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Immunolocalisation of Angiogenic Growth Factors in the Ovine uterus during the Oestrus Cycle and in response to Steroids

Running title: Ovarian steroids regulation of angiogenesis in sheep uterus

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(v) **Abstract**

The vascular changes associated with endometrial maturation in preparation for embryo implantation depend on numerous growth factors, known to regulate key angiogenic events. Primarily, the vascular endothelial growth factor (VEGF) family promotes vascular growth, whilst the angiopoietins maintain blood vessel integrity. The aim was to analyse protein levels of VEGFA ligand and receptors, Angiopoietin-1 and 2 (ANG1/2) and endothelial cell receptor tyrosine kinase (TIE2) in the ovine endometrium in the follicular and luteal phases of the oestrus cycle and in response to ovarian steroids. VEGFA and its receptors were localised in both vascular cells and non-vascular epithelium (glandular and luminal epithelium) and stroma cells. VEGFA and VEGFR2 protein were elevated in vascular cells in follicular phase endometrium, compared to luteal phase, most significantly in response to oestradiol. VEGFR1 was expressed by epithelial cells, endothelial cells, and was stimulated in response to oestradiol. In contrast, Ang-1 and Ang-2 proteins were elevated in luteal phase endometrium compared to follicular phase, and in response to progesterone, evident in vascular smooth muscle cells and glands which surround TIE2-expressing blood vessels. Our findings indicate that VEGFA is stimulated by oestradiol, most predominantly in follicular phase endometrium, and Ang-1 and 2 are stimulated by progesterone and were increased during the luteal phase of the oestrus cycle, during the time of vascular maturation.

Key words: VEGF, TIE2, oestradiol, progesterone, immunohistochemistry

(vi) **Introduction**

Throughout the oestrus cycle, continuous growth and regeneration of the endometrium is essential in providing a healthy maternal environment for the establishment of pregnancy (Sagsoz et al. 2011). Cyclical, morphological changes in the endometrium are accompanied by extensive remodeling of the vasculature to produce a receptive, vascularised environment (Torry et al. 2007, Ma et al. 2001). Such vascular remodeling is dependent on hormonally regulated angiogenesis (Walter et al. 2010). Under the influence of oestradiol, the follicular phase is associated with vascular growth, with rapid proliferation of endothelial cells (Boroujeni et al. 2016, Heryanto and Rogers 2002). As the vascular bed expands, angiogenic processes ensure stabilisation and maturation of vessels into the luteal phase of the cycle (Girling and Rogers 2009, Matsumoto et al. 2002), when progesterone is the dominant hormone. These cyclical changes in the ovarian steroids have been shown to correlate with the expression of angiogenic factors in the endometrium, essential to coordinate specific angiogenic events.

Angiogenic processes, including endothelial cell (EC) proliferation, migration and vessel formation, not only depend on the coordinate actions of oestradiol and progesterone, but interactions with several angiogenic growth factors (Meduri et al. 2000). Vascular Endothelial Growth Factor (VEGF) is a potent angiogenic mitogen for endothelial cells, which can also promote vascular permeability (Tasaki et al. 2010), by variable interactions with its receptors Vascular Endothelial Growth Factor Receptor 1 (VEGFR1; Flt-1) and Vascular Endothelial Growth Factor Receptor 2 (VEGFR2; KDR) (Fan et al. 2008). The mitogenic effect of VEGF on EC proliferation is known to be transduced via VEGFR2 (Thomas 1996), however, the exact role of VEGFR1 in the endometrium remains elusive. Although VEGFR1-mediated signalling is essential for vascularization, the higher binding affinity of VEGF for VEGFR1, only elicits a weak

angiogenic signal that does not stimulate endothelial cell proliferation (Tasaki et al. 2010). Furthermore, a non-vascular function for VEGF is proposed, as it is evident that the primary cellular source of VEGF in the uterus is the endometrial epithelium (Girling and Rogers 2009). This observation supports a non-angiogenic role for VEGF in epithelial cell function, in addition to a pro-angiogenic paracrine mechanism for VEGF.

Several groups have examined VEGF and receptors in pregnant and non-pregnant uterus and found cycle-dependent expression in rabbits (Das et al. 1997), pig (Winther et al. 1999), humans (Moller et al. 2001), marmosets (Rowe et al. 2003) and cows (Sagsoz and Saruhan 2011). Most studies show that oestradiol elicits its tropic effects on uterine angiogenesis by elevated expression and activity of VEGF. The first clear demonstration of oestradiol-induced effect of VEGF was from a study in the rat uterus, in which VEGF expression increased in response to oestradiol treatment (Cullinan-Bove and Koos 1993). Further studies by the same group went on to show the mechanism by which oestradiol stimulated VEGF expression resulting in increased microvascular permeability, in both isolated uterine epithelial cells and whole rat uteri (Kazi et al. 2009), (Kazi and Koos 2007). This effect was dependent on the simultaneous recruitment of hypoxia-inducible factor and (HIF1) and estrogen receptor alpha (ESR1) to the VEGFA gene promoter. Oestradiol enhanced VEGF in endometrial cells of mice (Shweiki et al. 1993), Baboons (Niklaus et al. 2003), rhesus macaques (Nayak and Brenner 2002) and humans (Mueller et al. 2000), with less known about the response of VEGF to progesterone.

Far less is known about another key group of angiogenic growth regulators, the angiopoietins, in the endometrium. Both angiopoietin-1 and 2 (Ang-1/2) act via EC tyrosine kinase receptor (TIE-2), and have opposing roles depending on the availability and interactions with VEGF

(Hirchenhain et al. 2003). Ang-1 is an agonist of TIE-2, which promotes interactions between endothelial cells and vascular smooth muscle cells, thereby contributing to the structural integrity and stabilisation of a newly formed vascular network (Tsuzuki et al. 2013). In contrast, Ang-2 is a natural antagonist of Ang-1 activity, competing at receptor binding sites, and its opposing functions are dependent on the presence of VEGF (Guo et al. 2012a).

A small number of studies report on the angiopoietins in the endometrium and are limited to humans and mice (Tsuzuki et al. 2013, Guo et al. 2012b). In human endometrium, immunoreactivity for Ang-1 was strongest in the glands with weaker staining in vascular smooth muscle cells (VSMCs) and ECs, throughout the menstrual cycle (Lash et al. 2012). Whereas, Ang-2 increased in the mid-late secretory phase, compared with early secretory or follicular phase. In early pregnant mouse uterus, Ang-2 and 3 increased by day 5 in response to the ovarian steroids, and were implicated in processes of decidualization (Guo et al. 2012a). No previous studies report on the expression of the angiopoietins in the cyclic ovine uterus, although one study showed increasing Ang-1 and 2 mRNA levels with the progression of early stage pregnancy in ovine maternal placenta (Grazul-Bilska et al. 2010). Another study investigated the effects of oestradiol regulation on several angiogenic growth factors in the endometrium, using an ovariectomised ewe model (Johnson et al. 2006). An increase in Ang-1, Ang-2 and TIE-2 mRNA expression in the endometrium was shown two hours post-oestradiol treatment. However, the importance of further study to clarify the expression and potential functions of the angiopoietins in the endometrium is essential.

Although a previous study reported on VEGF in ovine caruncular tissue during later pregnancy (Ruiz-Gonzalez et al. 2013), there is a distinct lack of knowledge regarding the localisation of VEGF family and the angiopoietins in the ovine endometrium and their regulation by steroid

hormones. Thus, the aim of the present study was to identify the temporal and spatial distribution patterns of VEGF and receptors and the angiopoietin family in the cyclic ovine endometrium, and in response to the ovarian steroids. Defining the expression pattern of these factors may provide further evidence to elucidate their physiological functions in the ovine uterus. To the best of our knowledge this is the first study, which thoroughly explored and demonstrated VEGF/receptors and ANG1/2 and TIE2 expression in both follicular and luteal phases of the oestrous cycle in the sheep model.

Materials and Methods

Animals and Tissue Collection

All experimental procedures complied with regulations in the UK Animal (Scientific Procedures) Act, 1986 and were approved by the College's Ethics and Welfare Committee. Thirty, proven fertile non-pregnant Welsh mountain ewes were used in this study, housed indoors and fed with hay and concentrate diet. Eight ewes were synchronized to a common day of oestrus using intravaginal Chronogest sponge (Intervet UK Ltd, Cambridge, UK) for 11 days and treated with 200 IU Pregnant Mare's Serum Gonadotrophin (PMSG; Intervet UK, Ltd, Buckinghamshire, UK) intramuscularly at the time of sponge removal. Having observed oestrous 24 h following sponge removal, reproductive tracts were collected following euthanasia on days 9 for luteal phase (n=5) and days 16 for follicular phase (n=3). Blood samples were also collected from intact ewes at the time of killing. The remaining 22 animals were ovariectomised as previously described by (Raheem et al. 2013). To remove the effect of endogenous gonadotrophins, luteinising hormone and follicle stimulating hormone (LH and FSH), ewes were administered Buserelin acetate (gonadotrophin agonist; Surefact, Aventis Pharma Ltd, Kent, UK) continuously by an osmotic pump (ALZET model, DURECT Corp,

125 Cupertino, US), inserted subcutaneously in the forelimb at the time of ovariectomy. Buserelin
126 was administered at 2mg in 2 ml normal saline and pumps were designed to secrete 2.5µl/h
127 for 28 days. Following an 8-day recovery period, ovariectomised ewes were randomly
128 assigned to receive daily intramuscular injections of one of the three treatments: i) 2ml corn
129 oil alone (also used as a vehicle for the hormonal treatments) for 10 days (ovariectomised;
130 OVX; n=6), ii) 25 mg progesterone for ten consecutive days (ovariectomised progesterone
131 treated; OVX P; n=8), or iii) 6 µg of 17β-oestradiol and 25 mg progesterone for 3 days followed
132 by progesterone only (25 mg) for the remaining 7 days (ovariectomised estradiol +
133 progesterone treated; OVX EP; n=8). Blood samples were collected on alternate days
134 following the first day of treatment; plasma was separated and stored at -20^{o}C until hormone
135 assays were performed for progesterone and oestradiol. Animals were euthanized 24 h after
136 the last hormone injection was administered and uterine horns were collected and several
137 dissected transverse sections were fixed in 4% formalin (BDH, Poole, UK), stored in 70%
138 ethanol and later embedded in paraffin wax blocks for immunohistochemistry.

139 ***Measurements of oestradiol and progesterone levels in plasma***

140 Progesterone plasma concentrations were analysed using ELISA kits (Ridgeway Science,
141 Cirencester, UK) according to manufacturer's instructions using standards prepared in
142 charcoal-stripped sheep plasma ranging from 0.5 to 20 ng/ml. Oestradiol plasma
143 concentrations were determined by RIA as previously described (Robinson et al. 2002). The
144 intra-assay coefficient of variation was 3.2%. The inter-assay coefficient of variation was not
145 calculated as all samples were analysed in a single assay.

146 ***Immunohistochemistry***

147 Formalin fixed paraffin wax embedded sections (5µm) of uterine tissue from each treatment
148 group were mounted on superfrost slides (VWR International Co, Leicestershire, UK),
149 dewaxed in histoclear (National Diagnostics, UK) and rehydrated through a descending series
150 of alcohols. Antigen retrieval for unmasking epitopes was performed by microwave boiling in
151 0.01 M sodium citrate buffer (pH 6.0) for 20 min. Slides were rinsed in PBS (phosphate
152 buffered saline; 1X, pH 7) and endogenous peroxidase activity was inactivated in 3% (v/v)
153 hydrogen peroxide in methanol for 20 min. Following further PBS washes, non-specific
154 protein binding was blocked in PBS buffer containing 10% (v/v) goat serum (Dako, Glostrup,
155 Denmark) + 4% (w/v) BSA for 60 min in a humidified chamber. Slides were incubated with
156 primary antibodies against VEGFA (polyclonal rabbit anti-human VEGF; 1:300), VEGFR1
157 (polyclonal rabbit anti-human VEGFR1; 1:300), VEGFR2 (polyclonal rabbit anti-mouse
158 VEGFR2; 1:50), ANG_1 (polyclonal rabbit Ang-1; 1: 50), ANG_2 (polyclonal rabbit anti-human
159 $Ang_{2;}$ 1:100), Tie-2 (polyclonal rabbit anti-human Tie-2; 1:100), all purchased from Santa Cruz,
160 Inc, Biotechnology, Inc, Santa Cruz, CA, USA) diluted in PBS, overnight at 4^{o}C in a humidified
161 chamber. Negative control slides were treated in the same manner except the replacement
162 of rabbit IgG diluted in PBS in the absence of primary antibody. On the following day, slides
163 were rinsed in PBS and incubated with the secondary antibody biotinylated goat anti-rabbit
164 (1:200, Dako) for 60 min at room temperature in a humidified chamber. Slides were rinsed
165 prior to application of the avidin-biotin complex solution (Vectastain Universal Elite ABC kit,
166 Vector Laboratories, Peterborough, UK) for 30 min at room temperature and detection of the
167 primary antibody was visualised using 3, 3'-Diaminobenzidine (DAB; Dako, Glostrup,
168 Denmark) until colour development. Slides were counterstained in Harris Haematoxylin,
169 dehydrated through an ascending series of alcohols and mounted using DPX, coverslipped
170 and images were captured under an Olympus BX60 microscope (Olympus, Essex, UK).

171 ***Semi-quantitative analysis of immunostaining by HSCORE***

172 The intensity of immunoreactivity for each antibody was assessed in cellular compartments
173 within sections by a four-point semi-quantitative scoring system as follows: negative (0), weak
174 (1), moderate (2) and strong (3). The H-score manual method of visual analysis is a double
175 graded system in which the total sum of a graded average intensity score is multiplied by the
176 percentage area expression. This obtains a score range from 0 to 300 by multiplying the
177 intensity of the stains (score 0-3) and the percentage of area stained (0-100) as previously
178 described (Ponglowhapan et al. 2008). Intensity of staining was considered separately in
179 endometrial compartments in luminal epithelium, glands, stromal cells and vasculature.
180 Percentage expression reflects the percentage area immuno-stained in each layer. The
181 estimate was based on ten random fields per section from all the animals in each treatment
182 group. The images were scored by two independent experienced assessors blinded to the
183 corresponding experimental groups under scoring. Data were analysed from a minimum of
184 at least three animals per experimental group using Graphpad Prism one-way analysis of
185 variance test with post hoc Tukey's test for multiple comparisons. $P<0.05$ was considered to
186 indicate a statistically significant difference.

187 **Results**

188 ***Plasma oestradiol and progesterone concentrations***

189 At the time of uteri sample collection, plasma progesterone levels were higher in luteal phase
190 animals (2.3 ± 0.49 ng/ml) in comparison to follicular phase ewes (below detection levels of
191 the assay; < 0.05 ng/ml). Progesterone was non-detectable in the OVX group, but significantly
192 increased in both OVX P and OVX EP groups in response to progesterone (P_4) or estradiol +
193 progesterone (E_2+P_4) treatment respectively (Fig. 1A). Plasma oestradiol levels at sample

collection from follicular phase ewes were 6.1 ± 1.69 pg/ml (Fig. 1B). In both OVX and OVX P ewes, oestradiol levels were below assay detection levels, whereas in OVX EP animals, oestradiol levels began a steady increase following injections administered daily for 3 consecutive days, and continued to increase until day 10 of sample collection.

Effects of oestrous cycle on protein expression of VEGF in the ovine endometrium

Protein expression of VEGFA/R1/2, Ang-1/2 and TIE2 was examined by immunohistochemistry and semi-quantified using HSCORE method. For each protein analysed, variation in the intensities of immunostaining detected in the cytoplasm of all cellular layers of the endometrium studied is summarised in Table 1. VEGFA immunostaining was evident in all cellular layers examined in the endometrium in follicular and luteal phases (Fig. 2A and B). The non-vascular cells of the epithelium stained strongest for VEGFA as compared to vascular cells in both follicular and luteal phases. VEGFA protein was localised to the cytoplasm of both luminal epithelium (LE) and was more strongly evident in glandular epithelium (GE) of the upper zone. No significant differences were detected for VEGFA in luminal and glandular epithelium between cycle phases ($P>0.05$). VEGFA staining in the stromal compartment was not cycle-dependant, with low level HSCORE values exhibited in both follicular and luteal phases. In the vasculature, VEGFA levels were shown to be cycle-dependant with stronger staining intensity in the endothelial cells in follicular phase sections, compared to the luteal phase ($P<0.05$). Positive VEGFA staining was observed in both vascular endothelial cells and smooth muscle cells surrounding blood vessels (BV), arterioles and capillaries.

Effects of ovarian steroids on protein expression of VEGFA in the ovine endometrium

Overall, in OVX sections due to a loss of ovarian steroids as a result of ovariectomy, the depth of the luminal epithelium and the diameter of uterine glands were markedly reduced compared to OVX EP/P tissues. In OVX tissue, there was a lack of positive VEGFA immunostaining to almost undetectable levels (Fig.2E). Treatment with the ovarian steroids induced a significant up-regulation of VEGFA protein, in the luminal and glandular epithelium, and in vascular cells (P<0.05; Fig. 2H). Steroid-treated OVXP (Fig. 2D) and OVXEP ewes exhibited significantly increased HSCORE values for VEGF compared to OVX tissue. Combined OVXEP treatment produced the greatest up-regulation of VEGF in all cell types examined, above that of progesterone alone (P<0.05; Fig. 2C).

Effects of oestrous cycle on protein expression of VEGFR1 and 2 in the ovine endometrium

VEGFR1 exhibited a staining distribution pattern comparable to that of VEGFA. Cells of the luminal and glandular epithelium in the superficial endometrium were most strongly stained for VEGFR1 with no significant differences observed between follicular and luteal phases (Fig. 3A and B). In the vasculature, VEGFR1 stained stronger in follicular phase, evident in both endothelial cells and smooth muscle cells of capillaries, and arterioles, although there was no significant effect compared to luteal ewes (Fig. 3A and B). Overall staining indices for VEGFR2 were relatively lower than VEGFR1 in the endometrium, reflected by an approximate 2-fold higher HSCORE value for VEGFR1 than VEGFR2 in luminal and glandular epithelium and vascular cells (Fig. 3G and 4G). Cell specific oestrus cycle-dependent differences in VEGFR2 expression were detected. In luteal phase, VEGFR2 was significantly higher in luminal epithelium compared to follicular phase, but not in the glands and stroma (P<0.05; Fig. 4A and 4B). In the vasculature, VEGFR2 was predominantly localised to the endothelial cells of blood vessels, with significantly stronger levels in the follicular phase (P<0.05). Only a small number

239 of cells in the stromal compartment stained positively for VEGFR2 from follicular and luteal
240 animals.

241 ***Effects of ovarian steroids on protein expression of VEGFR1 and 2 in the ovine endometrium***

242 Treatment with the ovarian steroids (OVXP/EP) induced a significant increase in both VEGFR1
243 and VEGFR2 compared to OVX ewes in all epithelial and vascular cells but not in the stroma
244 (Figs. 3H and 4H). The effects of combined OVXEP treatment induced the strongest intensity
245 staining for VEGFR1 in luminal and glandular epithelium, comparable to HSCORE values in
246 vascular cells (Fig. 3C). Progesterone treatment alone induced an increase in VEGFR1 in all
247 cell types examined, however this was not a significant effect (Fig. 3D). VEGFR2
248 immunoreactive protein was also induced in uterine sections in response to the ovarian
249 steroids (Fig. 4H). Combined OVXEP treatment produced the most marked increase in
250 VEGFR2 in both luminal and glandular epithelium, and most significantly in vascular
251 endothelial cells (Fig. 4C; $P<0.001$). VEGFR2 immunoreaction intensity was weaker along the
252 luminal epithelium and in glands and vascular cells in response to progesterone alone (Fig.
253 4D).

254 ***Effects of oestrous cycle on protein expression of angiopoietins in the ovine endometrium***

255 Ang-1 and Ang-2 proteins were both expressed in a cycle dependent manner and were
256 detected in all cellular layers examined at varying intensities (Figs. 5A, B and 6A, B). In the
257 blood vessels, Ang-1 is strongly evident in the supportive smooth muscle cells and in
258 endothelial cells. Ang-1 is also detected in non-vascular luminal and glandular epithelium and
259 in stroma. Ang-1 immunostaining was significantly reduced in follicular phase compared to
260 luteal phase in epithelial cells and blood vessels. In luteal phase, Ang-1 is significantly
261 increased in luminal epithelia, superficial glands and the vasculature, but not stroma ($P<0.05$;

Fig. 5B). Ang-2 protein exhibited a similar spatial distribution in uterine sections to Ang-1 (Fig 6A). Ang-2 protein was detected in the luminal epithelium, and stained stronger in glands of the superficial endometrium. Ang-2 was strongly evident in smooth muscle cells of the vasculature and in endothelial cells. Immunostaining for Ang-2 was consistently higher in luteal phase animals in all cells examined except the stroma ($P<0.05$; Fig. 6B).

Effects of ovarian steroids on protein expression of the angiopoietins in the ovine endometrium

Both ANG1 and ANG2 protein exhibited an increase in response to steroid treatments compared to OVX tissue (Fig. 5 and 6). Overall, Ang-1 and 2 were distinct and significantly higher ($P<0.05$) in all three cellular layers, although less elevated in the stroma, in response to progesterone treatment (Fig. 5D and 6D). Ang-1 and Ang-2 immunostaining was weak in response to combined OVXEP treatment, and did not induce a significant effect in comparison to OVX tissues, reflected by HSCORE values approximately 4-fold lower than for progesterone alone (Fig. 5C and 6C).

TIE-2 protein was strongly associated with blood vessels in both follicular and luteal phases, with strong staining localised to both endothelial and smooth muscle cells of capillaries, arterioles and venules (Fig. 7A and 7B). TIE-2 immunoreactivity was highly detectable in the superficial glands, in follicular phase and most significantly in luteal phase. TIE-2 staining was undetectable in cells of the luminal epithelium in the luteal phase (Fig. 7B), and almost at undetectable levels in follicular phase (Fig. 7A). TIE-2 was up-regulated in response to both progesterone and combined oestradiol and progesterone treatments, in cells of the vasculature ($P<0.0001$) and in the superficial glands ($P<0.0001$).

Discussion

In the present study, VEGFA protein and receptors were demonstrated by immunohistochemistry in the ovine endometrium, which have cycle-dependent variations in specific cell types and in response to the ovarian steroids. The main findings of our study demonstrated that epithelial, stromal and vascular cells produce VEGFA, and immunoreactivity in the vasculature was highest in the follicular phase. VEGF receptors were expressed by ECs of blood vessels, and specifically VEGFR2 vascular expression was highest in the follicular phase. VEGFR1 and VEGFR2 immunostaining was also present in the epithelial and stromal cells of the superficial endometrium. The distinct period of elevated VEGFA vascular expression in the follicular phase is in accordance with findings of (Punyadeera et al. 2006) who demonstrated increased VEGF expression in early proliferative human endometrium, and (Maas et al. 2001) who observed that early-proliferative phase endometrium has higher angiogenic activity than late proliferative phase.

The present study also described that in our ovariectomised model, the influence of oestradiol and progesterone induced high VEGFA expression in epithelial and vascular cells. Although oestradiol injections were administered only on days 1-3 of treatments, followed by progesterone thereafter, circulating levels of plasma oestradiol remained high until the final day 10 of treatment. It is likely that this results from a delay in absorption of oestradiol from the muscle at the site of injection. Thus, high circulating levels of oestradiol were maintained and endometrial samples were influenced by oestradiol until the day of sample collection. This is an important point to consider when interpreting data from this study.

Previous studies have shown that VEGF mediates processes of angiogenesis, vascular permeability and endothelial cell proliferation via VEGFR2. The highest expression of VEGFR2 in vascular cells shown in the follicular phase in this study may be indicative of the

requirement for VEGFR2-VEGFA mediated mitogenic activity of endothelial cells, during a period characterised by accelerated vascular growth. Further studies are required in the ovine endometrium, to determine the functionality of VEGFA in specific phases of the oestrus cycle. In accordance with previous studies in primates (Rowe et al. 2003), cattle (Sagsoz and Saruhan 2011) and in humans (Charnock-Jones et al. 1993), VEGFA immunostaining was stronger in luminal and glandular epithelial cells than in ECs. Endometrial epithelial and stromal cell proliferation and differentiation are controlled by the actions of oestradiol and progesterone, mediated by several growth factors, including VEGFA (Gabler et al. 1999). In this study, the marked increase in VEGFA expression induced in epithelial cells by combined oestradiol and progesterone treatment, suggests oestradiol regulation of VEGFA in these cell types, which may act to promote endometrial growth. Although no significant differences in VEGFA expression were observed across cycle phases in epithelial cells, a trend towards stronger staining in both glandular and luminal epithelial cells in mid-luteal phase was notable. In bovine uterus (Sagsoz and Saruhan 2011) reported a stronger VEGF signal in luminal and glandular epithelium in luteal phase, but no significant differences were detected in stromal or smooth muscle cells. (Tasaki et al. 2010) showed that VEGF mRNA was highest at estrus and VEGF protein peaked at early luteal phase in bovine endometrium. The significance of VEGF in these cells types at this time may be required in stimulating the increase in secretory activity of epithelial cells during the luteal phase.

VEGFR1 and VEGFR2 were highly localised to ECs of blood vessels, and are known to mediate VEGF signalling in the endometrium. VEGFR2 expression was cycle-dependant, highest in follicular phase, and stimulated by oestradiol treatment. This is indicative of oestradiol regulation of VEGFR2 in endothelial cells during the oestradiol-dominant follicular phase of the oestrous cycle. VEGFR1 did not change significantly between the follicular phase and

luteal phase, although overall VEGFR1 levels were higher in all cell types examined than VEGFR2. Given the higher binding affinity of VEGF for VEGFR1, this suggests a significant role for VEGFR1-VEGF mediated signalling. Similar to VEGFR2, VEGFR1 immunoreactivity was high in ECs in blood vessels in the superficial endometrium, and was upregulated in epithelial and vascular cells in response to oestradiol. Since VEGF is secreted by epithelial and stromal cells, a paracrine mechanism is likely, with VEGF binding to receptors on endothelial cells to stimulate different angiogenic functions. Although VEGF signalling via VEGFR1 cannot mediate endothelial cell proliferation, it has been implicated to facilitate vascular permeability and endothelial cell migration (Meduri et al. 2000). Intense immunostaining was detected in the ECs of blood vessels in follicular phase and was still highly detectable in mid-luteal phase endometrium. During this cycle phase, the characteristic changes associated with angiogenesis include vessel hyperpermeability and rapid expansion of the vascular bed towards the base of the epithelium in preparation for blastocyst attachment.

The functions of the angiopoietins in the endometrium and during implantation are not well understood. In this study, it was demonstrated that Ang-1 was high during the luteal phase, and that progesterone induced a ~5-fold increase in epithelial and vascular cells. In a primate model, during induced menstrual cycles in rhesus macaques, progesterone withdrawal in the early secretory phase induced a decline in Ang-1 mRNA in the glands and vascular smooth muscle cells in the endometrium (Nayak et al. 2005). The progesterone-dominant luteal phase is associated with processes of vascular maturation, characterised by increased interactions between endothelial cells and supportive smooth muscle cells. Ang-1 is the primary agonist for TIE-2 receptors, and functions via paracrine signalling, to bind and phosphorylate TIE-2, thereby promoting associations between vascular cell types, to maintain blood vessel stability (Woolf et al. 2009). Ang-1 was localised to smooth muscle cells of blood

vessels, highly expressed in the luteal phase. Our data shows TIE-2 immunoreactivity is largely restricted to ECs of blood vessels, with no significant differences between cycle phases. As Ang-1 is a secretory glycoprotein, and we show Ang-1 expression by epithelial and vascular cells in the superficial endometrium, whereas its receptor, TIE-2 is expressed by ECs, we suggest Ang-1 may stimulate processes of increased vascular stability in a paracrine fashion. Ang-1 is shown to function by paracrine signalling in other tissue types, including glomeruli of the kidney, where Ang-1 is expressed by podocytes and upregulates TIE-2 receptors on endothelial cells of capillaries (Woolf et al. 2009).

Similarly, Ang-2 peaked in the luteal phase and was stimulated in response to progesterone. Ang-2 antagonises Ang-1 binding to TIE2 and does not induce signal transduction. In doing so, Ang-2 can disrupt the associations between endothelial cells and peri-vascular support cells, to promote apoptosis and vessel regression (Woolf *et al.* 2008). Such processes may be advantageous during early gestation when placentation requires remodelling of an existing vascular network and formation of new branching vessels (Kappou et al. 2014). Pro-angiogenic effects of Ang-2 are thought to be dependent on available local VEGF expression. It has been indicated that Ang-2 may promote the accessibility of ECs to VEGF, thereby promoting neovascularisation. Notably, a previous study in an OVX-ewe model, reported that oestradiol-induced up-regulation of Ang-1/2 and TIE-2, occurred at 2 h post-treatment (Johnson *et al*. 2006). In fact this response time was shorter than the oestradiol-induced up-regulation of VEGF, observed at 4 h. In the present study, the effects of oestradiol were also stimulatory on angiopoeitin expression, however progesterone alone exhibited a greater response. Of interest, would be to compare progesterone treatment in the aforementioned

groups OVX-ovine model. However, differences in results between studies may be due to analysis at the protein level, in the present study, and at the mRNA level in the previous study.

Although no staining was evident in the luminal epithelium or stromal cells, TIE-2 was unexpectedly evident in glandular epithelium. Analysis of these results should be approached with some degree of caution, given that TIE-2 is an endothelial specific marker. Possibly, there is a lack of specificity of the polyclonal antibody used, which could prove a limitation of this study. However, there is some previous evidence of non-vascular TIE-2 expression in epithelial cells and a distinct population of monocytes (Coffelt et al. 2010). Future endometrium-specific studies of TIE-2 expression are required to discern these possibilities.

It is noteworthy to highlight the limitations of this study, in which antibodies with higher specificity may have been selected, and a more state of the art software analysis could have greater benefit. However, it is also critical to highlight that there is very limited evidence for angiogenic factors in the sheep. Although many other species have been studied in this area, this study provides novel data, which is no doubt crucial, and may highlight species-specific differences in implantation and placentation, that would subsequently be reflected in differences in angiogenic factor expression. Studies in ruminant species of economic relevance like sheep are critical, as evidence is limited on VEGF in cyclic ovine endometrium but has only been demonstrated in pregnant uterus (Grazul-Bilska et al. 2010). Early embryonic loss is a limiting factor which compromises reproduction, reflected in the fact that high fertilisation rates (70-80%) do not necessarily equate to similar pregnancy rates (Bridges et al. 2013). Deficient development and/or function of the vasculature may be a contributing factor to such early pregnancy loss.

In conclusion, this study provides evidence that VEGF and receptors VEGFR1/2 are expressed by uterine epithelial, vascular and stromal cells in the ovine endometrium, in a cycle-dependant manner, predominantly induced by oestradiol. The angiopoietin system is dominant in the luteal phase, and regulated by progesterone may be involved in processes of vessel integrity and stabilisation of a newly formed maternal vascular system.

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(vii) **Conflict of interest statement**

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

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547 (ix) Table 1: Synopsis of results

Protein	Stage of cycle / treatment	Luminal epithelium	Upper zone glandular epithelium	Upper zone stroma	Vasculature
VEGF	Follicular	++	++	+	+++
	Luteal	+++	+++	+	++
	OVXEP	+++	+++	+	++
	OVXP	++	++	+	+
	OVX	-	-	-	-
VEGFR1	Follicular	+++	+++	+	++
	Luteal	+++	+++	+	++
	OVXEP	++	++	+	++
	OVXP	++	++	+	+
	OVX	+	+	+	-
VEGFR2	Follicular	+	++	+	++
	Luteal	++	++	+	+
	OVXEP	+	+	+	++
	OVXP	+	+	+	+
	OVX	+	+	+	+
Ang-1	Follicular	+	+	-	+
	Luteal	+++	+++	+	+++
	OVXEP	+	+	-	+
	OVXP	+++	+++	+	+++
	OVX	-	-	-	-
Ang-2	Follicular	+	+	-	+
	Luteal	+++	+++	+	++
	OVXEP	+	+	-	+
	OVXP	+++	+++	+	++
	OVX	-	-	-	-
TIE-2	Follicular	-	++	+	+++
	Luteal	-	+++	+	+++
	OVXEP	-	++	+	++
	OVXP	-	++	+	++
	OVX	-	-	-	-

548 Staining intensity: +++ = strong, ++ = medium, + = weak, -no staining

(x) **Figure Legends:**

Figure 1: Plasma concentrations of progesterone (A) and oestraidol (B) in ovariectomised ewes, treated with corn oil (vehicle) (OVX; n=6), or progesterone (OVX P; n=9) or oestradiol + progesterone (OVX EP; n=8). Values represent mean values ± SEM.

Figure 2: Immunohistochemical localisation and quantification of *VEGF* in ovine endometrium. VEGF in the follicular and luteal phases (A, B) of the oestrous cycle, and ovariectomised ewes treated with oestradiol and progesterone (OVXEP; C), progesterone (OVXP; D), or vehicle (OVX; E). Immunostaining with isotype antibody for negative control (F). VEGF is visualised as brown staining (DAB) and nuclei are counterstained with hematoxylin. HSCORE values represent mean ± SEM (G, H). *significance ($P< 0.05$; n=3). LE; luminal epithelium, GE; glandular epithelium, BV; blood vessel, ST; stroma. Scalebar: 50 µm.

Figure 3: Immunohistochemical localisation and quantification of *VEGFR1* in ovine endometrium. VEGFR1 in the follicular and luteal phases (A, B) of the oestrous cycle, and ovariectomised ewes treated with oestradiol and progesterone (OVXEP; C), progesterone (OVXP; D), or vehicle (OVX; E). Immunostaining with isotype antibody for negative control (F). VEGFR1 is visualised as brown staining (DAB) and nuclei are counterstained with hematoxylin. HSCORE values represent mean ± SEM (G, H). *significance ($P< 0.05$; n=3). LE; luminal epithelium, GE; glandular epithelium, BV; blood vessel, ST; stroma. Scalebar: 50 µm.

Figure 4: Immunohistochemical localisation and quantification of *VEGFR2* in ovine endometrium. VEGFR2 in the follicular and luteal phases (A, B) of the oestrous cycle, and ovariectomised ewes treated with oestradiol and progesterone (OVXEP; C), progesterone (OVXP; D), or vehicle (OVX; E). Immunostaining with isotype antibody for negative control (F). VEGFR2 is visualised as brown staining (DAB) and nuclei are counterstained with hematoxylin.

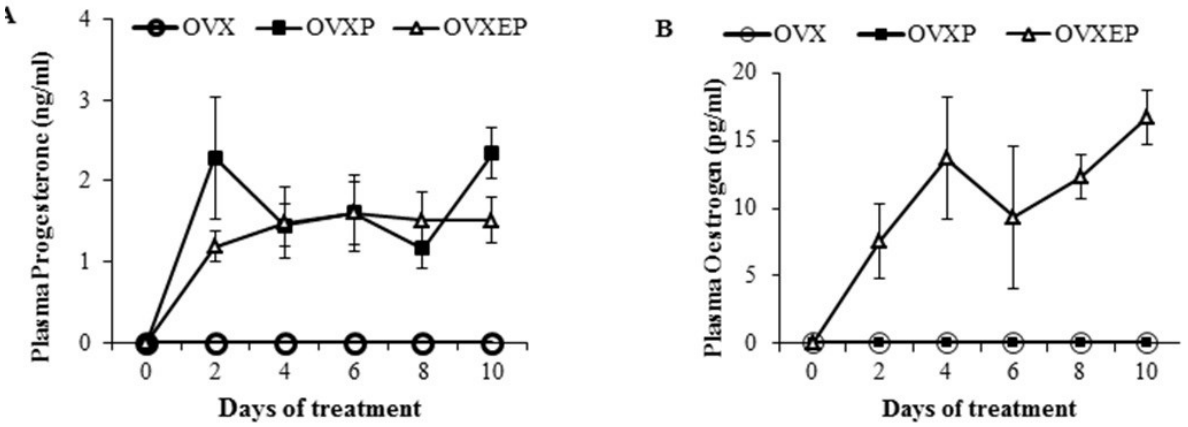
572 HSCORE values represent mean ± SEM (G, H). *significance (*P*< 0.05; n=3). LE; luminal
573 epithelium, GE; glandular epithelium, BV; blood vessel, ST; stroma. Scalebar: 50 µm.

574 **Figure 5:** Immunohistochemical localisation and quantification of *Ang-1* in ovine
575 endometrium. Ang-1 in the follicular and luteal phases (A, B) of the oestrous cycle, and
576 ovariectomised ewes treated with oestradiol and progesterone (OVXEP; C), progesterone
577 (OVXP; D), or vehicle (OVX; E). Immunostaining with isotype antibody for negative control (F).
578 Ang-1 is visualised as brown staining (DAB) and nuclei are counterstained with hematoxylin.
579 HSCORE values represent mean ± SEM (G, H). *significance (*P*< 0.05; n=3). LE; luminal
580 epithelium, GE; glandular epithelium, BV; blood vessel, ST; stroma. Scalebar: 50 µm.

581 **Figure 6:** Immunohistochemical localisation and quantification of *Ang-2* in ovine
582 endometrium. Ang-2 in the follicular and luteal phases (A, B) of the oestrous cycle, and
583 ovariectomised ewes treated with oestradiol and progesterone (OVXEP; C), progesterone
584 (OVXP; D), or vehicle (OVX; E). Immunostaining with isotype antibody for negative control (F).
585 Ang-2 is visualised as brown staining (DAB) and nuclei are counterstained with hematoxylin.
586 HSCORE values represent mean ± SEM (G, H). *significance (*P*< 0.05; n=3). LE; luminal
587 epithelium, GE; glandular epithelium, BV; blood vessel, ST; stroma. Scalebar: 50 µm.

588 **Figure 7:** Immunohistochemical localisation of *TIE-2* in ovine endometrium. TIE-2 in the
589 follicular and luteal phases (A, B) of the oestrous cycle, and ovariectomised ewes treated with
590 oestradiol and progesterone (OVXEP; C), progesterone (OVXP; D), or vehicle (OVX; E).
591 Immunostaining with isotype antibody for negative control (F). TIE-2 is visualised as brown
592 staining (DAB) and nuclei are counterstained with hematoxylin. HSCORE values represent
593 mean ± SEM (G, H). Significance (**P*< 0.05, ****P*< 0.0001; n=3). LE; luminal epithelium, GE;
594 glandular epithelium, BV; blood vessel, ST; stroma. Scalebar: 50 µm.

595 Figure 1



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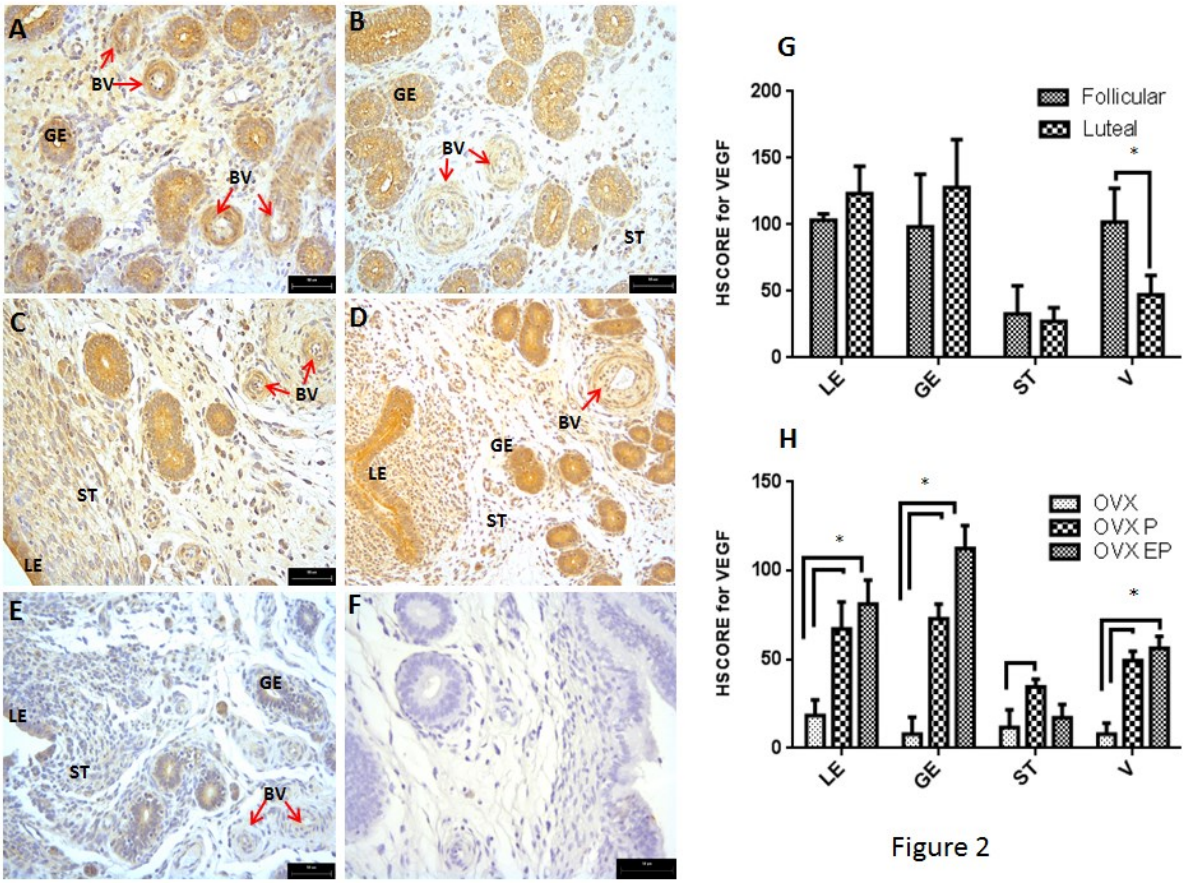

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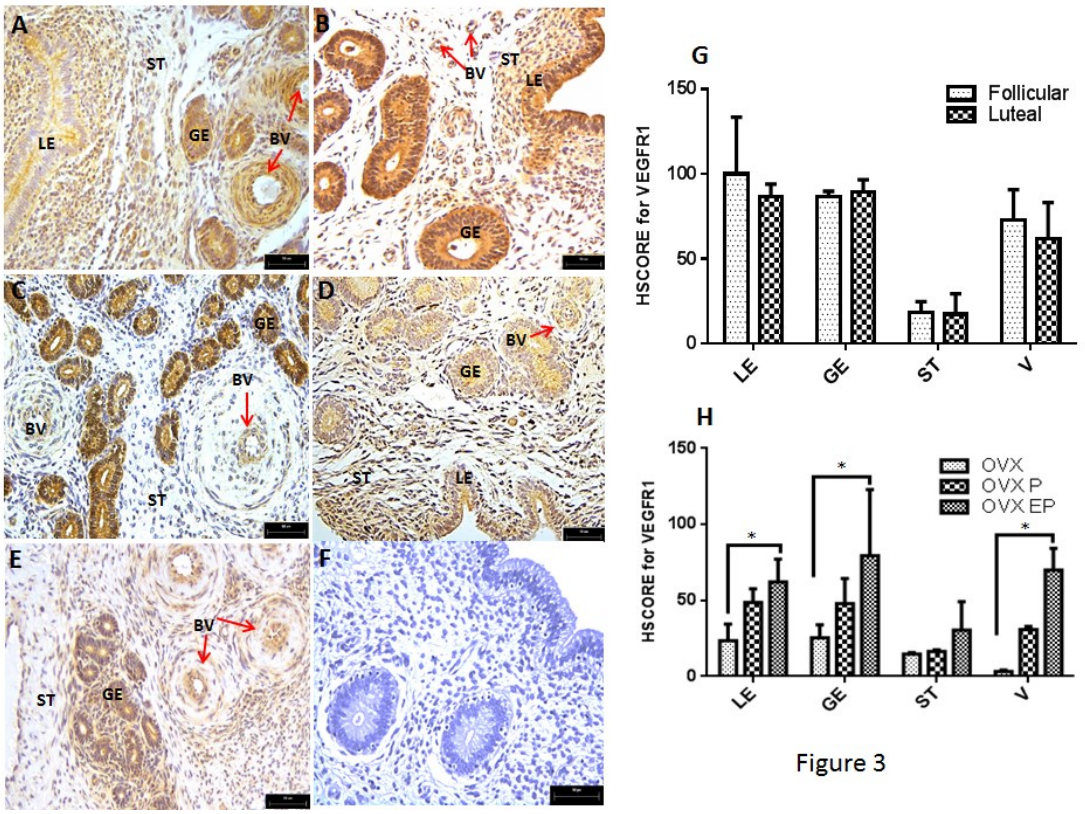

Figure 3

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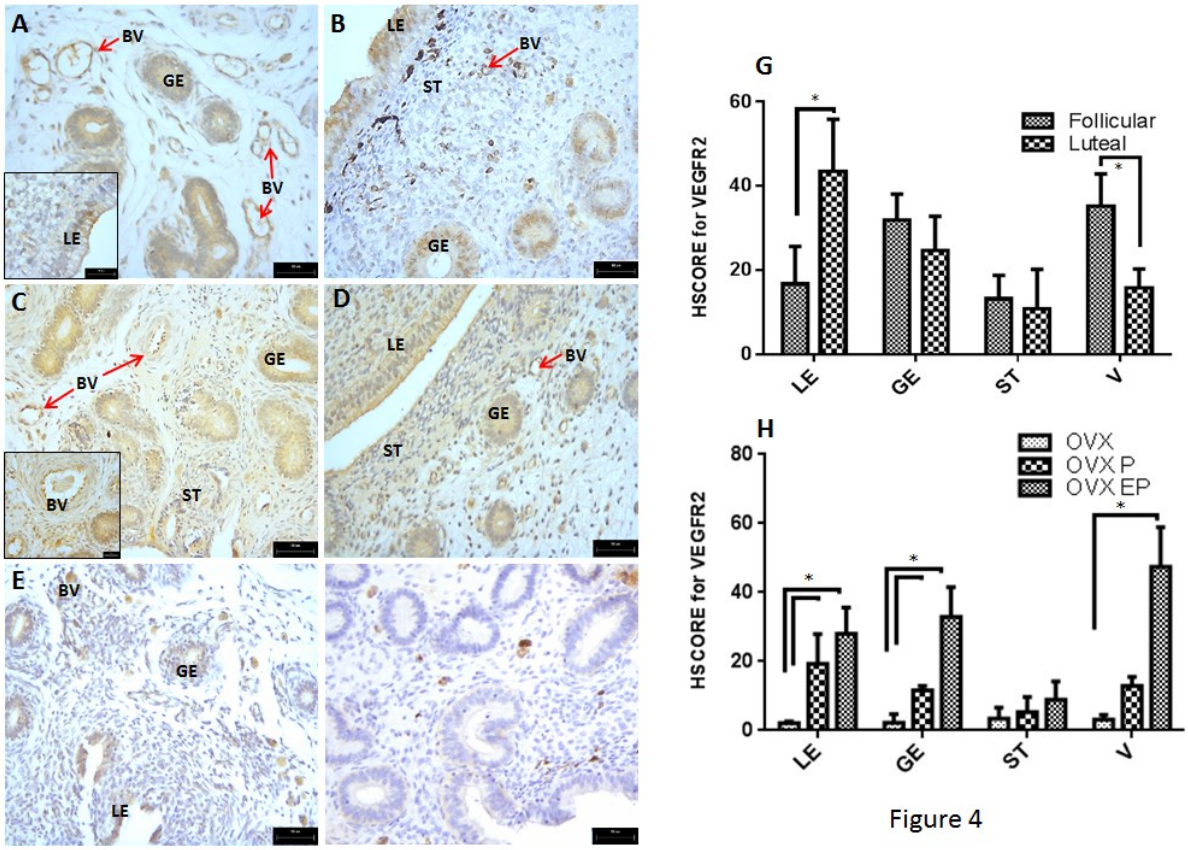

Figure 4

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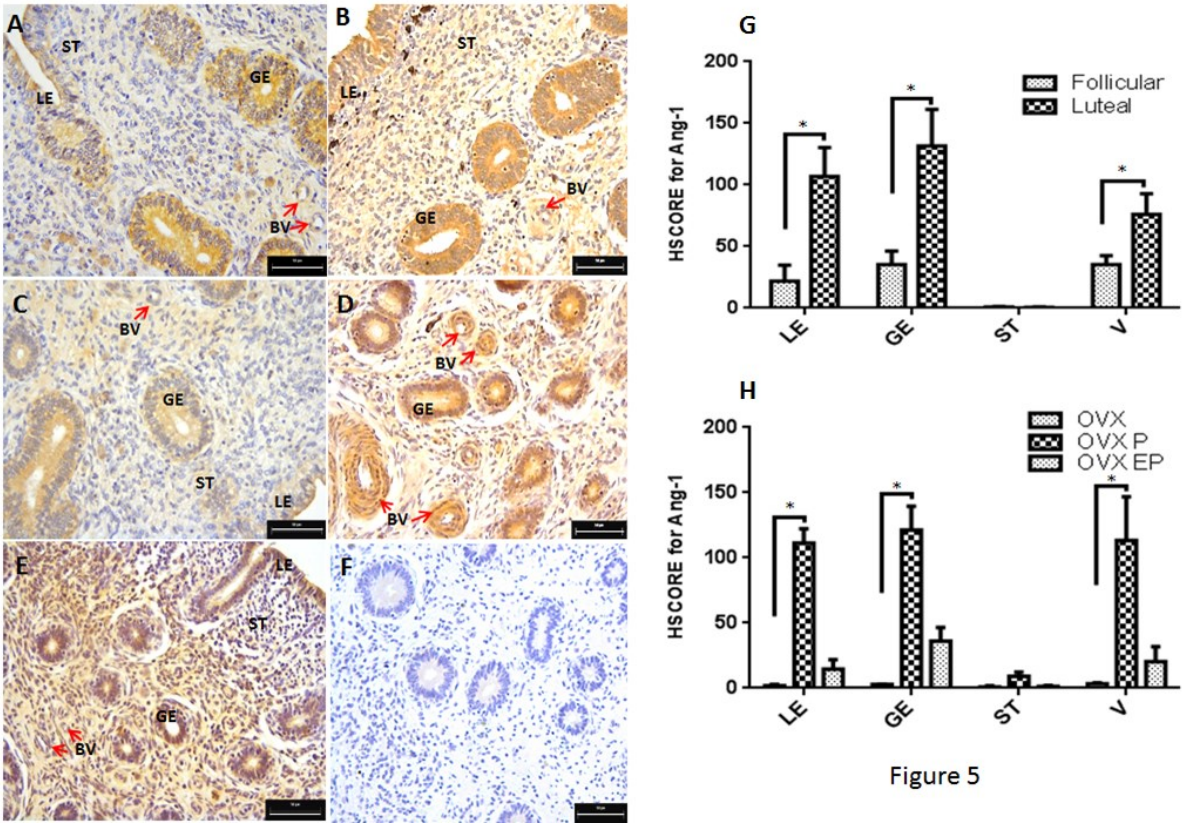

Figure 5

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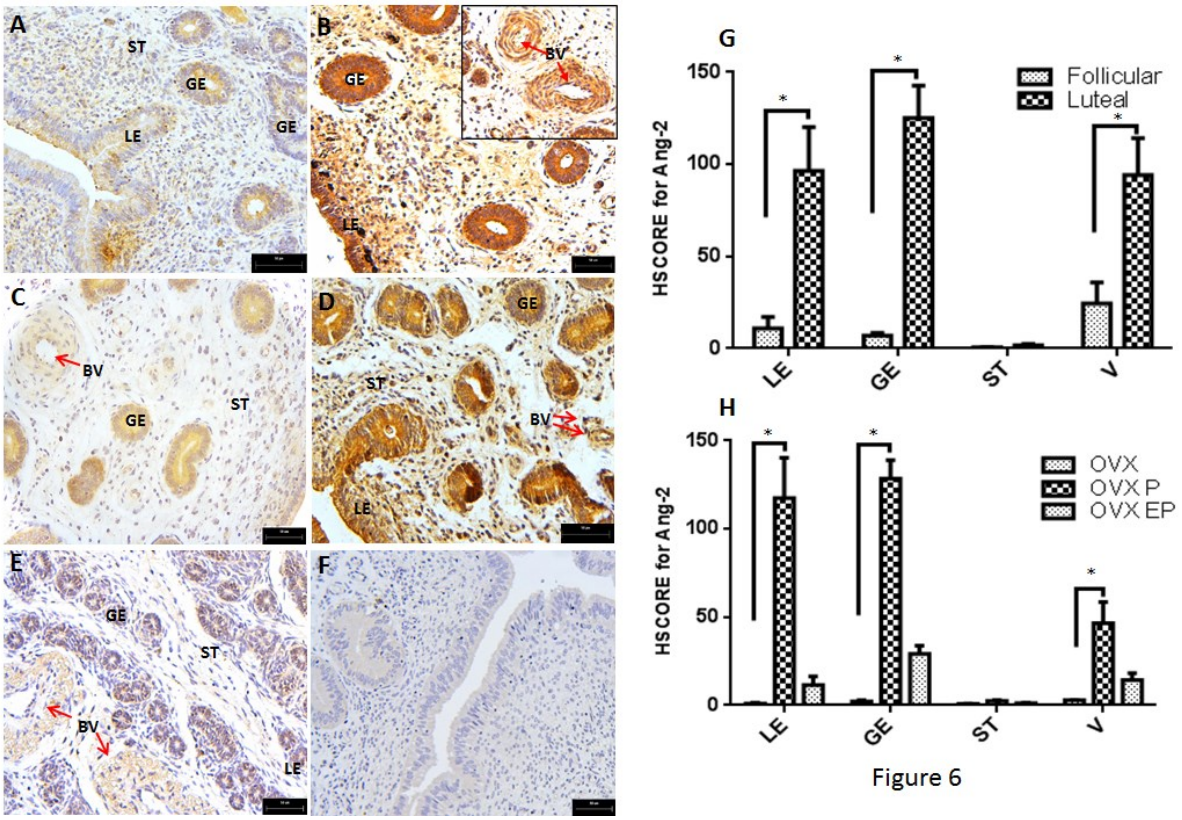

Figure 6

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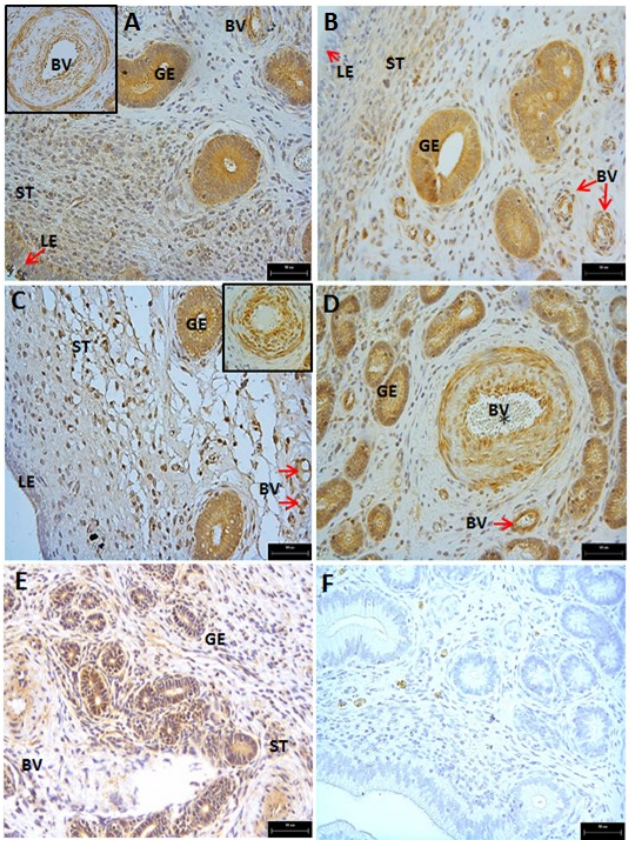

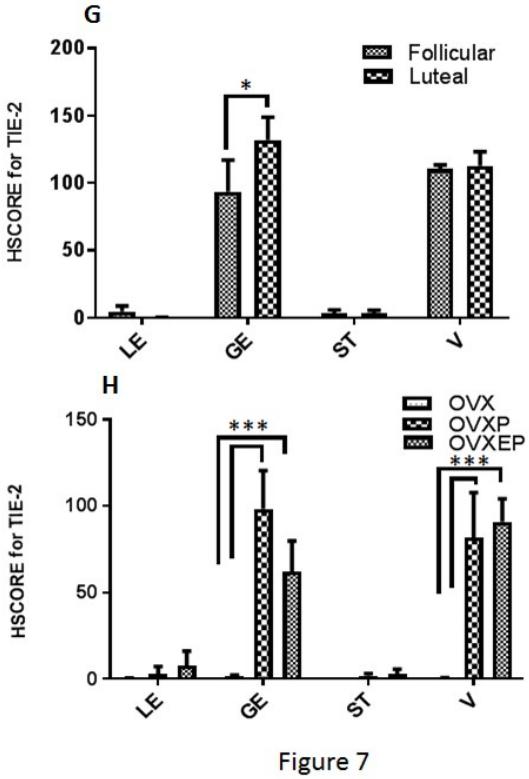

Figure 7