animals, extraembryonic membrane luminal to trophoblast, probably allantois, expressed moderate to high levels of osteopontin (Fig. 4E,4F,4G).

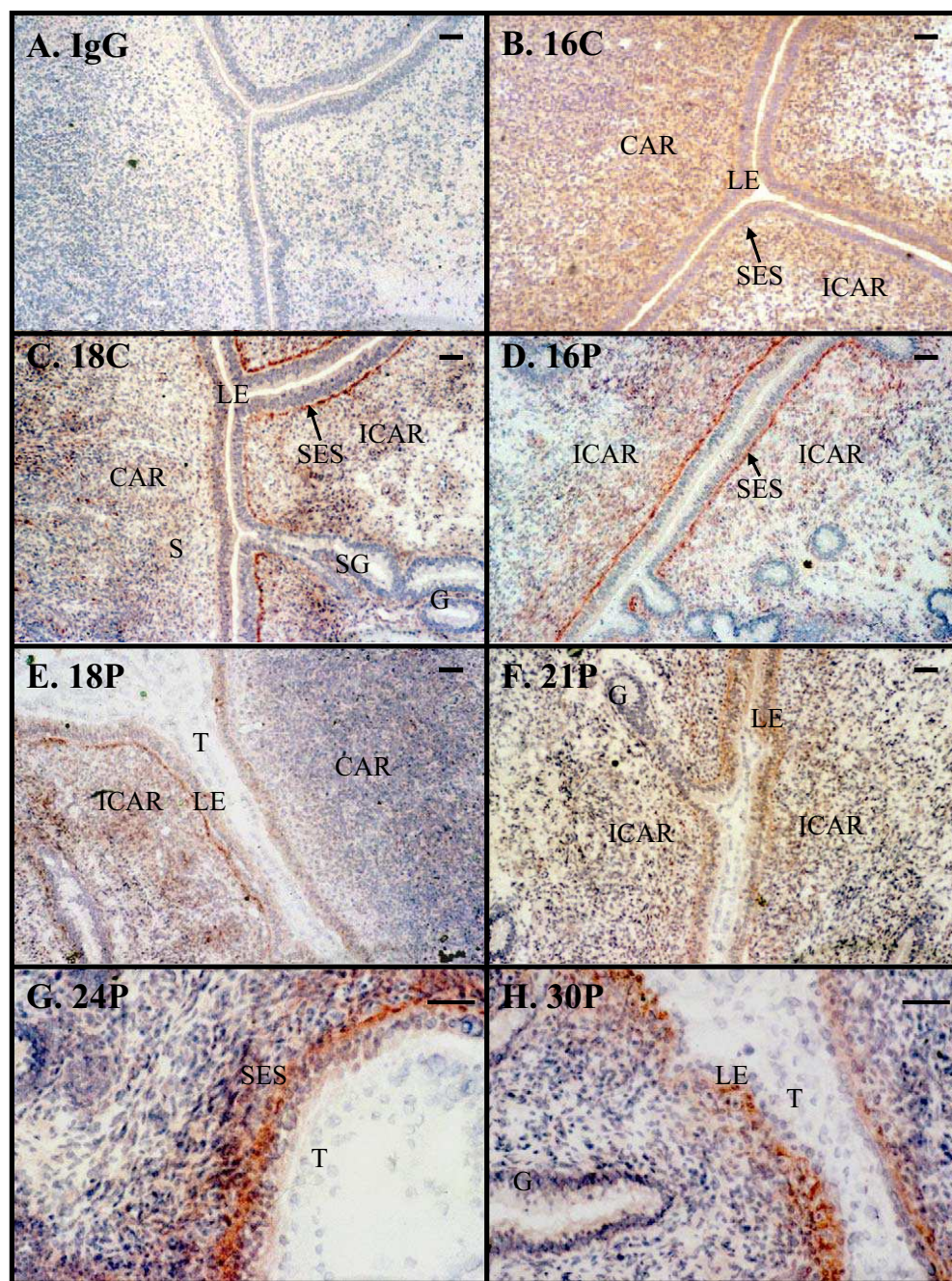
### **Distribution of osteopontin in ovine endometrium**

Similar to the staining patterns observed in cows, in sheep osteopontin was immunolocalized to the apical surfaces of uterine epithelium, the smooth muscle of blood vessels, and when present, trophoblast (Fig. 6). No difference in reactivity was observed between ewes regardless of pregnancy status ( $P > 0.05$ ).

## Discussion

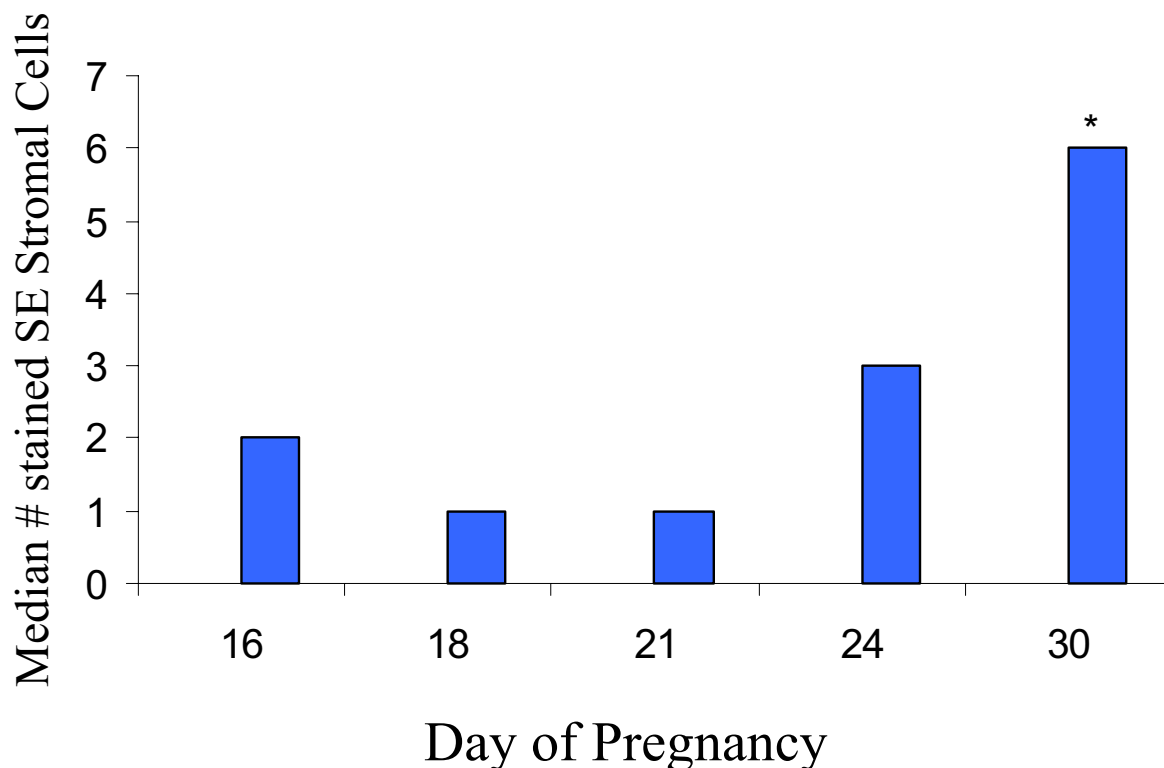
In pregnant bovine endometrium reactivity scores for integrin  $\alpha_v\beta_3$  were always highest in the stroma underlying the luminal epithelium of intercaruncular tissues, and weak or no staining was detected in the caruncles or stroma underlying the glands. This concurs with our previous study of cycling cattle [14]. In cows, integrin  $\alpha_v\beta_3$





**Figure 1**

Immunohistochemical localization of integrin  $\alpha_v\beta_3$  in cryostat cross sections of bovine endometrial tissue during the estrous cycle (**A** and **C**, day 18 cyclic, **B**, day 16 cyclic) and at different stages of pregnancy (**D**, day 16 pregnant, **E**, day 18 pregnant, **F**, day 21 pregnant, **G**, day 24 pregnant, **H**, day 30 pregnant). Positive antibody reactivity (shown as brown) was strongest in intercaruncular (ICAR) subepithelial stroma (SES) as indicated by the arrow, except at day 16 of the estrous cycle (**B**) as reported previously 14. Note the low reactivity in the caruncles (CAR), glands (G), superficial gland (SG) and trophoblast (T). A section treated with mouse IgG instead of primary antibody (negative control) is shown in panel **A**. Bar = 50  $\mu\text{m}$ .



**Figure 2**

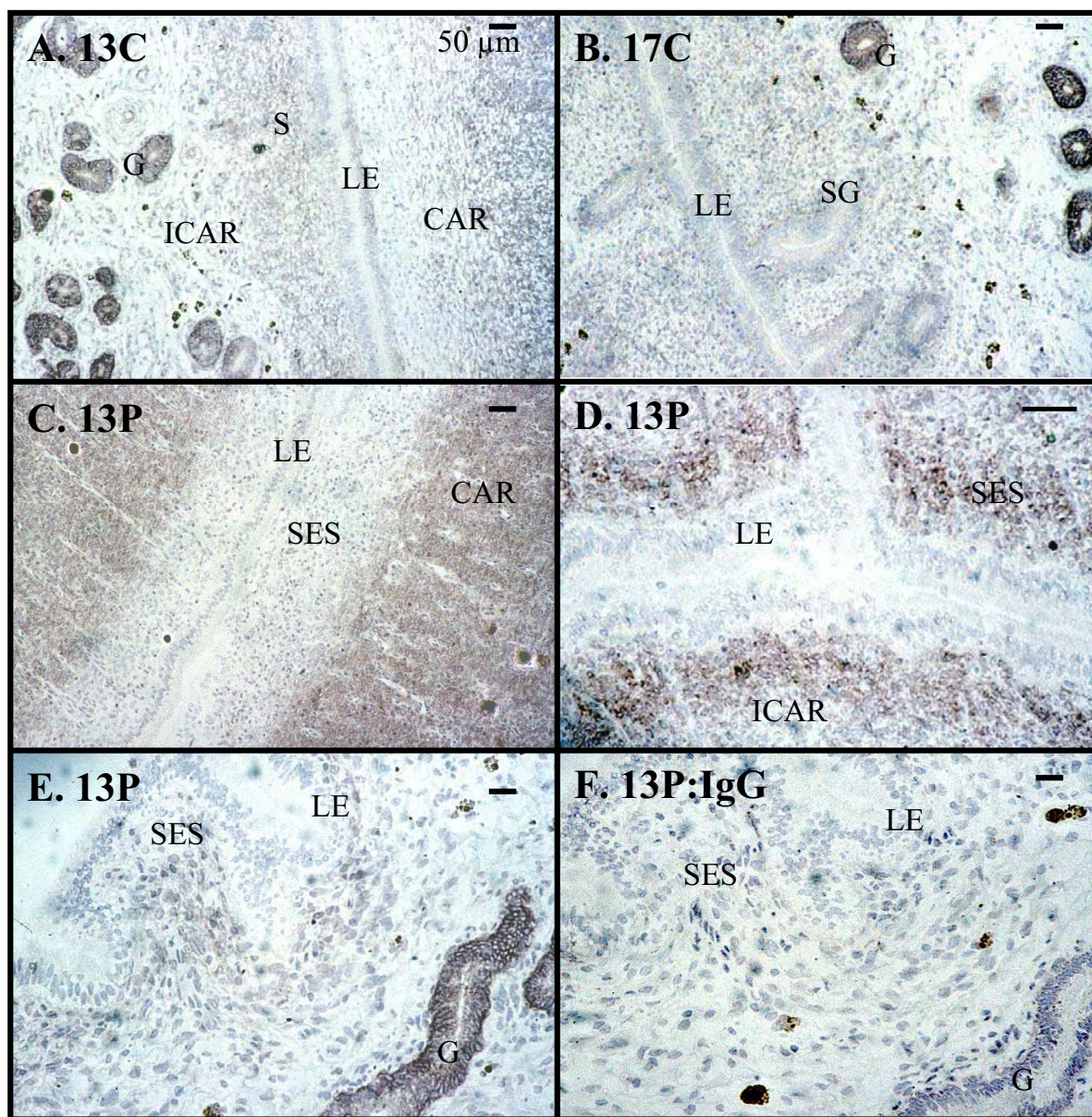
Effect of pregnancy stage on the median number of rows of subepithelial (SE) stromal cells showing strong reactivity to anti- $\alpha_v\beta_3$  antibody. Acetone fixed cyrostat cross sections from three cows each at days 16, 18, 21, 24 and 30 of pregnancy were used. The number of rows of positively stained SE stromal cells (score  $\geq 4$ ) underlying the intercaruncular luminal epithelium were counted by two independent observers and analyzed to determine if there was an effect of stage of pregnancy. \* indicates the median was significantly different from other medians ( $P < 0.05$ ).

may be involved in constraining trophoblast and endometrium in the intercaruncular regions, since the growth of both tissues to form the chorionic villi and intervening maternal septae occurs only in the caruncles. We hypothesize that subepithelial stromal integrin  $\alpha_v\beta_3$  is involved in regulating the behavior of the overlying epithelium and adjacent trophoblast. Such a signal from stroma underlying the uterine epithelium is logical, since the epithelium itself is modified rapidly after attachment by trophoblast binucleate cell migration and fusion with maternal epithelium to form giant cells [6]. This migration is maximal about day 24, then subsides to the extent that trinucleate, rather than giant cells, form and there is some regeneration of the luminal epithelium [6]. The observation in the current study that this is preceded by

increased expression of stromal integrin  $\alpha_v\beta_3$  provides support for a role of this integrin in constraining invasion.

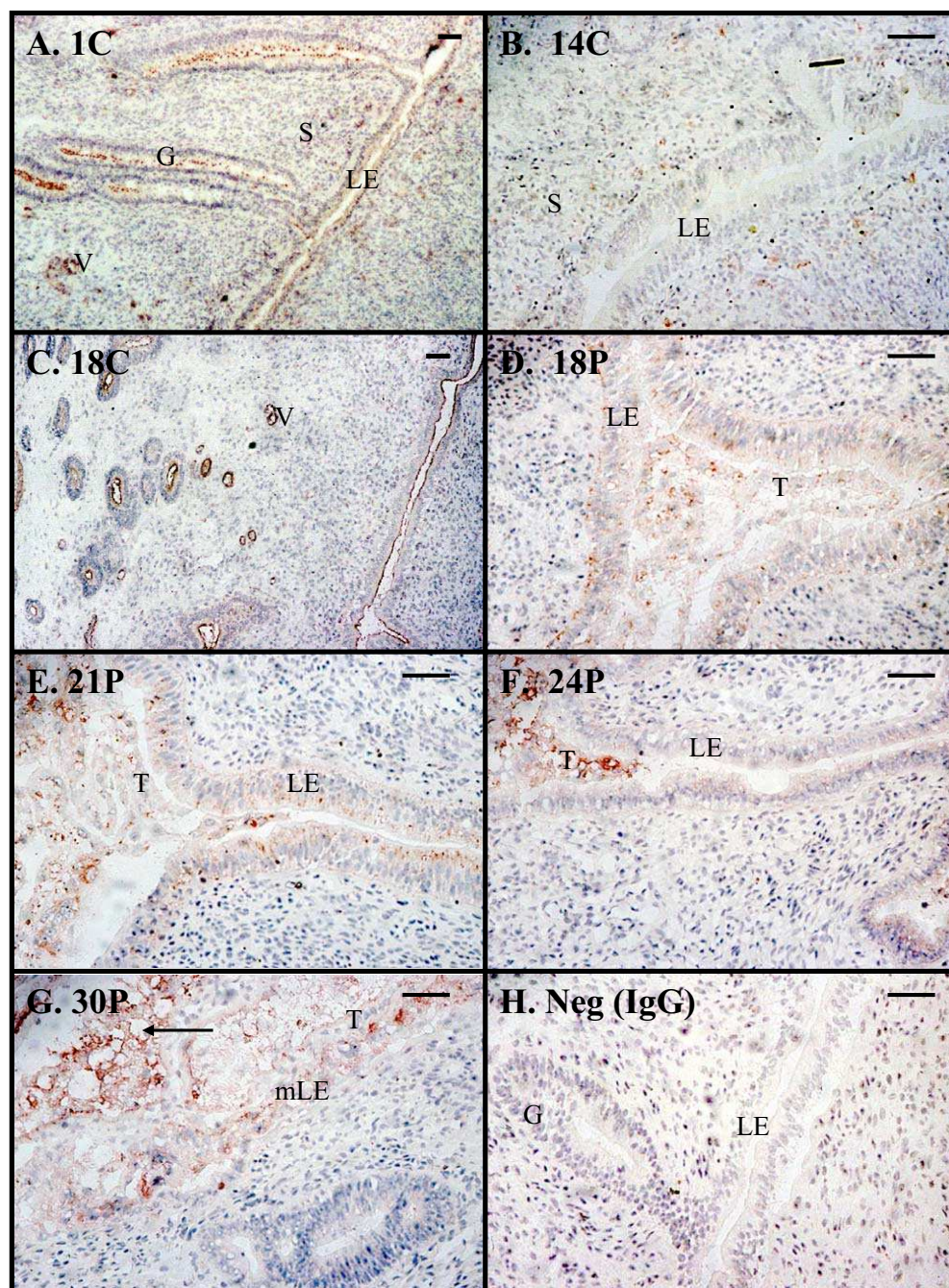
Integrin  $\alpha_v\beta_3$  reactivity was not concentrated in the basal lamina region of the subepithelial stroma in sections from ewes in this study (Table 1) or in the sheep endometrium examined by Johnson *et al.* [16]. This difference between sheep and cattle is interesting and may be attributable to the slightly more invasive attachment process that occurs in sheep in comparison to cattle. In sheep there is more extensive degeneration of luminal epithelium from migrant trophoblast binucleate cells than in cattle, that results in a syncytium that persists throughout pregnancy. In addition, cytoplasmic processes penetrate the basal lamina of ovine fetomaternal hybrid epithelium to contact the underlying stroma [8,35]. Although speculative, it



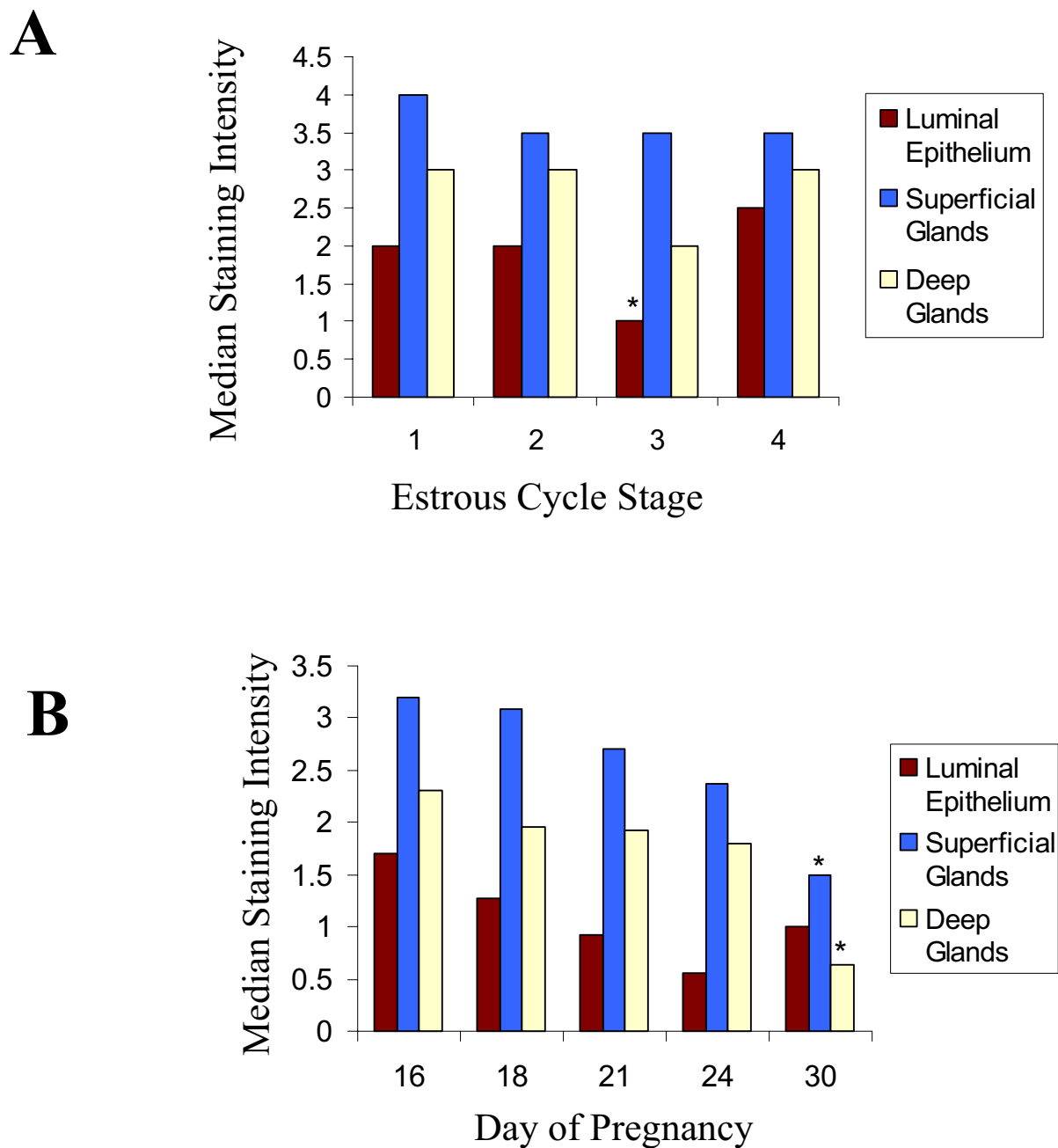
**Figure 3**

Immunohistochemical localization of integrin  $\alpha_v\beta_3$  in cryosections of ovine endometrial tissue during the estrous cycle (**A**, day 13 cyclic, **B**, day 17 cyclic) and at day 13 of pregnancy (**C-F**, day 13 pregnant). Positive reactivity (brown) was strongest in the glands (G) and blood vessels, while very weak or no reactivity was detected in the superficial glands (SG) or luminal epithelium (LE). Panels **A**, **C** and **D** illustrate the differences in subepithelial stroma (SES) reactivity to anti-integrin  $\alpha_v\beta_3$  in caruncular (CAR) and intercaruncular (ICAR) regions at different magnifications. Intercaruncular SES reactivity varied among the sheep, ranging from moderate (**D**) to relatively weak and diffuse as seen in panel **E**. Control sections (**F**) were treated with mouse IgG substituted for primary antibody. Stroma (S). Bar = 50  $\mu$ m.

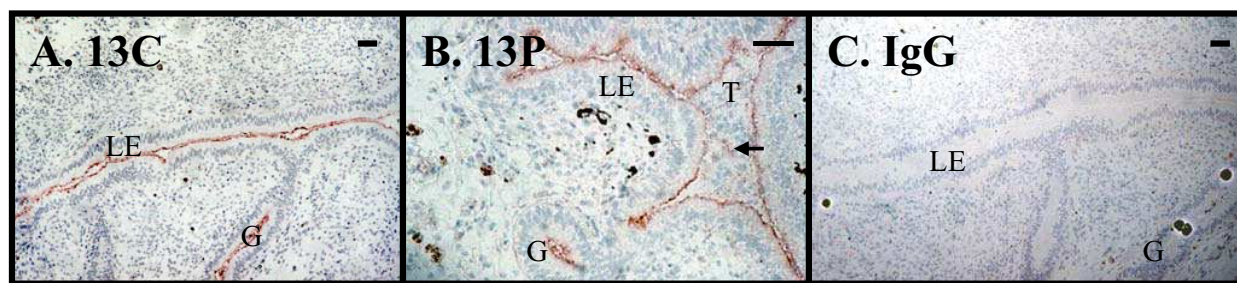


**Figure 4**

Immunohistochemical localization of osteopontin in cryostat cross-sections of bovine endometrial tissue during the estrous cycle (**A**, day 1 cyclic, **B**, day 14 cyclic, **C**, day 18 cyclic) and early pregnancy (**D**, day 18 pregnant, **E**, day 21 pregnant, **F**, day 24 pregnant, **G**, day 30 pregnant). Osteopontin (positive reactivity is red) was detected at the apical surface of glandular (G) and luminal epithelium (LE), associated with blood vessels (V) and on extraembryonic membranes (arrow) luminal to the trophoblast (T), as well as trophoblast itself. A representative control section, treated with rabbit IgG instead of primary antibody, is shown in **H**. mLE – LE modified by binucleate cell migration. Bar = 50  $\mu$ m.

**Figure 5**

Estrous cycle (**A**) and pregnancy (**B**) effects on osteopontin levels in bovine endometrium. Tissue sections from stages 1 (metestrus), 2 (early diestrus), 3 (late diestrus), and 4 (proestrus/estrus) of the estrous cycle and from early pregnancy (days 16, 18, 21, 24 and 30) were scored by two independent observers for staining intensity of the luminal epithelium, superficial glands and deep glands. Osteopontin reactivity in luminal epithelium decreased in late diestrus. As pregnancy progressed, apical cell reactivity to osteopontin antibody decreased in the glands. \* means significantly different from other stages ( $P < 0.05$ ).

**Figure 6**

Immunohistochemistry for detection of osteopontin in endometrial tissue collected from cyclic (**A**, day 13) and pregnant (**B**, day 13) sheep. Moderate to strong apical cell reactivity to osteopontin antibodies (shown in red) was detected on glands (G), luminal epithelium (LE) and extraembryonic membranes (arrow). No reactivity was observed on trophoblast (T). A representative control section treated with rabbit IgG substituted for primary antibody is shown in **C**. Bar = 50  $\mu$ m.

**Table 1: A comparison of the distribution of integrin  $\alpha_v\beta_3$  and osteopontin in ovine and bovine endometrium.**

Species	Protein	Apical surface LE	Trophoblast	Subepithelial stroma	Deep glands
Cow	Integrin $\alpha_v\beta_3$	—	—	+	—
Ewe	Integrin $\alpha_v\beta_3$	—	—	—	+
Cow	Osteopontin	+	+	—	+
Ewe	Osteopontin	+	+	—	+

Abbreviations: LE = luminal epithelium

is possible that subepithelial integrin  $\alpha_v\beta_3$  in the cow somehow acts to restrict trophoblast penetration whereas the relative lack of integrin  $\alpha_v\beta_3$  in sheep subepithelial stroma permits a higher degree of invasion.

In cattle, the transient disappearance of subepithelial integrin  $\alpha_v\beta_3$  on day 16 of the cycle, but not day 16 of pregnancy, suggests a possible role for this integrin in the onset of luteolysis. Our more recent studies have shown that this temporary downregulation may be mediated by estrogen [30], a known stimulator of the luteolytic mechanism [36]. Expression of IGF-1, like integrin  $\alpha_v\beta_3$ , is high at estrus and throughout the cycle is restricted to the subepithelial stroma [37]. Furthermore, in tissue from day 16 cycling cows expression of IGF-1 mRNA in this region reached a cycle low which preceded the proestrous increase beginning on day 17 [37]. Similar patterns of IGF-1 protein expression were observed by Ohtani and coworkers [38] in cyclic heifers, although the downregulation was observed on day 14–15, rather than day 16–17,

consistent with the shorter estrous cycle generally observed in heifers compared to cows. Again, similar to what was observed for integrin  $\alpha_v\beta_3$  in the current study, Robinson *et al.* [37] found that pregnant cows were more likely to express stromal IGF-1 mRNA on day 16 than nonpregnant animals, although the results were not conclusive. IGF-1 mRNA expression in sheep was evident in caruncular as well as intercaruncular stroma [39]. In other cell systems IGF-1 and integrin  $\alpha_v\beta_3$  are known to have cooperative signaling roles [22]. Both proteins have been linked to prostaglandin production via cyclooxygenase 2 [40–43], which has been localized to the luminal epithelium of cows [44]. Differences in expression patterns of IGF-1 and integrin  $\alpha_v\beta_3$  may mark a subtle difference in the mechanisms regulating prostaglandin release between sheep and cows.

In this study integrin  $\alpha_v\beta_3$  was absent from bovine trophoblast, but was expressed at moderate intensity in the glandular epithelium, and in the luminal epithelium until



it was modified by binucleate cell migration. In ovine endometrium, glandular epithelium exhibited a strong pericellular expression but no reactivity was observed in the luminal epithelium (Table 1). The pattern of integrin  $\alpha_v\beta_3$  expression we observed in ovine endometrium was not only different from cows, but also from what has previously been reported for the ewe [16]. This difference in localization patterns could be attributable to the antibodies used. In this study we used two antibodies that targeted different epitopes of the integrin heterodimer  $\alpha_v\beta_3$ , whereas the earlier study used antibodies targeting the individual integrin subunits,  $\alpha_v$  and  $\beta_3$ . Johnson et al. [16] reported antibody reactivity for these subunits on the apical surfaces of uterine epithelium and trophoblast. Monomeric integrin subunit protein is not normally expressed at the cell surface [9,10], so it is possible that other integrin heterodimers, such as integrins  $\alpha_v\beta_1$  or  $\alpha_v\beta_5$ , were being detected.

Moderate but consistent osteopontin expression was observed in uterine epithelium and trophoblast of sheep and cattle. It has been proposed that osteopontin or another matrix molecule could act as a "bridge" for integrin  $\alpha_v\beta_3$ , connecting trophoblast and uterine epithelium during embryo attachment [27,45,46]. Integrin  $\alpha_v\beta_3$  is thought to be the functionally most important receptor for osteopontin in bone and vascular tissue [47,48], and experiments with the Ishikawa endometrial cell line suggest that this is also true for human endometrial cells [15]. Since in this study integrin  $\alpha_v\beta_3$  was not detected on trophoblast or the apical surface of the luminal epithelium of either sheep or cattle, another integrin may be anchoring the bridge. The integrins  $\alpha_v\beta_1$ ,  $\alpha_v\beta_5$ ,  $\alpha_4\beta_1$ ,  $\alpha_8\beta_1$  and  $\alpha_9\beta_1$ , as well as CD44, can bind osteopontin [28,29]. Bovine trophoblast and uterine epithelium express  $\beta_1$  integrins [17,49], but the alpha subunits associated with  $\beta_1$  in these tissues have not been fully characterized. We have not been able to detect the integrin  $\alpha_4$  subunit at the fetomaternal interface [50], therefore it is unlikely that integrin  $\alpha_4\beta_1$  is the interaction partner for osteopontin in bovine uterine epithelium.

The pattern of expression of osteopontin was generally similar in bovine endometrium to what has been observed in other species, including ovine (Table 1) and human endometrium [13,25,51]. However, unlike what has been observed in those species, the scores for osteopontin reactivity in luminal epithelium decreased during the luteal phase in cyclic heifers, and strong apical reactivity was not observed on trophoblast, luminal or glandular epithelial cells during implantation as has been observed in sheep [current study, [25]]. Although the human and murine promoters for osteopontin possess progesterone response elements, the role of progesterone is complex in endometrium. The downregulation we

observed occurred when progesterone levels are high but expression of progesterone receptors in epithelium is low [52,53]. Sheep infused with progesterone showed increased osteopontin mRNA in the glands, however no progesterone receptors were expressed by these cells [26]. These experiments suggest that progesterone may act in concert with local modulators of osteopontin expression, which include hepatocyte growth factor, transforming growth factor  $\beta$  1 and epidermal growth factor [15,47,54], perhaps via a stromal mediated paracrine mechanism.

In human placenta, the chorionic villus cytotrophoblasts but not the overlying syncytiotrophoblast [55] produce osteopontin. It has been proposed that differentiated syncytiotrophoblasts control a paracrine communication loop with underlying cytotrophoblasts by releasing progesterone to stimulate secretion of osteopontin, which in turn binds and activates integrin  $\alpha_v\beta_3$  on the adjacent syncytial trophoblast [56]. Engagement of integrin  $\alpha_v\beta_3$  may then signal increased adhesion, or other intracellular events that promote normal tissue function. In the cow, there is no syncytial trophoblast per se but there are syncytial plaques and trinucleate cells formed by migration and fusion of trophoblast binucleate cells with maternal luminal epithelium [6]. These are exposed to progesterone from the trophoblast [56] and showed reactivity to osteopontin antibodies in the current study. However, integrin  $\alpha_v\beta_3$  was not expressed on trophoblast and expression decreased in the fetomaternal hybrid epithelium as it formed from day 24 to 30. On the other hand the underlying stroma of the hybrid epithelium showed increased expression of integrin  $\alpha_v\beta_3$  through this period. Whether this is related to a paracrine loop similar to what occurs in human placenta and/or a response to extracellular matrix changes occurring at this time [17] remains to be determined.

## Conclusions

Integrin  $\alpha_v\beta_3$  in the subepithelial stromal cells of bovine endometrium may have a role in the epithelial-stromal signaling events that regulate remodeling of pregnant epithelium and trophoblast during attachment in the cow. The endometrial distribution of integrin  $\alpha_v\beta_3$  we observed in sheep endometrium differed from the distribution in cows. Apparently at the molecular level, the signaling mechanisms between the epithelium and stroma that lead to formation of the synepitheliochorial placenta of sheep are not the same as in cows. At the fetomaternal interface in both species, the distribution patterns of integrin  $\alpha_v\beta_3$  and osteopontin did not overlap. This suggests that, unlike in primates and rodents, in ruminants these proteins could not act together to facilitate embryo attachment.



## Authors' contributions

SK carried out the synchronization of animals and tissue collection. SK and HL performed the immunohistochemistry experiments. LM and SK designed and coordinated the studies and their analysis. All authors read and approved the final manuscript.

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