Effects of bovine pregnancy-associated glycoproteins on gene transcription in bovine endometrial explants

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled:

Effects of bovine pregnancy-associated glycoproteins on gene transcription in bovine endometrial explants

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and hereby certify that, in their opinion, it is worthy of acceptance.

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
CL	Corpus luteum
TE	Trophectoderm
LE	Luminal epithelium
IG	Integrin
IFN	Interferon
IFNτ	Interferon-tau
MUC1	Mucin-1
ECM	Extracellular matrix
SPP1	Secreted phosphoprotein 1/Osteopontin
GE	Glandular epithelium
RGD	Arg-Gly-Asp
MRP	Maternal recognition of pregnancy
ESR1	Estrogen receptor alpha
CG	Chorionic gonadotropin
LH	Luteinizing hormone
OXY	Oxytocin
PGF2α	Prostaglandin F2α
PGE2	Prostaglandin E2
OXTR	Oxytocin receptor
BNC	Binucleated cells
UGKO	Uterine gland knockout
ECM	Extracellular matrix
MMP	Matrix metalloproteinase
EnJSRN	Endogenous Jaagsiekte sheep retrovirus
Env	Envelope
Berve-A	Bovine endogenous retrovirus envelope element-like transcript-A
PAG	Pregnancy-associated glycoprotein

PL	Placental lactogen
PSPB	Pregnancy-specific protein B
PSP60	Pregnancy-specific protein of sixty thousand molecular weight
boPAG	Bovine pregnancy-associated glycoprotein
ELISA	Enzyme-linked immunosorbent assay
GCP2	Granulocyte chemotactic protein-2
CXCL6	C-X-C motif chemokine ligand 6
CXCL5	C-X-C motif chemokine ligand 5
ELR	Glutamate-Leucine-Arginine
SERPINA14	Uterine serpin
NK	Natural killer cells
TCR	T-cell receptor
BCR	B-cell receptor
HSC	Hematopoietic stem cell
PLC	Protein lipase C
MCP	Monocyte chemoattractant protein
MIP	Macrophage inflammatory protein

EFFECTS OF BOVINE PREGNANCY-ASSOCIATED GLYCOPROTEINS ON GENE TRANSCRIPTION IN BOVINE ENDOMETRIAL EXPLANTS

ABSTRACT

Pregnancy-associated glycoproteins (PAGs) are a complex gene family, whose members are expressed by trophoblasts of ruminants and related species. In cattle, the PAGs accumulate at the trophoblast-uterine interface and many can enter the maternal circulation. However, very little is known about the role they play in pregnancy although preliminary results suggest that PAGs at the placenta-uterine interface play roles involving matrix turnover and immune modulation. This study was designed to provide further insight into the biological roles of bovine PAGs by measuring changes in endometrial transcript abundance for some matrix metalloproteinases (MMPs) and chemokines/cytokines. PAGs for these experiments were purified from mid-gestation bovine placental extracts by affinity chromatography. Heifers were synchronized and bred by artificial insemination with high fertility semen (n = 14) or dead semen (n = 5). Heifers were slaughtered at day 18 post-insemination and the reproductive tracts were obtained and flushed to determine if a conceptus was present. Endometrial explants were collected and split between 4 groups: pregnant with and without 15 µg/ml PAG (n = 10) and nonpregnant with and without 15 µg/ml PAG (n = 9). Endometrial explants were cultured with or without added PAGs for up to 96 hours at 37°C and 5% CO_2 and samples were harvested at 24 hour intervals for extraction of RNA and fixation. This study focuses on the 48 and 72 hour collection points. Transcript abundance for target genes was analyzed in the endometrial tissue by quantitative PCR. The normalization control transcript was peptidylprolyl isomerase A (PPIA). After 48 and 72 hours, significant increases in *CXCL1*, *CXCL2*, and *CXCL5* as well as *MMP1*, *MMP3* and *MMP13* were measured in the PAG-treated endometrium from both pregnant and non-pregnant animals ($P<0.05$). *CCL11* was upregulated at both time points in the pregnant endometrium but only after 72 hours in the nonpregnant endometrium. There were also significant decreases in message for *CCL2*, *CCL8* and *CCL16* in the PAG-treated groups from both pregnant and non-pregnant animals at each time

point (P<0.05). Significant decreases in *CXCL10*, *CXCL12*, and *Regakine* message were seen only in PAG-treated endometrium from pregnant animals (P<0.05). Structural differences in the luminal and glandular epithelium were seen in the PAG-treated biopsies from both non-pregnant and pregnant heifers. These results suggest that PAGs are capable of inducing structural changes as well as changes in transcript abundance in bovine endometrial explants, which suggests that this model system might be useful to assess PAG function at the placenta-uterine interface.

CHAPTER I

LITERATURE REVIEW

Early Embryo Development

Blastocyst formation and conceptus elongation

In cattle, the morula stage embryo enters the uterus 4-5 days after mating (2.5-3 days in sheep). By days 7-8 the blastocyst has formed and on days 9-11 in cattle and days 7-8 in sheep, hatching from the zona pellucida occurs (Guillomot 1995; Hafez 1993). Because ruminant trophoblasts do not degrade or invade the maternal endometrium, it is believed that the ruminant conceptus compensates for a lack of direct access to maternal blood by occupying as much surface area as possible to increase the amount of exchange that can take place. Toward these ends, the embryo undergoes considerable expansion wherein it transforms from a 3mm spherical/tubular conceptus to a 250mm filamentous conceptus between days 13-17 in cattle (days 11-14 in the sheep). By day 18 (day 14 in sheep) the conceptus completely occupies the full length of the uterine horn ipsilateral to the corpus luteum (CL) and even extends into the contralateral horn (Guillomot 1995; Hafez 1993; Senger 2005; Spencer et al. 2004). Day 19 and day 15 after mating in cattle and sheep, respectively, marks the end of the pre-attachment phase and the beginning of the apposition phase. The conceptus will continue to elongate, however, until at least day 21 and day 16 in cattle and sheep, respectively.

Conceptus Attachment and signaling

Maternal recognition of pregnancy

Pregnancy maintenance requires that progesterone levels remain elevated beyond the length of a typical estrous or menstrual cycle. This means that the CL must be maintained throughout the entire pregnancy or until such time when the placenta can provide enough progesterone to support the

pregnancy. For this to occur, the conceptus must signal its presence to the maternal system to prevent luteal regression. This process is known as *maternal recognition of pregnancy* (MRP; Spencer et al. 2004a). MRP signaling from the conceptus usually falls into two categories: 1) luteotropic, i.e. directly stimulating the CL to maintain luteal function and 2) anti-luteolytic, i.e. prevention of luteolytic signals such as prostaglandin $F_{2\alpha}$ (PGF2α; Bazer et al. 2009). Human and primate embryos produce chorionic gonadotropin (CG) as a luteotropic signal. CG is similar to luteinizing hormone (LH) and it can bind to the same receptors on the CL, thereby promoting luteinization rather than luteolysis (R. Michael Roberts, Xie, and Mathialagan 1996; Fazleabas 2007; Fuller W. Bazer et al. 2009). Rodents maintain the CL through mating-induced release of prolactin from the anterior pituitary; this mechanism maintains the CL until about day 12 of pregnancy at which point placental lactogens from trophoblast giant cells take over the role (Soares 2004; Fuller W. Bazer et al. 2009). In pigs, the exact signal for MRP is unclear. It has long been accepted that conceptus-derived estrogens act as the primary MRP signal (Geisert et al. 1987 and 2015) by diverting PGF2α secretion away from the uterine vasculature and into the uterine lumen (Bazer and Thatcher 1977). However, recent data suggest that conceptus estrogens are necessary but not sufficient for pregnancy recognition and establishment (Meyer et al. 2019).

In ruminants, the signal for MRP is interferon-tau (IFNτ), a Type-1 interferon secreted by the trophoblasts of the peri-attachment conceptus (Helmer et al. 1987; Imakawa et al. 1987; Roberts et al. 1992; Roberts 2007). IFNτ Mrna and protein expression in the blastocyst is low but starts to increase around the time of conceptus elongation. Around day 14-15 in cattle, and day 12-13 in sheep, Mrna expression rises dramatically accompanied by an increase in protein production. The dramatic rise in IFNτ expression is probably due to the proliferation of the trophectoderm during conceptus elongation. This allows for large accumulations of IFNτ to interact across a large surface area of its likely target tissue (Ealy and Yang 2009). One study reported that a single filamentous ovine conceptus could produce up to 500ng of IFNτ per hour (Ashworth and Bazer 1989). This increase in expression is also significant

because it is occurring at the time in which MRP is required for pregnancy to be establish. In cattle, this is around day 16 and conceptus *IFNT* Mrna expression peaks around day 15-16 (Ealy and Yang 2009).

At the end of a normal ruminant estrous cycle when a viable conceptus is not in the uterus, the CL is regressed via a positive feedback loop between oxytocin (OXY) and $PGF_{2\alpha}$. OXY, initially released from the posterior pituitary, induces the secretion of uterine $PGF_{2\alpha}$ from the uterus. The $PGF_{2\alpha}$ enters the uterine venous drainage from where it quickly enters the ovarian artery via a counter-current exchange mechanism arising from the arrangement of the uterine venous and ovarian arterial vasculatures. The $PGF_{2\alpha}$ binds to its receptor on luteal cells, which induces the release of oxytocin from them. The oxytocin circulates and binds to its receptors on the uterus to induce a new pulse of $PGF_{2\alpha}$. The $PGF_{2\alpha}$ arising from this positive feedback loop induces a series of responses that suppress progesterone production from luteal cells and induce the functional demise of the corpus luteum. IFNτ acts as an anti-luteolytic signal by downregulating the expression of estrogen receptor α (ESR1) in the endometrium. This change decreases estrogen-induced OXY receptor (OXTR) expression. Without OXTR expressed in the endometrium, OXY cannot induce the pulsatile release of luteolytic $PGF_{2\alpha}$ from the endometrium and thus prevents regression of the CL (Ealy and Yang 2009). IFNτ may also prevent luteolysis through another mechanism. At high concentrations, IFNτ can increase production of PGE_2 from bovine endometrial epithelial and stromal cells (Asselin, Bazer, and Fortier 1997). PGE_2 has long been regarded as a luteotrophic and luteoprotective prostaglandin (Shelton et al. 1990). Therefore, it is not unreasonable to infer that IFNτ may be both inhibiting luteolysis and promoting luteinization for sustained CL function. Overall, there is strong evidence to support the hypothesis that IFNτ is a crucial factor in the recognition and establishment of pregnancy in ruminants.

Apposition and Adhesion

The initial stage of implantation, when the trophectoderm (TE) of the growing conceptus begins to closely associate with the uterine luminal epithelium (LE), is called the *apposition* phase. The apposition phase is marked by close association between the endometrial LE and the conceptus TE, loss of anti-adhesive components of the uterine glycocalyx, such as mucins, and subsequent unmasking of underlying adhesion proteins, such as integrins, to promote conceptus attachment to the LE. At this stage several morphological changes are also occurring within the LE and TE including cytoskeletal reorganization, microvilli formation, and epithelial interdigitation. The apposition phase is followed by the *adhesion* phase where more stable interactions are made between the two epithelia, which results in the TE becoming more fully adhered to the endometrial LE. This is mediated by adhesion proteins and interdigitation of endometrial and chorionic villi. This begins around day 21 in cattle and day 16 in sheep (Guillomot 1995; Spencer et al. 2004).

1. Adhesion proteins: Mucins

The anti-adhesive properties of the endometrial glycocalyx are due in part to the apical expression of mucins, primarily MUC1 and MUC4. Mucins are heavily glycosylated surface glycoproteins that are present on the surface of mammalian mucus membranes throughout the body, including the female reproductive tract. MUC1 is a main mucin found in several mucosal epithelia in mammals; among its many known functions, it also has been shown to play a crucial role in conceptus-uterine interactions. Mucins contain highly extended extracellular domains that can project 200-500 nm from the apical cell surface. By comparison, typical cell surface adhesion proteins, such as integrins, may only extent 10-30 nm from the cell surface (Thathiah and Carson 2002). MUC1 expression in the uterine LE is normally high prior to apposition and attachment. The abundance of MUC1 prevents the embryo from interacting intimately with other cell surface receptors. However, under the influence of progesterone

in pigs and sheep, MUC1 expression decreases, which then allows the conceptus to interact with integrins and other adhesion proteins on the LE surface (Johnson et al. 2001; Thathiah and Carson 2002). In sheep, uterine LE MUC1 protein is very abundant on the apical surface from Days 1-7 of the estrous cycle. Expression then begins to decline though Day 15 of the estrous cycle. This decline in MUC1 expression correlates with increasing progesterone levels and the onset of implantation. Furthermore, MUC1 expression on the LE of pregnant sheep is very low and is barely detectable by Day 17 of pregnancy when intimate contacts between the LE and conceptus are being made (Johnson et al. 2001). A similar expression pattern has also been seen in pigs (Bowen, Bazer, and Burghardt 1996).

MUC1 expression in the uterus is regulated by ovarian steroid hormones. In mice, MUC1 expression is greatly stimulated by estrogen while progesterone appears to antagonize the actions of estrogens (Surveyor et al. 1995). Ovariectomized gilts treated with either estrogen, progesterone, or estrogen + progesterone showed high MUC1 immunostaining of the uterine LE in response to estrogen. However, in the presence of progesterone (with or without estrogen) MUC1 staining was barely detectable (Bowen, Bazer, and Burghardt 1996). Treatment of mice with antiprogestins restored MUC1 expression (Surveyor et al. 1995) and inhibited embryo implantation (Vinijsanun and Martin 1990). Collectively, these data demonstrate that loss of MUC1 on the uterine LE is required for embryo/conceptus implantation (DeSouza et al. 2000; Thathiah and Carson 2002).

2. Adhesion proteins: Integrins

As mucins and other anti-adhesive components of the endometrial glycocalyx are altered via steroid hormone regulation and/or the actions of local or blastocyst-derived proteases, the underlying adhesive proteins are unmasked. These alterations allow adhesive proteins on the LE and TE to interact. Integrins (IGs) are a family of heterodimeric transmembrane glycoproteins that facilitate cell-cell and cell-extracellular matrix (ECM) adhesion as well as mediate intracellular signaling, cellular

differentiation, and cell motility (Y. Takada, Ye, and Simon 2007). IGs are present on both the uterine LE and the conceptus TE and are thought to play a crucial role in adhesion of these two epithelia during implantation. For example, null mutations in IG subunits α_v, α_5, β_1, or β_5 in mice lead to implantation failure, failure of the chorion and allantois to associate, and embryo lethality (Hynes 1996). Some species show spatial and/or temporal regulation of IGs in the endometrium throughout the estrus cycle, suggesting regulation by ovarian steroid hormones. Pigs constitutively express α_v, α_3, α_4, α_5, β_1 and β_3 subunits on the apical LE throughout the estrous cycle. However, α_4, α_5, and β_1 show spatial and temporal regulation (Bowen, Bazer, and Burghardt 1996). In cattle, α_3, α_4, and β_1 are constitutively expressed in the endometrium while expression of α_6 and the dimer $\alpha_v\beta_3$ changes throughout the cycle (Kimmins and MacLaren 1999). In contrast to cattle, sheep do not appear to modify temporal or spatial expression of IGs in the endometrium. Variations in IG expression among these closely related species suggests differences in implantation mechanisms (Kimmins and MacLaren 1999; Johnson et al. 2001).

Often, similar IGs are expressed on both the endometrial LE and conceptus TE. In pigs and sheep, IGs α_v, α_4, α_5, β_1, β_3, and β_5 are expressed on both the LE and TE around the time of conceptus attachment (Bowen, Bazer, and Burghardt 1996; Johnson et al. 2001). Similar IG expression may suggest common ligands, potentially serving as bridging molecules for conceptus adhesion. Osteopontin (Secreted phosphoprotein 1/SPP1) is one such bridging molecule. SPP1 is a secreted phosphoprotein of the endometrial glandular epithelium (GE) and is a component of the uterine histotroph. Studies in pigs (Garlow et al. 2002), sheep (Johnson et al. 2001; Johnson, Burghardt, Joyce, et al. 2003), and cattle (Kimmins, Lim, and MacLaren 2004) have shown that SPP1 protein increases in the endometrium and/or in uterine flushing around the time of implantation and may remain high throughout pregnancy. This is notable because SPP1 contains an Arg-Gly-Asp (RGD) sequence motif that mediates binding to integrins such as $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_4\beta_1$, $\alpha_4\beta_5$, and $\alpha_5\beta_1$, some or all of which have been identified in the endometrium of domestic animal species (Bowen, Bazer, and Burghardt 1996; Johnson et al. 2001; Johnson, Burghardt,

Joyce, et al. 2003; Thomas E. Spencer et al. 2004; Fuller W. Bazer et al. 2010; Johnson, Burghardt, and Bazer 2014; Johnson, Burghardt, Bazer, et al. 2003). Taken together, these data suggest that SPP1 could serve as a bridging ligand between IGs on the endometrial LE and the conceptus TE to facilitate conceptus adhesion and implantation. Other potential IG bridging molecules that could mediate or facilitate conceptus implantation include GlyCAM-1 (Giblin et al. 1997; Hwang et al. 1996; Thomas E. Spencer et al. 2004), galectin-15 (Kimber and Spanswick 2000; C. A. Gray et al. 2004; Thomas E. Spencer et al. 2004), and ECM components such as fibronectin, vitronectin, and laminin (Johansson et al. 1997; Burghardt et al. 2002; Wennerberg et al. 1996; Bowen, Bazer, and Burghardt 1996). These observations are suggestive that multiple adhesion mechanisms are involved in implantation and the establishment of a successful pregnancy.

Tissues at the fetal-maternal interface

Conceptus attachment not only involves changes in cell surface protein expression but also requires structural changes in the endometrium and TE to facilitate subsequent processes associated with placentation. Humans, primates, and rodents have an invasive type of placentation that requires extensive endometrial remodeling, such as LE erosion, stromal decidualization, and vascular remodeling (Enders and Blankenship 1999; Fazleabas 2007; Furukawa, Kuroda, and Sugiyama 2014). Ruminants and pigs exhibit a non-invasive type of implantation and placentation where little, if any, of the LE is lost. Instead, an extensive network of villous interdigitation can be seen between the LE and TE as well as vascular and glandular remodeling. The following sections will discuss these topics within the context of *ruminant* attachment.

Changes in the epithelia at the interface

The epithelial changes that occur during conceptus attachment are similar between ruminant species, the main differences are associated with the timing of such events. The following section will discuss the timing of events in relation to bovine pregnancy.

As discussed previously, during apposition the LE and TE become closely associated with each other. This is accommodated by a reduction of the apical microvilli along the surface of the TE. Unlike in other species, however, loss of endometrial microvilli does not appear to occur in ruminants (Guillomot et al. 1981; Guillomot et al. 1982; Spencer et al. 2004). Prior to the start of apposition (around day 12 of pregnancy in cattle), the intercaruncular (described later in 'Placentation') LE starts to develop large, bulbus cytoplasmic protrusions that resemble the 'pinopods' observed in rodents. The function of these cytoplasmic protrusion is not well understood, unlike rodents, however, they are not predicted to perform pinocytotic functions like those of rodents. It's thought that they may, instead, play a role in exocytosis and secretion of endometrial products during early pregnancy for support of the growing conceptus (Guillomot et al. 1986). This also occurs in cyclic animals, however, when no conceptus is present the protrusions regresses (Guillomot and Guay 1982). Around the start of apposition (about day 18 in cattle) the caruncular (described later in 'Placentation') LE begins to take on a folded appearance. Simultaneously, the conceptus trophoblasts that are normally rounded and covered in microvilli along their apical surface start to lose their surface microvilli and take on a more spindle-like shape. Their surface becomes irregularly rigid and perhaps slightly folded (Guillomot, Fléchon, and Wintenberger-Torres 1981; Guillomot and Guay 1982; Wooding, Staples, and Peacock 1982). By day 20, when firmer contacts are beginning to be made between the LE and TE, the TE is completely devoid of microvilli. This process appears to happen sequentially along the length of the conceptus starting in the TE closest to the embryo because non-adherent trophoblasts in the uterine

horn contralateral to the CL are still covered in microvilli at the same stage (Guillomot and Guay 1982; M. Guillomot 1995).

As apposition progresses and the two epithelia are pulled closer to each other, the microvillar surface of the uterine LE presses against the TE which gives the appearance of trophoblast projections into the 'crypts' between the uterine microvilli (Figure 1A). The rigid, folded surface of the TE also accommodates the invagination of the TE into the uterine microvillar crypts (Lawn, Chiquoine, and Amoroso 1969; Guillomot, Fléchon, and Wintenberger-Torres 1981). Localized areas of the TE begin to proliferate and form large columns made exclusively of trophoblasts known as 'papillae'. These trophoblastic papillae form in the intercotyledonary regions of the TE and project deep into the uterine glands (Figure 1B and C). It's thought that this is meant to aid in adhering the conceptus to the uterine LE by acting as an anchoring point. The papillae could also be privileged sites of endocytosis of glandular secretions to supply the peri-attachment conceptus with histotrophic nutrition (Guillomot and Guay 1982; Wooding, Staples, and Peacock 1982). These structures are short-lived, however, and start to degenerate around the start of conceptus adhesion.

Another characteristic change in the uterine epithelium of ruminants during attachment is the formation of a fetal-maternal syncytium. The syncytium is formed from the migration of a unique trophoblast cell type known as a giant binucleate cell (BNC). BNCs differentiate from mononucleate trophoblasts. They first appear in the TE between day 18 and 20 and shortly thereafter begin to migrate across the fetal-maternal interface (Wooding and Wathes 1980; Wooding 1982b). When BNCs cross the interface into the uterine LE, they fuse with a uterine epithelial cell to create a hybrid trinucleate cell. The trinucleate cells can continue to fuse with migrating BNCs until a large multinucleated syncytium is formed. These syncytial masses seem to only occur within the caruncular regions. In the cow, the syncytium is only transiently present and is usual gone by day 40 of pregnancy. However, in the sheep

and goat the syncytium is present throughout pregnancy. More on BNCs and syncytial formation will be discussed later in this review.

Figure 1

Endometrial Glands and histotroph in the establishment of pregnancy

Due to the non-invasive properties of the ruminant conceptus and the prolonged pre-attachment period, the nutrition for the early conceptus is through endometrial secretions known as 'histotroph'. Uterine histotroph contains substances that are essential for the growth and development of the fetus. The main tissues responsible for producing histotroph are the uterine LE and glands.

Uterine glands are a very important feature of the endometrium. They serve many important roles in the uterus during pregnancy including 1) synthesizing, secreting, or selectively transporting histotrophic substances into the uterine lumen (Bazer 1975); 2) promoting conceptus elongation by producing embryotrophic factors (Simintiras, Sánchez, McDonald, and Lonergan 2019c); and 3) assisting in blastocyst attachment as discussed previously (Wooding 1982a). Uterine gland development, or adenogenesis, begins postnatally in most domestic animals. It involves the differentiation and budding of the LE, which forms the GE, followed by invagination, coiling, and extensive branching throughout the uterine stroma (C. A. Gray, Bartol, et al. 2001). These steps are mediated by intrinsic factors such as changes in gene expression, cell proliferation, and cell-cell interactions as well as extrinsic factors such as pituitary and ovarian hormones (Kelleher, DeMayo, and Spencer 2019). Progesterone and estrogen are known to be especially important in endometrial adenogenesis. Blocking estrogen action with prolonged exposure to progesterone from birth to puberty creates a uterine gland knockout (UGKO) model, i.e. an animal that has substantially fewer than normal glands (Bartol et al. 1997; C. A. Gray et al. 2000; Filant, Zhou, and Spencer 2012). UGKO ewes are completely infertile. When bred, they carry morphologically normal blastocysts up until the time of attachment, at such time conceptus growth becomes retarded (Gray, Taylor, et al. 2001; Gray, Bazer, and Spencer 2001; Gray et al. 2002). This would suggest that the failure of the conceptus to elongate is due to the absence of embryotrophic factors originating from the uterine glands (Gray, Bartol, et al. 2001; Spencer, Kelleher, and Bartol 2019).

What are the embryotrophic factors in the uterine histotroph? Currently, a single embryotrophic factor cannot be determined. This is most likely due to the fact that the uterine histotroph is incompletely defined and because of its dynamic properties (Bazer 1975; Gray et al. 2001a; Mullen et al. 2012; Spencer et al. 2019). It is likely that there is no single embryotrophic factor, rather a dynamic heterogeneous mixture of growth factors, macromolecules and enzymes that supports growth and development of the trophoblasts. In addition to the composition of uterine histotroph itself, the composition of the secreted material is constantly altered by enzymes that are present (Simintiras et al. 2019c; Simintiras et al. 2019d) Not unexpectedly, histotroph production and secretion is regulated by steroid hormone actions. Current research suggests that the influence of progesterone alters histotroph composition to promote membrane biogenesis (Simintiras et al. 2019a), amino acid metabolism (Simintiras et al. 2019b), as well as glucose and nucleotide metabolism (Simintiras et al. 2019c). All of these data support the theory that uterine histotroph supports and/or promotes conceptus elongation by supplying the building blocks, metabolic energy and growth stimulates needed for trophoblast proliferation.

Role of MMPs in attachment and placentation

The morphological changes occurring during early pregnancy that have been discussed here all involve remodeling of the extracellular matrix (ECM). To support the attaching conceptus, and later the growing placenta, a delicate balance of degradation and formation of the extracellular environment must be regulated. This is done largely through the regulation of a family of enzymes known as the matrix metalloproteinases (MMPs). The MMPs are a gene family of more than 26 enzymes that are broadly grouped based on their preferred substrates (e.g. collagenases, gelatinases, stromelysins) or unique expression patterns (membrane-type-MMPs) (Birkedal-Hansen et al. 1993; Nagase and Woessner 1999; M. F. Smith et al. 2002). They are zinc- and calcium-dependent enzymes that are responsible for the degradation of the proteinaceous components of the ECM such as collagen I, II, III,

and IV, fibronectin, gelatin, and laminin (M. F. Smith et al. 2002). Remodeling of a tissue relies on the regulation of these enzymes at the level of transcription, through activation of the latent enzymes, and by directly inhibiting proteolytic activity of the mature enzyme (Smith et al. 1999; Smith et al. 2002). Most MMPs are secreted as proenzymes and require further processing from other enzymes such as serine proteinases (L. L. Espey and Lipner 1994), mast cell proteinases (Suzuki et al. 1995), and by other MMPs (Lijnen et al. 1998; Ruangpanit et al. 2002; Ra and Parks 2007)However, some MMPs are activated prior to secretion by the serine protease furin. These include MMP-11, MMP-23, and all membrane-type (mt-) MMPs (Strongin et al. 1995; Cao et al. 1996; Nagase and Woessner 1999; Sato et al. 1999). The activated enzymes are predominantly regulated by a small family of inhibitors known as tissue inhibitors or metalloproteinases (TIMPs -1, -2, -3, and -4). TIMPs bind noncovalently to the catalytic domain of most activated MMPs with high affinity and a 1:1 stoichiometry (Nagase and Woessner 1999; M. F. Smith et al. 2002). Contrarily, it is predicted that TIMP-2 is involved in the activation of proMMP-2 in vivo (Z. Wang, Juttermann, and Soloway 2000).

Regulation of tissue remodeling is primarily dependent upon the ratio of MMPs and their inhibitors (M. F. Smith et al. 2002). For species with invasive implantation, MMPs are especially important in aiding of degradation of the LE as well as the surrounding matrix to accommodate the invading trophoblasts (Hulboy 1997). During conceptus attachment, MMPs are expressed in the bovine endometrium and the TE throughout pregnancy. In particular, MMP-2 and -9 have been shown to play a role in attachment/implantation in most species (Harvey et al. 1995; Menino et al. 1997; Salamonsen, Nagase, and Woolley 1995; Franek, Salamonsen, and Lopata 1999; Jeziorska et al. 1996). In ruminants and pigs, TIMPs are also produced by both the TE (Menino et al. 1997; Hirata et al. 2003) and the endometrium (Salamonsen, Nagase, and Woolley 1995). They are thought to be acting to limit the invasive activity of the TE by limiting MMP activity (R. M. Roberts, Xie, and Trout 1993; Menino et al. 1997; Salamonsen, Nagase, and Woolley 1995). The ruminant endometrium has the capacity to produce

a range of MMPs. Of note, MMP-1, -2, 3, -9, and -14 have been shown to be expressed by the endometrium during attachment (Salamonsen, Nagase, and Woolley 1991; Salamonsen et al. 1993; Walter and Boos 2001). The endometrium also secretes TIMP-1 and -2 (Hampton et al. 1995). MMP-2 may be the most abundant of these MMPs and it is often colocalized with MMP-14 and TIMP2, both of which are involved in the activation of proMMP-2 (Z. Wang, Juttermann, and Soloway 2000; Uekita et al. 2004; Hashizume 2007). The TE also secretes MMP-2 and -9 as well as TIMP-2. Interestingly, TIMP-2 is very abundant within BNCs. This perhaps demonstrates a regulatory function of the BNCs in matrix remodeling (Hirata et al. 2003). There is also a lot of vascular remodeling occurring in the endometrium and in the chorion during conceptus attachment. MMPs are known to play a large role in vascular remodeling by degrading the endothelial basement membrane and remodeling the surrounding ECM to allow endothelial migration (Rundhaug 2005). During pregnancy, MMP-2 and -9 are localized around blood vessels in the caruncular and intercaruncular endometrium as well as in the chorion (Walter and Boos 2001). This would suggest a role for these MMPs in vascular remodeling during pregnancy in ruminants. Though we've been able to observe changes in endometrial ECM components, remodeling of the connective tissue, and vascular remodeling during attachment and placentation in ruminants, the exact mechanism by which this occurs is still unclear (M. Guillomot 1995; 1999; Bairagi et al. 2016).

Matrix remodeling is a continuous process in the endometrium and the placenta throughout pregnancy. However, there is a pause in matrix remodeling prior to parturition (Gross, Williams, and Russek-Cohen 1991). During this time MMPs increase in the TE, most likely to begin the process of utero-placental separation (Dilly et al. 2011). An increase in trophoblastic apoptosis is also seen in the final stage of pregnancy. Degradation of the placental ECM by MMPs is not followed by ECM deposition, which leads to apoptosis of the trophoblasts and eventually detachment of the placenta and fetal membranes at the time of parturition (Boos, Janssen, and Mulling 2003).

Placentation

Placental classifications

Many centuries of evolution has allowed mammals to develop a unique way to retain and sustain the fertilized egg through all necessary stages of development until birth (R Michael Roberts, Green, and Schulz 2016). This is accomplished through the development of a chorioallantoic placenta in a subset of animals called the Eutherians (placental mammals). In his monograph on comparative placentation, Mossman defined the placenta as an "apposition or fusion of the fetal membranes to the uterine mucosa for physiological exchange" (as cited by Roberts, Green, and Schulz 2016). He went on to describe the process of placentation as "an approximation or combination of an embryo's tissues with those of its natural or surrogate parent for physiological interchange" (as cited by Allen C Enders and Blankenship 1999). From these definitions we can take away two key concepts: 1) the placenta is derived from both fetal and maternal tissues, 2) these tissues must form close interactions to facilitate appropriate nutrient exchanges for a successful pregnancy. In particular, the placenta facilitates exchange of water, nutrients, and gasses; in addition, it eliminates fetal metabolic waste (R Michael Roberts, Green, and Schulz 2016). Due to the importance of these functions, it might be assumed that the placenta would be structurally conserved among eutherian mammals. This, however, is not the case. There are many morphologically distinct placental variations between mammalian species, but there are also several shared characteristics. This diversity among placental structures created a need for classification systems. Two particularly useful classification systems that were developed were based on 1) gross placental structure and 2) the cell layers present at the interface (P. Wooding and Burton 2008; Furukawa, Kuroda, and Sugiyama 2014). Other placental characteristics can be use in

addition to these criteria such as whether any tissue (fetal or maternal) is lost at parturition and whether any accessory placental structures happen to be present (P. Wooding and Burton 2008).

Gross structure

The gross structure of the placenta refers to the area of fetal-maternal exchange. More specifically, it refers to the distribution of villi or folding between the endometrium and the chorion where exchange takes place. It defines whether that area of exchange is found over the entire chorionic surface or is restricted to certain specialized regions (P. Wooding and Burton 2008; Furukawa, Kuroda, and Sugiyama 2014).

(1) Diffuse – A diffuse placenta is characterized by folds/villi that are present over the entire chorionic surface. These vascularized villi are in contact with nearly the entire luminal surface of the endometrium (Figure 2A). This placental structure is most notably found in pigs and horses.

(2) Cotyledonary – A cotyledonary placenta is characterized by localized areas of vascularized chorionic villi known as 'cotyledons' that intercalate into aglandular regions of uterine endometrium known as 'caruncles' (Figure 2B). Together, the combined structure consisting of a cotyledon and a caruncle is a 'placentome' (G. J. King 1993). Depending on the species there could be up to 150 placentomes on a single placenta (P. Wooding and Burton 2008). This type of placenta is found in ruminant ungulates.

(3) Zonary – A zonary placenta is characterized by a restricted region of complex chorionic folding that forms a band wrapped around the middle of the conceptus (Figure 2C). This creates an equatorial 'zone' of fetal-maternal exchange, hence the name 'zonary'. This placental structure is most notably found in carnivores.

(4) Discoid – This type of placenta is characterized by chorionic villi that are restricted to one or more distinct discs (Figure 2D), hence the name 'discoid' (P. Wooding and Burton 2008; Furukawa, Kuroda, and Sugiyama 2014). This placental structure is found in many species, most notable in rodents, lagomorphs and higher primates.

Tissue organization at the placenta-uterine interface

While classifying a placenta based on gross structure is simple, it does not provide much functional insight into how physiological exchange takes place. Grosser was the first to propose a more relevant classification system based on the number of intact cell layers at the fetal-maternal interface (Grosser 1909; 1927; referenced by Mossman 1987 and Amoroso 1952). This approach is still considered one of the most useful and instructive methods for functionally describing placental types. Grosser developed four placental categories that were essentially describing the number and type of maternal tissue layers that were apposed to the chorion in the mature placenta. These categories were: epitheliochorial, syndesmochorial, endotheliochorial, and hemochorial (Figure 3). The 'syndesmochorial' classification was later replaced with 'synepitheliochorial' after it was demonstrated to be a more accurate description of this placental type present in ruminant species (Wooding 1982a; 1982b; 1992).

(1) Epitheliochorial – The epitheliochorial placental type is the least invasive form of placenta. There is generally little to no invasion of the endometrium, resulting in the uterine LE being in direct apposition to the fetal chorion (Figure 3A). Species with this type of placenta are able to reduce the distance required for physiological exchange by interdigitation of vascularized chorionic and uterine luminal microvilli. This allows the fetal and maternal vasculatures to lie in close proximity to each other, which serves to decrease diffusion distance (Enders and Blankenship 1999; Wooding and Burton 2008; Furukawa, Kuroda, and Sugiyama 2014). There are a total of 6 tissue layers separating fetal and

maternal blood: fetal endothelium, fetal connective tissue, fetal TE, uterine LE, uterine connective tissue, and maternal endothelium.

Epitheliochorial placentation is found in many species of the Artiodactyla (e.g. pigs, whales, camels) and Perissodactyla (e.g. horses, rhinos) orders. This form is also represented in prosimian primates and Pangolins (Philidota order). Even within this category, there are distinct adaptations. The horse serves as a good example. In the horse, there is transient invasion of the LE and subepithelial stroma by a subset of trophoblasts known as chorionic girdle cells. These cells aggregate and differentiate into structures known as endometrial cups. The uterine LE is briefly lost during the migration of these cell but it is quickly recovered. Later in pregnancy the endometrial cups are destroyed by the maternal immune system. Invasion of the endometrium by girdle cells starts around day 36 of pregnancy; endometrial cups are fully formed around day 70 and their destruction begins around day 100. Overall, the horse exhibits a modified epitheliochorial placenta for only about ¼ of the pregnancy (Allen, Hamilton, and Moor 1973; Hamilton, Allen, and Moor 1973; A. C. Enders and Liu 1991; Carter and Enders 2004; P. Wooding and Burton 2008)

(2) Synepitheliochorial – Ruminants within the Artiodactyla order are known to have a modified type of epitheliochorial placenta known as 'synepitheliochorial'. The 'syn-' prefix is derived from the distinguishing feature of this placenta: a fetal-maternal syncytium. This type of placenta was originally named 'syndesmochorial' because it was believed that the uterine LE was lost and the chorion was in direct contact with uterine connective tissue (Grosser 1909; 1927). Later work from Wooding and colleagues (F. B. Wooding 1982a; 1982b; 1984; F. B. P. Wooding 1992) demonstrated that the LE is not lost, rather, it is modified. In ruminant placentation, giant BNCs migrate across the trophoblast-LE interface and fuse with uterine luminal epithelial cells to form a unique trinucleated fetal-maternal hybrid cells. Depending on the species and/or the developmental stage, continued fusion of BNCs can lead to the formation of large multinucleated syncytial plaques. Only the caruncular uterine epithelium

is modified in this way, however (Figure 3B). The LE within the intercaruncular areas remains intact and the fetal-maternal interface in these areas resemble an epitheliochorial arrangement (Wooding 1982b; 1984; Wooding et al. 1996). Overall, the number of tissue layers separating fetal and maternal blood is the same as for epitheliochorial placentation.

In sheep and goats, the syncytial plaques can persist throughout the entire pregnancy. In cattle, however, syncytial formation is transient. By day 40 of pregnancy most, if not all, of the syncytial plaques have been overgrown by the residual rapidly dividing luminal epithelial cells. Throughout the rest of the pregnancy, the placenta resembles an epitheliochorial placenta. BNC continue to migrate across the interface and form trinucleated cells, however, these cells are short-lived and are soon phagocytosed back into the TE layer (Wooding 1992).

(3) Endotheliochorial – An endotheliochorial type of placentation is more invasive than the previously mentioned placental types. This type of placentation involves the breaching of both the uterine LE and its underlying connective tissue so that the chorion can be in direct apposition to the maternal endothelial basement membrane (Figure 3C). In this type of placenta the fetal and maternal capillaries are usually separated by 4 tissue layers: fetal endothelium, fetal connective tissue, fetal TE, and maternal endothelium. This type of placenta has be studied most extensively in carnivores, although it is widely represented in eutherians (Enders and Carter 2006; Wooding and Burton 2008; Furukawa, Kuroda, and Sugiyama 2014)

(4) Hemochorial – Hemochorial placentation is the most invasive type of placentation. In this form, the invasive trophoblasts can erode or circumvent the uterine LE, connective tissue, and the endothelium of the maternal vasculature to establish direct contact with maternal blood. This leaves only 3 tissue layers separating fetal and maternal blood: fetal endothelium, fetal connective tissue, and fetal TE (Figure 3D). Hemochorial placentation can be further subdivided to described how many layers

of trophoblasts lie between the maternal blood and fetal connective tissue. When the placenta only contains a single layer of trophoblasts between maternal blood and fetal connective tissue it is called 'hemomonochorial'. When the placenta contains 2 layers of trophoblasts (usually a layer of syncytiotrophoblasts and a layer of cytotrophoblasts) it is called 'hemodichorial'. When the placenta contains 3 layers of trophoblasts it is called 'hemotrichorial' (Enders 1965; Enders and Blankenship 1999; Wooding and Burton 2008). This type of placentation is found in rodents, lagomorphs and higher primates.

Figure 2

Figure 3

Additional placental classifications

There are additional ways to characterize a placenta that provide further insight into how physiological exchange is mediated. One way is by classifying the type of fetal-maternal interdigitation. The tissue at the fetal-maternal interface can be classified into 5 conformational groups: folded, lamellar, trabecular, villous, and labyrinthine (Figure 4). The simplest of these forms is the 'folded' type, an arrangement seen in swine placentation (Figure 4A). This type of interdigitation involves apposition of the folded surface of the TE to the primary and secondary folds of the uterine LE (G. J. King 1993). When these folds are further drawn out into tall, closely packed sheets, the result is a 'lamellar' conformation (Figure 4B). This forms an elaborate interdigitation of elongated, parallel chorionic and uterine lamellae that is observed in the placenta of carnivores (Leiser and Koob 1993). A 'trabecular' interface contains incomplete folds that engage in secondary branching (Figure 4C; B. F. King 1993). This conformation is observed in some primates. A 'villous' type interface consists of complex chorionic branching that results in a three-dimensional tree-like structure (Figure 4D). This type of configuration is observed in human placentation (Wooding and Flint 1994). Finally, the most complex interface is a 'labyrinthine' configuration. It is comprised of a complex, three-dimensional, interconnected meshwork of chorionic villi which creates cavities that can fill with maternal blood for placental exchange (Figure 4E; Pijnenborg et al. 1981). This type of interface is seen in the placenta of rodents.

Placentae can also be characterized by the loss of maternal tissue at parturition. A 'deciduate' type of placentation results in a variable loss of maternal tissue with the fetal membranes at parturition. This generally correlates with placentas that involve erosion of maternal tissue (e.g. endotheliochorial and hemochorial). A 'non-deciduate' type of placenta separates at the fetal-maternal interface at parturition with very little to no loss of maternal tissue.

Finally, placentae can be classified by specialized accessory placental structures. Hemophagous zones are one such specialized structure that is defined as an 'area where the chorion and maternal tissue are widely separated by stagnant, but not necessarily clotted, maternal blood' (Wooding and Burton 2008). Trophoblasts in hemophagous zones are usually large and full of vacuoles containing phagocytosed erythrocytes. It is hypothesized that digestion of hemoglobin in these zones provides a source of fetal iron (Baker and Morgan 1973). Areolae are another type of specialized placental structure that are similar to hemophagous zones except, rather than filling with maternal blood, they fill with glandular secretions for endometrial glands. Areolae, most commonly found in epitheliochorial type placentation, consist of areas of columnar trophoblasts overlying the mouths of endometrial glands (A. C. Enders and Carter 2006; P. Wooding and Burton 2008). The areolar trophoblasts phagocytose the uterine glandular secretions known as 'histotroph' to supply the developing placenta with nutrients such as iron, amino acids, carbohydrates, proteins, and lipids (A. C. Enders and Carter 2006; Thomas E. Spencer 2014).

Figure 4

Binucleate cells

Ruminant ungulates possess a cotyledonary synepitheliochorial placenta. This form appears to be an adaptation of the epitheliochorial placenta (Roberts, Green, and Schulz 2016). As previously described, a large area of this placenta has retained an epitheliochorial phenotype, consisting of areas of simple apposition of the fetal and maternal tissues without loss of any maternal tissue. However, the 'syn' designation is derived from the multinucleated syncytium that is formed within specialized structures known as placentomes (the combined structure consisting of a maternal caruncle and a fetal cotyledon). The syncytium in ruminant ungulates is derived from the fusion of migrating fetal BNC with maternal endometrial epithelial cells (Wooding 1984; and 1992). The BNCs are very important for the structure and function of the ruminant placenta. In the following section, this review will attempt to describe the origins and the functional and structural significance of the BNC in the ruminant placenta.

Binucleate cell origin, structure, and differentiation

BNCs first appear in the ruminant placenta around the start of conceptus attachment. In cattle and goats this is around day 18 post coitus (Greenstein, Murray, and Foley 1958; Wango, Wooding, and Heap 1990) and in sheep this is around day 16 post coitus (Boshier 1969). Eventually, BNCs make up 15-20% of the TE of the mature ruminant placenta (Wooding 1992). Of the BNC population, 15-20% of those are migrating across the microvillar junction at any given time (Wooding 1983; Wooding et al. 1986).The origins of BNCs are unclear. There are no indications of stem cells or reserves in the TE that give rise to giant BNCs. However, the common assumption is that they originate from the columnar mononucleate cells (Wooding 1992; Wooding et al. 1994). Indeed, any mononucleate trophoblast appears to be capable of producing a BNC. It has been proposed that BNC are generated first through asymmetrical mitoses of mononucleate trophoblasts that gives rise to two distinct cells: a normal mononucleate trophoblast that incorporates into the TE and an irregular mononucleate cell that no

longer rests on the basement membrane or participates in apical tight junctions. This cell would then undergo acytokinetic mitosis, resulting in a binucleate cell with two diploid nuclei (Wooding and Flint 1994; Klisch et al. 1999). Klisch and colleagues were able to visualize irregular mitoses in 'free floating' trophoblasts that did not result in cytokinesis, thereby supporting the previous theory.

There is also little known about what drives syncytial formation during synepitheliochorial placentation. Research on this area suggests that endogenous retroviruses may be involved in placental growth, BNC differentiation, and BNC fusion (Dunlap et al. 2005 and 2006). In sheep, the endogenous beta retrovirus Jaagsiekte sheep retrovirus (enJSRV) envelope (*env*) RNAs have shown to be expressed throughout gestation in the endometrium and in the conceptus. However, when enJSRV *env* was knocked down in the endometrium and conceptus, conceptus growth was notably retarded by day 16 and there were a lack of BNC in the TE (Dunlap et al. 2005). This would suggest that this endogenous retrovirus plays a major role in conceptus growth and elongation as well as BNC formation. Bovine trophoblasts also express endogenous retroviral RNAs, most notably is *bovine endogenous retrovirus envelope element-like transcript-A* (*Berve-A)*, which is similar to human syncytin-1 (Koshi et al. 2011 and 2012). *Berve-A* as well as *Berve-K1 env* are detected in bovine trophoblasts during the peri-implantation period and their expression is enhanced during BNC induction. Like *enJSRV* in the sheep, these endogenous retroviruses may be involved in the formation of BNC (Koshi et al. 2012).

Once a giant BNC is formed, it is considered terminally differentiated and has lost its ability to proliferate. However, there is still a maturation process that must occur. The maturing BNC not only increases in size but also develops an extensive network of rough endoplasmic reticulum and a large Golgi apparatus (Wooding 1992). The mature BNC produces a large amount of proteins packaged into cytoplasmic granules; not unexpectedly, they possess large endoplasmic reticulum and Golgi. The cytoplasmic granules can occupy more than 50% of the total cell volume of a mature BNC. Contained within these granules is a mixture of proteins and glycoproteins; most notable they contain placental

lactogen and pregnancy-associated glycoproteins (PAGs) (Wooding 1982a; Duello, Byatt, and Bremel 1986; Green et al. 2000; Nakano et al. 2001).

Interestingly, the contents of the BNC granules may differ at different times throughout pregnancy; the granule contents can also differ with the location of the BNC in the placenta. This led to the hypothesis that, while all BNC ultrastructure is very uniform, there may exist subpopulations of BNCs that contain different proteins within their cytoplasmic granules (Wooding 1992). Immunostaining of bovine placentae with the BNC-specific monoclonal antibody SBU-3 revealed that staining did not occur until day 30 of pregnancy, even though BNC are present in the TE by days 18-20 (Morgan et al. 1989). In the ovine placenta, cytoplasmic granules of BNC within the placentome have been shown to contain both placental lactogen and SBU-3 antigen, however, neither of these were present in the interplacentomal BNC (Lee, Wooding, and Brandon 1986a, b, c; Lee, Gogolin-Ewens, and Brandon 1986). Furthermore, spatial differences in potential BNC subpopulations have been noted in deer. A higher concentration of SBU-3 positive BNC were present in the chorionic villous tips compared to the base of the villi (Lee, Gogolin-Ewens, and Brandon 1986). The SBU-3 antigen(s) are probably likely PAGs and more than likely a specific sub-group of PAGs (Atkinson et al. 1993). If that is the case, then the staining patterns seen with this antibody may suggest that different BNC produce different PAGs based on stage of pregnancy and location within the placenta.

Binucleate cell function: Migration and cell fusion

BNCs have two characteristic functions; 1) They migrate across the microvillar junction from the TE into endometrial epithelium, fuse with an endometrial epithelial cell to form a trinucleate cell, and eventually form a syncytium that is either transient (cattle) or persistent throughout pregnancy (sheep). 2) BNCs synthesize and deliver their secretory products into the uterine stroma after fusion with an endometrial epithelial cell (Wooding 1992).

After conceptus attachment, at any time during pregnancy BNCs can be seen migrating across the microvillar junction. About 20% of the total BNC population are actively migrating at any given time (Wooding 1983). In sheep and cattle, the earliest occurrence of BNC migration has been recorded as day 18 and 20, respectively. The free BNCs are not anchored to the TE by the basement membrane. Pseudopodia on the BNCs form that push through the TE apical tight junctions and make contact with the apical surface of an endometrial epithelial cell. The contact surface made between the BNC pseudopodium and the epithelial cell appears flat and without microvilli. The cytoplasm in the pseudopodium is devoid of organelles but abundant in small vesicles. This creates the initial fusion surface for the formation of a trinucleate cell (Wooding et al. 1994; Klisch et al. 1999). As the cytoplasm of the two cells mix during fusion, the cytoplasmic granules of the BNC move towards the maternal size of trinucleate cell. The secretory granules are shortly after exocytosed from the trinucleated cell towards the endometrial stroma where the contents either accumulate or then diffuse into the maternal circulation (Wooding et al. 1994).

This process is continuously occurring throughout pregnancy and its continuation is what ultimately forms the fetal-maternal hybrid syncytium that is characteristic of synepitheliochorial placentae. In sheep the syncytium can be quite extensive, consisting of syncytial 'plaques' with 20-24 nuclei. The syncytium in sheep also persists throughout pregnancy, whereas in cattle it is transient and usually disappears by day 40 (Wooding 1992). After day 40 of pregnancy in cattle, migration and fusion of BNC is still occurring, however, the trinucleate cells that are formed are short-lived and are quickly phagocytosed back into the TE (Wooding and Wathes 1980). Whether the syncytium is persistent or short-lived, continued migration and fusion of the BNC contributes to the development and growth of the placenta throughout pregnancy.

Products of binucleate cells

Trophoblastic BNC are known to produce many proteins and glycoproteins. With their large extensive networks of endoplasmic reticulum and large Golgi they can make large quantities of proteins and package them into secretory granules (Wooding 1992). One such protein is placental lactogen (PL), also known as chorionic somatomammotropin. PL is a placental polypeptide hormone with a molecular weight of 30-32 kDa (Murthy et al. 1982). It can be measured in both the maternal and fetal circulation of cattle, sheep, and goats during pregnancy and is immunolocalized to the BNC. More specifically, it is localized in the cytoplasmic granules of the BNC (Duello, Byatt, and Bremel 1986). PL hormones (there are at least 2 identified in cattle) exhibit both lactogenic and somatogenic properties in the mother and the fetus (Alvarez-Oxiley et al. 2008). Binding sites for PL are present in the fetal tissues, especially the fetal liver, which has given rise to the belief that PL may act as a fetal growth hormone (Thordarson et al. 1987).

BNCs also produce some steroid hormones. Progesterone is essential for pregnancy establishment and also for maintaining pregnancy. Cattle primarily rely on the CL as the main source of progesterone throughout pregnancy. However, sheep depend on placental progesterone for the last two-thirds of the pregnancy. Placental progesterone primarily comes from trophoblasts, both mononucleate and binucleate. Perhaps because of their size, BNC produce significantly more progesterone per cell than mononucleate trophoblasts. Enriched suspensions of bovine BNC produced large amounts of progesterone over a 4 hour period. They also produced PGI_2 and PGE_2. Both PGI_2 and PGE_2 are important in maintenance of the CL during pregnancy (Reimers, Ullmann, and Hansel 1985). Sheep appear to rely more on placental progesterone and probably benefit from the high production capacity of the BNC. Cattle probably benefit more from the BNC prostaglandins because the CL continues to be the main source of progesterone during pregnancy.

Lastly, the major products of the BNCs are the PAGs. A more detailed descriptions of these BNC products is discussed in the next section of this review. Briefly, the PAGs are a large family of placental aspartic proteinases produced by both the mononucleated and BNC trophoblasts of the ruminant and porcine placenta (Butler et al. 1982; Xie et al. 1991; Zoli et al. 1991). Ruminant PAGs can be put into two main categories: ancient and modern. The modern PAGs are exclusively produced by BNC (Figure 5B). Ancient PAGs can be produced by either the mononucleate trophoblasts or BNC or both (Figure 5A, 5C, 5D; Xie et al. 1991; Green et al. 2000). Like PL, some PAGs can enter the maternal circulation during pregnancy because of the migratory and fusogenic properties of the BNC. The PAGs in the BNC granules that get exocytosed into the uterine stroma either accumulate in the uterine stroma or diffuse into nearby endometrial capillaries (Green et al. 2000 and 2005; Wooding, Roberts, and Green 2005). Accumulation of PAGs in the maternal circulation forms the basis for most modern pregnancy tests for cattle and sheep (Green et al. 2005a). There is evidence to suggest that there may be subpopulations of BNCs that produce different PAGs based on the stage of pregnancy and location in the placenta (Lee, Wooding, and Brandon 1986a; 1986c; Lee, Gogolin-Ewens, and Brandon 1986; Wooding 1992). Currently, the function(s) of the PAGs are unknown. There is evidence to suggest that the PAGs may play a role in matrix remodeling at the interface and/or in the endometrium (R. M. Wallace et al. 2019), they may serve as luteotrophic agents during pregnancy (Weems et al. 2007), or they may be serving immunomodulatory roles (Hoeben et al. 1999). The next section of this review will attempt to give a more detailed overview on this complex family of placental glycoproteins with highlights on the gene family, its expression patterns, and proposed functions.

Figure 5

Bovine Pregnancy-Associated Glycoproteins

Overview

The pregnancy-associated glycoproteins (PAGs) are products of a gene family of placental aspartic proteases that are present in members of the *Cetartiodactyla* order (even-toed ungulates and cetaceans; Green and Hennessy 2018). They are produced exclusively by the trophoblasts of the placenta (mononucleated and BNC) throughout pregnancy. They were originally identified by three independent research groups from purified bovine placental extracts (Butler et al. 1982; Sasser et al. 1986; A P Zoli et al. 1991; S. C. Xie et al. 1991; Mialon et al. 1994). The protein that was initially isolated was named pregnancy-specific protein B (PSPB) and pregnancy-specific protein of sixty thousand molecular weight (PSP60). Today we know this protein as the first member of the bovine PAG family, PAG1 (Guruprasad et al. 1996). Since then, the characterization of this gene family has revealed an unanticipated complexity in the number and placental expression of the PAGs. At least two dozen distinct PAGs have been identified in cattle and sheep. Other species within Bovidae have comparable numbers of PAG genes; while there are fewer in pigs (Suidae) and whales (Cetacea). Although we still don't know much about the roles of the PAGs, they likely play important roles in placental function and pregnancy in even-toed ungulates. This review of the bovine PAGs will attempt to provide the reader an overview of the gene family, its products, and the potential roles they may play in pregnancy.

The PAG family

The PAGs are believed to have arisen as the result of a duplication and subsequent expansion of an ancestral *pepsin F*-like gene around the time that the *Cetartiodactyla* order arose (Hughes et al. 2000). This *pepsin F*-like gene itself is represented in most mammals and it is thought to have arisen from a duplication of *pepsin A*. Pepsin F and pepsin A, are members of an enzyme family known as aspartic proteinases. Phylogenetic analyses of the PAGs shows them clustering with other known

aspartic proteinases such as the cathepsin D, cathepsin E and renin (Hughes et al. 2003; Green and Hennessy 2018). Phylogenetic analyses also show a segregation of the ruminant PAGs into two distinct groups termed 'ancient' and modern' based on the times at which they arose during *Artiodactyl* evolution (Hughes et al. 2000). The ancient PAGs are predicted to have emerged approximately 87 million years ago and consist of bovine PAG (boPAG) -2, -8, 10, -11, and -12 (Hughes et al. 2000). The modern PAGs are by far the most numerous and make up the rest of the PAG family. They are predicted to have arisen approximately 52 million years ago (Hughes et al. 2000) and have only been observed in the *Ruminantia* (B. P. V. Telugu, Walker, and Green 2009). The *Bovidae* (cattle, sheep, etc.), in particular, have experienced a large expansion of the PAGs with at least two dozen confirmed PAG genes plus numerous pseudogenes, adding to the complexity of this gene family.

The PAGs belong to the aspartic proteinase group of enzymes and they share the common structural features of these enzymes. They consist of a symmetrical bi-lobed, bean shaped structure with a cleft in between the lobes that forms the peptide binding/catalytic domain. Within the binding cleft, each lobe contributes a catalytic aspartic acid residue within a conserved sequence motif (Asp-Thr-Gly-Ser/Thr-Ser/Thr) that is preceded by two hydrophobic amino acid residues (Dunn 2002; Green and Hennessy 2018). The catalytic aspartic acid residues give rise to the name of this group of enzymes: 'aspartic' proteinases (Davies 1990). The catalytic aspartic acids participate in the hydrolysis of peptide bonds by orientating and stabilizing a water molecule between them. This water molecule can then serves as a nucleophile during hydrolysis of the bound peptide (Dunn 2002; Green and Hennessy 2018). The majority of the PAGs contain these conserved aspartic acid motifs, along with other

conserved regions found in this enzyme class. Consequently, most have the potential to function as active aspartic proteinases. This activity has been demonstrated in some PAGs such as boPAG2, boPAG12 and porcine PAG2 (Telugu and Green 2008; Telugu et al. 2010) However, there are a few notable exceptions. For example, the first of the bovine PAGs to be cloned, boPAG1, has the catalytic aspartic acid residues but one of the invariant glycine residues in the N-terminal catalytic domain is replaced with an alanine (Xie et al. 1991). BoPAG10 also has an amino acid substitution in its C-terminal catalytic domain. Further cloning of the PAGs has revealed multiple bovine and ovine PAGs with mutations within the conserved catalytic domains that may render these proteins enzymatically inactive (Guruprasad et al. 1996; Sancai Xie et al. 1997; Green et al. 2000). At present, it is unclear if the enzymatic activity is relevant to their biological role(s) *in vivo*.

Temporal and spatial expression of PAGs

1. Expression at the interface

The complexity of the PAG family not only comes from the large number of family members but also in their expression patterns throughout pregnancy. Because of the large expansion of the PAGs in *Ruminantia,* the expression patterns of various PAGs have been well studied throughout pregnancy. Initially the characterization of PAG expression was done using *in situ* hybridization in placentomes of cattle, sheep, and goats (Garbayo et al. 2000; Green et al. 2000). These studies revealed differences in cell-specific expression patterns of the PAGs. The majority of the PAGs studied were expressed solely by the BNCs. However, a few of the PAGs were expressed by all trophoblasts (mononucleated and binucleated). The cell-type expression seemed to correspond with the 'ancient' versus 'modern' divisions of ruminant PAG phylogeny;. In general, the modern PAGs are BNC-specific. Both Mrna and

protein expression of these PAGs are restricted to the BNC (Green et al. 2000). The ancient PAGs are a bit more complicated because they can be expressed by the mononucleated trophoblasts, BNC, or both. For example, boPAG1 is a modern PAG that is expressed solely by the BNCs (Xie et al. 1991). *BoPAG2* is an ancient PAG that is expressed uniformly by all trophoblasts (Sancai Xie et al. 1994). Immunolocalization of boPAG2 agrees with the *in situ* localization (R. M. Wallace et al. 2015), however, one immunolocalization study did not observe boPAG2 staining in the BNC (Touzard et al. 2013). *BoPAG8* is also an ancient PAG but it expressed only by the mononucleated trophoblasts (R. M. Wallace et al. 2015). In contrast, with *in situ* hybridization *boPAG10* (an ancient PAG) appears to have a broad trophoblastic distribution (Green et al. 2000) but immunolocalization localizes this PAG specifically to BNCs (R. M. Wallace et al. 2015). These expression patterns do not apply to all species, however. There are exceptions, such as deer PAG3 (a modern PAG) that is expressed in both mononucleated trophoblasts and BNC (Brandt et al. 2007).

With the exception of the *Tragulidae* family (mouse deer), which have a diffuse placenta (Kimura et al. 2004), *Ruminantia* members have developed a unique strategy for utero-placental exchange. As discussed previously in this review, the ruminant placenta consists of two distinct areas of exchange; 1) the intercotyledonary regions where the exchange surface is that of an epitheliochorial placenta, and 2) the cotyledonary regions where the exchange surface is increased by networks of cotyledonary and caruncular villi and syncytial cells or multinucleated syncytial plaques. While BNCs are present in both of these regions, only in the placentome do they seem to cross the interface into the endometrial epithelium (Wooding, Morgan, and Adam 1997). This provides a new delivery mechanism for the BNC-produced PAGs that is distinct from the intercotyledonary regions of the placenta. The population of PAGs expressed between these two regions often differs as well as the accumulation of PAGs at the interface or within the tissues. *BoPAGs1, -2, 3, -15, -17*, and *-21* expression is greater in the cotyledons than in the intercotyledonary TE (Touzard et al. 2013). Even within the cotyledons there may

be uneven spatial distribution of the PAGs. Immunolocalization of boPAG2 revealed that this protein is not equally distributed within the placentome. BoPAG2-positive trophoblasts were seen primarily in the secondary and tertiary cotyledonary villi and little to no staining in the villous stems or the villous base (Touzard et al. 2013). In contrast, *boPAG8, -10, -11,* and *-12* expression is greater in the intercotyledonary TE throughout pregnancy. Western blots of boPAG11 (an ancient BNC-specific PAG) extracted from cotyledonary and intercotyledonary TE agrees with the Mrna expression, however, immunostaining labeled boPAG11-positive BNCs in both tissues. The intercotyledonary TE showed an even distribution of boPAG11 but in the cotyledons, boPAG11 BNCs were restricted to the chorionic plate and were absent in the secondary and tertiary villi (Touzard et al. 2013). Some PAGs are capable of accumulating in the uterine stroma. As discussed previously in the binucleate cell section of this review, BNCs within the placentome can migrate across the utero-placental interface to enter the uterine LE and fuse with an epithelial cell. Fusion of the BNC with a LE cell allows the BNC granules to be exocytosed into the endometrial stroma (Wooding 1992). It's very likely that the PAGs produced by the BNCs within the placentome can enter and accumulate in the uterine stroma as a result. Wooding et al. were the first to report a systematic evaluation of the boPAGs by immunolocalization (Wooding, Roberts, and Green 2005). They reported that immunostaining of some boPAGs, primarily modern PAGs, also stained the endometrial connective tissue of the caruncular villi as early as day 30 of pregnancy. This pattern of villous connective tissue staining was seen throughout the rest of pregnancy only for the modern PAGs and within the placentome. In the intercotyledonary TE, immunostaining for modern PAGs was restricted to BNCs and no staining of the endometrium was noted (Wooding, Roberts, and Green 2005). Because the ancient PAGs are often produced by mononucleated trophoblasts that do not migrate across the interface, no uterine connective tissue staining was ever observed. However, accumulation of these PAGs was observed along the microvillar junction as early as day 20 or pregnancy. This pattern is similar to what is seen in pigs, where fetal-maternal interdigitation is less extensive and

there is no BNC migration. Accumulation of these PAGs along the microvillar junction was observed throughout pregnancy (Wooding, Roberts, and Green 2005). It is clear that each PAG may possesses a unique spatial expression pattern within the placenta. Whether or not these expression patterns contribute to their functions is still unclear.

While the PAGs are not distributed evenly throughout the placenta at a given time, the same is true of their temporal expression throughout pregnancy. Early efforts to characterize the expression of the PAGs throughout pregnancy were done by Green et al. using Rnase protection assays to identify various PAG transcripts from day 25 of pregnancy to term. The authors demonstrated that each PAG seems to have a distinct temporal expression pattern. Some PAGs are expressed throughout pregnancy while expression of other may not turn on until a mature chorioallantoic placenta has formed. One commonality, however, is that expression of the BNC-specific PAGs studied was invariably absent in term placenta (Green et al. 2000). Patel and colleagues focused on *boPAG1* and *boPAG9* (modern PAGs) expression from days 30-250 of pregnancy. They showed that expression of *boPAG9* is up to 10 times greater in early pregnancy compared to *boPAG1* expression. *BoPAG1* Mrna abundance seemed to increase as pregnancy progressed, peaking in cotyledonary tissue on day 250 (Osman V. Patel et al. 2004). These results were consistent with the earlier study by Green et al. that showed that the earliest expression of *boPAG1* was seen on day 45 while *boPAG9* was highly expressed on day 25 (Green et al. 2000). Work by Telugu et al. focused on temporal expression of the ancient PAGs. They demonstrated that each of the ancient PAGs has a distinct temporal expression pattern that, with the exception of *boPAG10*, the ancient PAGs are most abundant in early pregnancy. *BoPAG10* displays an opposite expression pattern that is low in early pregnancy and peaks at term (Telugu, Walker, and Green 2009).

Overall, the PAGs express a range of expression patterns, both spatial and temporal, throughout pregnancy. In general, the ancient PAGs are usually expressed early in pregnancy at a greater abundance than the modern PAGs (Patel et al. 2004; Telugu, Walker, and Green 2009; Touzard et al.

2013). The modern PAGs vary considerably in their temporal expression but most of them appear to be absent or minimally expressed in term placenta (Green et al. 2000; Touzard et al. 2013). The cell-specific expression combined with the distinct spatial and temporal expression patterns of the PAGs sheds light on the complexity of this family of aspartic proteinases. It is reasonable to assume that the unique expression patterns of the PAGs is reflective of their functional role(s) during pregnancy. What those roles are remains unclear but several possibilities are discussed in the 'Possible functions' section of this review below.

2. Use of PAGs in pregnancy diagnosis

Pregnancy detection in cattle traditionally was done via rectal palpation. While this is still performed today, farmers and researchers recognize that this is not the most reliable method of pregnancy diagnosis, particularly when performed in early pregnancy. In the '70's the measurement of progesterone concentration in the milk became a popular tool and was found to be relatively accurate and this approach became a useful method for researchers and producers (Pennington, Spahr, and Lodge 1976; Pennington, Schultz, and Hoffman 1985; Laing and Heap 1971; Heap et al. 1976). However, there are a lot of factors that affect progesterone concentrations that often led to incorrect pregnancy diagnosis; these include 1) variations in the length of the estrous cycle (short vs. long cycle) and 2) embryonic mortality (Pennington, Schultz, and Hoffman 1985). In other species, assays for placental proteins, such as human chorionic gonadotrophin (Hcg) in humans, was commonly used to diagnose pregnancy. When the PAGs were first discovered (originally called PSPB) they became a prime candidate for pregnancy diagnosis. As discussed previously in the binucleate cells section of this review, the BNC-produced PAGs are released into the endometrial stroma after fusion of BNCs with LE cells. The PAGs released into the uterine stroma often accumulate around capillaries in the shallow stroma which allows them to enter the maternal circulation, making them a good candidate for placental-specific pregnancy diagnosis (Wooding, Roberts, and Green 2005). Sasser et al. were the first to develop

a radioimmunoassay targeting PSPB in the maternal serum of cows. They were able to reliably detect PSPB as early as day 30 of pregnancy and noticed that PSPB serum concentrations rose through pregnancy and peaked about two days before parturition (Sasser et al. 1986). Later studies were able to develop an enzyme-linked immunosorbent assay (ELISA) for the detection of PAGs in bovine and ovine sera (Green et al. 2005a; Egen et al. 2009). These studies were able to create a PAG serum profile that mirrored the profile recorded by Sasser et al. The bovine ELISA uses monoclonal anti-boPAG antibodies that detect a rise in serum PAG concentrations around days 23 of pregnancy and could diagnose pregnancy by day 28 with 93-96% accuracy (Silva et al. 2007; Pohler et al. 2013). In cattle, serum PAG concentration begins to increase to from day 23 to about day 36, reaching an average concentration of 8.75 ng/Ml by day 28 and 12.3 ng/Ml by day 35. Between days 36 and 60 PAG concentrations decline to about half of that on day 35, but then start to steadily increase through the remainder of pregnancy. During the last week of pregnancy, serum PAG concentrations rise dramatically and peak between 500ng/Ml to as much as 5ug/ml, depending on the assay that is employed. Concentrations decline after parturition, but persistent circulating PAGs post-partum can limit this method of pregnancy detection for a subsequent pregnancy. PAGs in the postpartum circulation creates a potential for false positives if an animal were to be rebred too soon after calving. The ELISA established by Green et al. attempted to address this scenario by using monoclonal antibodies that target PAGs that were present early, were less abundant at term, and exhibited a shorter half-life (Green et al. 2005a). Overall, this ELISA has become a useful tool for ruminant pregnancy diagnosis. Today various forms of this assay have been developed commercially and are regularly used by companies to provide pregnancy diagnostic services.

In addition to pregnancy diagnosis, PAGs circulating in maternal blood have also been used to characterize fetal number, fetal health, parity, lactation status, and early embryonic loss (O. V. Patel et al. 1995; J. M. Wallace et al. 1997; Chavatte-Palmer et al. 2006; López-Gatius et al. 2007; Constant et al. 2011; Pohler et al. 2013). Additionally species differences are indicated between genotypes (Lobago et

al. 2009; Mercadante et al. 2013). For example, circulating PAGs in cattle with *Bos indicus* genetics are higher than cattle with *Bos taurus* genetics at a mean gestational age of 53 days (Mercadante et al. 2013). Placental insufficiencies, such as those from pregnancies of somatic clones, are also known to cause elevated systemic PAG concentration. Somatic clone pregnancies have been known to present with higher circulating PAG concentrations from days 24-50 (Chavatte-Palmer et al. 2006) and day 62 of pregnancy (Constant et al. 2011).

Possible functions of the PAGs

1. Matrix remodeling and adhesion

Though the PAG family has been extensively studied over the last forty years, little is known about the role(s) that the PAGs play during pregnancy. Speculation have been made based on their localization patterns during pregnancy and their predicted proteolytic activity, if any. Some of the PAGs are known to be proteolytically active. BoPAG2, boPAG12, and porcine PAG (poPAG)-2 have all been shown to cleave a common aspartic proteinase FRET substrate (Telugu and Green 2008; Telugu, Walker, and Green 2009). These PAGs have been shown to accumulate along the microvillar junction, so it is reasonable to predict that some PAGs could be acting to proteolytically process proteins at this location. Latent growth factors at the interface are potential substrates for aspartic proteases such as PAGs (Munger et al. 1998; Rifkin et al. 1999; Moussad et al. 2002). If PAGs at the interface are proteolytically active, then it's likely that PAGs accumulating in the uterine stroma could be proteolytically active as well. It's also possible that the PAGs could affect matrix remodeling by upregulating expression of MMPs. In bovine endometrial explants, treatment with PAGs (a mixture of modern PAGs) increased the endometrial expression of several MMPs (R. M. Wallace et al. 2019). Whether this is a direct result of PAG action or a downstream effect of the PAGs is unclear. It is also not known whether this same response occurs *in vivo*.

Another possible role for the PAGs is as adhesion proteins. All PAGs contain an amino acid binding cleft and several glycosylation sites on the protein's surface. The binding cleft could allow for interactions with other proteins, especially proteins at the microvillar junction, such as integrins. The carbohydrates could conceivably interact with lectins, thereby permitting the PAGs to act as bifunctional linking molecules (Klisch et al. 2007; Wooding, Roberts, and Green 2005). So long as the Ph at the microvillar junction is not too low, the enzymatically active PAGs could also serve as binding/linking molecules (aspartic proteinase activity is typically greatest at low Ph). If the Ph were to drop and the PAGs were to become activated, which might occur around parturition, then the active PAGs could begin to cleave their bound proteins and sever the protein-protein connections between the placenta and endometrium (Green and Hennessy 2018). Very little research has been done on this potential aspect of the PAGs so this is all speculative.

2. Luteotrophic

Many PAGs are first expressed by the placenta during conceptus attachment, the time when most early embryonic loss occurs (Short 1969; Martal et al. 1979; Godkin et al. 1984). Therefore, it is not unreasonable to predict that the PAGs may play a role in early pregnancy maintenance. Early studies demonstrated that PAG/PSPB could increase production of PGE_2 (Del Vecchio, Sutherland, and Sasser 1996), which in turn could increase progesterone production by bovine luteal cells *in vitro* (Del Vecchio, Sutherland, and Sasser 1995). Later studies by Weems et al. showed that PAGs increased the production of PGE_2 relative to PGF_{2a} in cultured bovine luteal and endometrial cells and explants (Weems et al. 1998; 2003; 2007). This is relevant because PGE_2 is considered to be a luteotrophic prostaglandin (Shelton et al. 1990; Arosh et al. 2004) and its concentration in relation to PGF_{2a} is important in determining the fate of the CL (Christenson et al. 1994; Ziecik 2002; Kaneko and Kawakami 2009). Therefore, it is has been predicted that the PAGs may serve a luteotrophic role during early pregnancy by regulating PGE_2 and PGF_{2a} production by the CL and/or the endometrium.

3. Immunomodulatory

The PAGs have also been implicated in modulation of the maternal immune system in cattle. Circumstantial evidence was recorded by Dosogne et al. who noticed that peak plasma PAG concentrations at parturition immediately preceded a decrease in circulating polymorphonuclear cell (PMN) phagocytosis (Dosogne et al. 1999). Reports from the same group reported that a decrease in the respiratory burst activity of circulating PMN coincided with an increase in plasma PAG concentrations in cattle (Hoeben et al. 2000). Experimental evidence from this group also demonstrated that treatment of immune cells with PAGs decreased the proliferative ability of bovine erythroid and myeloid cells (granulocytes and monocytes) *in vitro*, suggesting that high concentrations of circulating PAGs around parturition could contribute to a decrease in innate immune responses and an increased susceptibility of cattle to mastitis (Hoeben et al. 1999). PAGs have also been linked to chemokine release from the endometrium. An early study identified an 8 kDa protein that was secreted by bovine endometrial explants in response to treatment with PSPB and IFNτ (Austin et al. 1999; T. R. Hansen et al. 1999). This protein was identified as bovine granulocyte chemotactic protein-2 (GCP2). It is an alpha chemokine, also known as CXCL6, whose primary function is to recruit and activate neutrophils (Rajarathnam et al. 2019). In the current bovine genome build, this chemokine is now named CXCL5 as it more closely resembles human CXCL5 (more on this point in the 'Immune system' section of this review). More current research from our lab supports the results seen by Austin et al.; bovine endometrial explants treated with boPAGs for 24 and 96 hours showed a significant upregulation in *CXCL5* Mrna expression (R. M. Wallace et al. 2019). While the role of neutrophils during early pregnancy is not well studied in ruminants, current evidence for potential immunosuppressive and immune recruitment functions of the PAGs may indicate a role in regulating immunity and inflammation associated with attachment in ruminants.

Several alpha chemokines, such as CXCL5, not only recruit neutrophils to sites of inflammation but are notable players in tissue angiogenesis. Chemokines containing a Glu-Leu-Arg (ELR) amino acid motif have been shown to promote angiogenesis *in vitro* and *in vivo* (Strieter et al. 1995; Balestrieri et al. 2008). Angiogenesis is very important in the endometrium and in the developing placenta for facilitating transplacental exchange (Reynolds and Redmer 1995 and 2001). Since boPAGs can upregulate transcription and production of endometrial CXCL5, it may be likely that this function is not only important for immune regulation but also angiogenesis of the endometrium. Results from the current research of this thesis demonstrate that PAGs can induce an increase in expression of several angiogenic chemokines (CXCL1, CXCL2, and CXCL5), providing further support for this hypothesis. The role of chemokines in angiogenesis will be further discussed in the 'Immune system' section of this review.

Uterine serpin is another immunomodulatory protein that has been shown to interact with PAGs (Mathialagan and Hansen 1996). The serpins are a large family of serine proteinase inhibitors. One member of this family, in particular, is expressed in the endometrium of some mammals. Uterine serpin, also known as SERPINA14, is a serpin that is regulated by progesterone and appears to have lost its inhibitory activity towards serine proteinases (Padua and Hansen 2010). It is thought that the function of SERPINA14 may be species specific; in sheep it has been shown to have immunomodulatory properties. Ovine SERPINA14 can block lymphocyte proliferation induced by IL-2 (Skopets, Liu, and Hansen 1995; Peltier, Liu, and Hansen 2000), block ovine and murine NK cell activity (W.-J. Liu and Hansen 1993), and reduce antibody production in ewes immunized against a T-cell dependent antigen (Skopets, Liu, and Hansen 1995). While SERPINA14 does not bind to serine proteinases, it does have an affinity for aspartic proteinases and can weakly inhibit pepsin A and C. It can also bind ovine PAGs (Mathialagan and Hansen 1996). Circumstantial evidence in bovine endometrium demonstrated that endometrial expression of SERPINA14 and boPAG1 and -2 was inversely related. In cows artificially

infected with *N. caninum*, endometrial boPAG1 and -2 expression was elevated while SERPINA14 expression was reduced (Serrano-Pérez et al. 2016). Due to the fact that PAGs are produced by the placenta, measuring PAG Mrna expression in the endometrium usually means that separation of the fetal membranes from the endometrium was incomplete and there are still trophoblasts left over in the endometrium. That being said, PAG Mrna was consistently upregulated in the endometrium of *N. caninum* infected animals, perhaps suggesting an increase in trophoblast PAG expression and deposition in the endometrium in response to the infection (Serrano-Pérez et al. 2016). Increasing PAG expression coupled with decreasing SERPINA14 expression could signify an inverse or antagonistic relationship between these two proteins. If an active infection is present it would be counterintuitive to increase production of an immunosuppressant such as SERPINA14. Perhaps the PAGs both decrease immunosuppressive proteins and increase immune cell recruitment via chemokine signaling. Of course this is speculative and more research will need to be done to conclude this.

Lastly, circumstantial evidence may suggest that the circulating PAGs may modulate circulating immune cell activity. In the cow, systemic PAG concentrations peak around parturition (Green et al. 2005a) but circulating PMN activity is lowest around parturition (Kehrli et al. 1989; Saad et al. 1989; P. J. Hansen 2013). Immune cells treated with boPAGs *in vitro* showed decreased proliferative capacities of erythroid and myeloid lineages. If this also occurs *in vivo* then it might imply a role for the PAGs in the maternal circulation in immune tolerance and acceptance of the fetus (Hoeben et al. 1999). However, reduction of systemic PAGs in sheep through autoimmunization did not result in fetal rejection (Egen et al. 2009).

There is still quite a bit that is unknown about the PAG family, the biggest mystery being their function(s). Some of the immunomodulatory data for the PAGs seems conflicting, which further adds to the growing complexity of this family of placental enzymes. Are the PAGs inhibiting immune cell function or are they recruiting immune cells to the site of attachment through chemokine signaling?

Perhaps the chemokines are not recruiting immune cells at all, rather are they promoting angiogenesis instead. Are the PAGs helping to promote maternal tolerance to the semi-allogenic fetus? It is most likely that the answer is multifactorial. It is also possible that there are functional differences between the individual PAGs and even between species. The majority of research related to PAG function has been conducted *in vitro*, so perhaps more *in vivo* research is needed to understand the role of the PAGs during pregnancy.

Immune system

Overview

The first record of immunological observation is believed to date all the way back to 430 BC after a plague epidemic in Athens, a Greek historian noted that those who survived did not catch the disease a second time (Retief and Cilliers 1998). Many years later, Louis Pasteur postulated that disease is caused by unseen germs (Plotkin 2005). In contrast to popular belief at the time, this was shortly after proven to be true by Robert Koch, who went on to discovered several infections agents such as tuberculosis, cholera, and anthrax (King 1952). Pasteur and Koch are regarded as the fathers of modern immunology because their observations and discoveries set the groundwork for the characterization and study of the immune system (Sattler 2017). Today, we know the immune system as a biological system whose primary roles are to control inflammation and to prevent infection from harmful agents such as bacteria and viruses. It has to ability to distinguish 'self' from 'non-self'. This means that the material and/or microorganisms that make up the body and its many physiological systems (i. e. self) are tolerated, while those that are foreign (non-self) are quickly eliminated. This way, the immune system provides us with rapid, specific, and protective responses towards the many potentially pathogenic microorganism/substances that inhabit the world around us (Parkin and Cohen 2001; Paul 2003; De and Tomar 2014; Sattler 2017).

The immune system is comprised of many specialized cells collectively referred to as leukocytes. Leukocytes are all derived from hematopoietic stem cells in the bone marrow. Hematopoietic stem cells can differentiate into two types of progenitor cells that give rise to the two main classes of immune cells: myeloid and lymphoid. Myeloid progenitor cells further differentiate into many different cell types such as erythrocytes, megakaryocytes, mast cells, granulocytes, and monocytes. Lymphoid progenitor cells further differentiate into T-cells, B-cells, and natural killer (NK) cells (Huston 1997). Further descriptions of these two cell lineages (myeloid and lymphoid) will be discussed in more detail in the following sections of this review.

Immunity can be broken down into 2 main branches: innate and adaptive immunity. Innate immunity is considered to be phylogenetically more ancient than the adaptive immune system and is found in all plants and animals (De and Tomar 2014). Chaplin defined the innate immune system as "all aspects of the host's immune defense mechanisms that are encoded in their mature functional forms by the germ-line genes of the host" (Chaplin 2010). This includes physical barriers such as epithelial and mucosal barriers (such as those in the respiratory and digestive tracts), soluble proteins and other small molecules present in biological fluids (such as complement proteins), cytokines and chemokines, effector cells, and membrane bound receptors that bind pathogenic antigens (Chaplin 2010; Aristizábal and González 2013). The effector cells of the innate immune system are the first responders to sites of damage or infection. They act first because they don't require much, if any, time for terminal differentiation or clonal expansion in response to an immune stimulus, unlike the cells of the adaptive immune system. These include granulocytes, monocytes, dendritic cells, and NK cells (Medzhitov and Janeway 2009). Together all of these components work to control and contain infection and inflammation until the adaptive immune system is able to step in.

The adaptive immune system is made up primarily of lymphocytes, i.e. T-cells and B-cells. NK cells can also be considered an adaptive immune cell, though they possess qualities of both innate and adaptive

immunity. The adaptive immune system is activated by the innate immune system. Cells from the innate immune system present processed antigens to immature T-cells and B-cells within secondary lymphoid organs, primarily lymph nodes. Binding of the antigen to T-cell receptors (TCR) and B-cell receptors (BCR) activates the naïve lymphocyte and initiates a process of activation, differentiation, proliferation, and clonal expansion (Parkin and Cohen 2001; Medzhitov and Janeway 2009; Takada and Jameson 2009; Sun et al. 2020). The result is generation of a large population of lymphocytes that express cell surface receptors or produce antibodies that specifically target the foreign antigen. This process can take three to seven days to generate sufficient numbers of clones for an efficient immune response. Because of this, the adaptive immune response is slower than the innate response. However, the response generated is highly specific (Parkin and Cohen 2001; F. Liu and Whitton 2005; Kurtulus, Tripathi, and Hildeman 2013; De and Tomar 2014).

Lymphoid cell origins

Cells of the lymphoid lineage primarily consist of T-cells and B-cells. These cells are collectively called lymphocytes because of their lymphoid origin. Lymphocytes contribute specificity to the immune system. They possess receptors that are specifically made to target a single antigen, and through the process of clonal expansion, they can create thousands of copies of cells that express a single receptor specific for a single antigen (Adams et al. 2020). The ability to produce a large number of cells with high antigen specificities makes lymphocytes a very powerful too for the immune system in fighting potentially pathogenic substances.

Lymphopoiesis – the generation of lymphocytes – starts in the bone marrow. To make mammalian T-cells, hematopoietic stem cells (HSCs) in the bone marrow first differentiate into lymphoid progenitor cells. A subset of lymphoid progenitors that will be destined to become T-cells migrate to the thymus as thymocytes, where maturation and selection of T-cells takes place (Parkin and Cohen 2001;

Paul 2003; Kumar, Connors, and Farber 2018). Once in the thymus they undergo a series of complex rearrangements and splicing of cell surface receptors. First, the $CD4^-CD8^-$ thymocytes begin expression of these receptors to become $CD4^+CD8^+$ 'double positive' cells. The double positive cells then undergo a selection process that ultimately results in T-cells $CD4^+$ or $CD8^+$ 'single positive' cells that also co-express several other receptors such as CD45, CCR7, CD25, and Foxp3 (Hori, Nomura, and Sakaguchi 2003; Watanabe et al. 2005; Seddiki et al. 2006; Kumar, Connors, and Farber 2018). The single positive cells then leave the thymus to take up residency in tissues or lymph nodes throughout the body. As previously mentioned, in the lymph nodes, antigen presenting cells can display pieces of processed antigens to the resident T-cells. The MHC-II–antigen–TCR complex activates the naïve T-cell and starts the process of maturation and clonal expansion (K. Takada and Jameson 2009; L. Sun et al. 2020). In most, if not all mammals, this process starts *in utero*. By the time humans are born they already have a full complement of T-cells sufficient for anti-pathogenic immunity. In mice, T-cells don't begin to populate the lymph nodes until the very end of gestation (Burt 2013).

The other major lymphoid cells are B-cells. Like T-cells, B-cell development starts in the bone marrow where HSCs differentiate into lymphoid progenitor cells. However, unlike T-cells, B-cells derived from the same common lymphoid progenitor cells do not migrate out of the bone marrow. Instead, cell surface receptor rearrangement, splicing, and cellular maturation takes place in the bone marrow (Hardy and Hayakawa 2001). Early B-cell precursors can be identified by expression of the B220 isoform of CD45 and a lack of expression of CD19, a molecule whose expression characterizes all mature B-cells (Li et al. 1996; Allman, Li, and Hardy 1999; Ogawa et al. 2000). Early B-cells also have little or no immunoglobulin rearrangement. At this stage they are referred to as Pro-B-cells or pre-Pro-B-cells (Hardy et al. 1991; Osmond et al. 1998). As the Pro-B-cells mature the BCR is assembled and the process of selection starts where only cells with BCR that are non-reactive towards self are allowed to live (Goodnow et al. 1988; Hartley et al. 1991; 1993). At this stage the B-cells can be recognized by

expression of cell surface immunoglobulin. When the BCR are fully assembled and autoreactive B-cells have been removed, the now mature B-cell can enter the circulation to take residence in the peripheral tissues and secondary lymphoid organs (Richard R. Hardy and Hayakawa 2001).

Myeloid cell origins

The other cell lineage derived from HSCs and that constitute the largest percentage of the total immune cells in the body is the myeloid lineage. Myeloid immune cells consist of monocytes and granulocytes. Erythrocytes and megakaryocytes are also derived from the myeloid lineage. Monocytes further differentiate into macrophages and dendritic cells. These cells are generally a little larger than the other leukocytes and have a large bean-shaped nucleus. Monocytes and dendritic cells are referred to as professional antigen presenting cells because their main job in the immune system is to process and present antigens to lymphocytes in order to stimulate the adaptive immune response (Rodgers and Rich 2013). Granulocytes are a group of myeloid cells so named for their characteristic cytoplasmic granules. This group consists of neutrophils, basophils, eosinophils, and mast cells. The granulocytes are considered to be 'first responders' to sites of infection. They provide a fast and efficient defense against an infectious agent and keep the infection under control until the adaptive response can take over (Parkin and Cohen 2001; Kawamoto and Minato 2004).

Like lymphocytes, myeloid cells originate from HSCs in the bone marrow. HSCs can produce myeloid progenitor cells which can differentiate into the many members of the myeloid lineage. Hematopoiesis – the formation of the cellular components of the blood – first occurs during embryonic development and continues throughout the life of the animal. The earliest waves of hematopoiesis during embryogenesis appear to be largely regulated by the transcription factors Gata1 and Pu.1 (Cantor and Orkin 2002; Cantor, Katz, and Orkin 2002). Gata1 is known for its major role in regulating erythropoiesis (formation of erythrocytes) but it is also known to be a negative regulator of myeloid cell

fate. *Gata1* knockdown in zebrafish resulted in a hematopoietic switch to myeloid lineage (Rhodes et al. 2005). In contrast, Pu.1 is a positive regulator of myeloid cell fate (Scott et al. 1994). Later waves of hematopoiesis (later in gestation and in the mature animal) are regulated by several other transcription factors such as Runx1 (Q. Wang et al. 1996), Wnt (Luis et al. 2011; 2012), and Notch (Guruharsha et al. 2012). Though it is currently not fully understood what determines the lineage commitment of the HSCs, it is predicted to be a combination of transcription factors and the tissue microenvironment (Kawamoto and Minato 2004). Other factors such as soluble cytokines and chemokines are believed to be the main drivers of terminal differentiation of the myeloid progenitor cells (Metcalf 1998). For example, cytokines such as G-CSF and M-CSF are important in maintaining homeostatic myelopoiesis (the formation of myeloid cells). Cytokines produced by T-cells, such as IL-3 and GM-CSF, are also crucial in maintaining myelopoiesis.

Role of chemokines

While the cellular component of the immune system is critical for the recognition and destruction of potentially harmful pathogens, secreted proteins from the immune cells and the surrounding tissues are just as crucial for the initiation and coordination of the immune response. One such family of secreted proteins is the chemokine family. Chemokines are part of a family of small 8-10 kDa chemotactic cytokines (hence the name 'chemokine') that play important roles in immune cell chemotaxis, activation, and in angiogenesis. There have been about 50 chemokines and 20 chemokine receptors identified in humans and mice (Mackay 2001). Chemokines have been well studied in humans and mice and have been sought after as potential therapeutic targets for various diseases, especially cancer (Gangur et al. 2002; Strieter et al. 2006). Studying chemokine functions can be difficult because of the promiscuous nature of the ligands and their receptors. A single chemokine may bind two or three different chemokine receptors. Likewise, a single chemokine receptor may be able to bind several different chemokines (Le et al. 2004). However, ruminants chemokine families and their functions have

not been as extensively studied. To date, there have been about 40 chemokines identified in cattle and they have been named based on their closest human homolog (Widdison and Coffey 2011). Assigning functions to the bovine chemokines based on amino acid homology is tricky, though. There has been little study of the direct biological roles of the bovine chemokines. Most of the functional research is circumstantial, using experimentally infected animals and measuring changes in immune markers, including chemokines (Santos et al. 2002; Buza et al. 2003; Zhang et al. 2003; Taubert et al. 2006). Phylogenetic and syntenic comparison of the bovine and human chemokine genome would suggest that bovine-specific gene duplication and/or mutations could result in chemokines with high sequence homology but very different functions (Widdison and Coffey 2011). However, there is some direct evidence that suggests some chemokines may indeed be functional homologs of their human counterparts (Rainard et al. 2008; Behrendt et al. 2008). Without further research into the direct functionality of the bovine chemokines, it is unwise to assume they are direct functional homologs of the human genes.

Chemokines fall into three main classes, CC (beta chemokines), CXC (alpha chemokines), and CX_3C (gamma chemokines), based on the arrangement of two conserved N-terminal cysteine residues, where the X represents any amino acid residue other than cysteine. The CXC chemokines can be further grouped based on the presence of the previously mentioned ELR amino acid motif preceding the first N-terminal cysteine. CXC chemokines that contain the ELR motif (ELR+) are known for recruiting neutrophils to sites of inflammation (Kobayashi 2008; Rajarathnam et al. 2019) as well as for promoting angiogenesis (Strieter et al. 2006a; Balestrieri et al. 2008). These include CXCL1, -2, -3, -5, -6, -7, and -8. Interestingly, most species only possess a CXCL5 or CXCL6 gene but not both. Humans and non-human primates appear to be the only species who have both genes. Phylogenetic analysis would suggest that a duplication event occurred post-speciation resulting in two duplicate genes in the human genome. All of the ELR+ CXC chemokines bind to the chemokine receptors CXCR1 and/or CXCR2 (Addison et al.

2000). This appears to be the basis for their neutrophil chemotactic properties because neutrophils express both of these receptors. Binding of an ELR+ chemokine to CXCR1 or CXCR2 on the surface of neutrophils activates G-protein and β-arrestin-mediated second messenger cascades (Richardson et al. 1998; 2003; Barlic et al. 2000; Molteni et al. 2009). Chemokine-activated PLC and Rac1 in neutrophils induces changes in actin dynamics that are crucial for formation of the leading edge of neutrophils during cellular migration (Hirayama et al. 2007; Xu et al. 2015; Sun et al. 2007). In regards to the role of ELR+ chemokines in angiogenesis, the ELR motif seems to be the key. A very elegant study by Strieter et al. demonstrated this by using a corneal micropocket angiogenesis assay. Rat corneas treated with recombinant CXCL5 or CXCL8 exhibited a significant increase in angiogenesis while corneas treated with CXCL9 or CXCL10 (non-ELR containing CXC chemokines) exhibited little to no angiogenic response. When only the ELR motif of CXCL8 was mutated to TVR (the amino acid sequence preceding the N-terminal cysteine of CXCL10), little to no angiogenesis was initiated. Additionally, when CXCL9 was mutated to contain the N-terminal ELR motif, it was able to induce angiogenesis similar to that of CXCL5 and CXCL8 (Strieter et al. 1995). These results demonstrate that the angiogenic activity of the CXC chemokines is likely to be dictated by the presence or absence of the ELR motif.

CXC chemokines that lack the ELR motif (ELR-) are generally not chemotactic towards neutrophils and some of them possess angiostatic activity (Belperio et al. 2000; Balestrieri et al. 2008). CXCL12 is an exception to this rule, however. CXCL12 is an ELR- chemokine however it plays important roles in chemotaxis and promoting angiogenesis (Kijowski et al. 2001; Salcedo and Oppenheim 2003; Bachelder et al. 2002). Three of the ELR- CXC chemokines, CXCL9, -10, and -11, are known to be induced by interferons, namely IFNγ, and are predicted to promote Th1/type 1 cytokine-mediated immune responses (Sharma et al. 2003; Hillinger et al. 2003). CXCL9, -10, and -11 not only promote angiostasis but are potent anti-angiogenic molecules. CXCL9 and CXCL10 can actively inhibit the angiogenic effects of the ELR+ CXCL5 and CXCL8 (Strieter et al. 1995). In corneal micropocket angiogenesis assays,

treatment with CXCL5 or CXCL8 (ELR+) together with CXCL9 or CXCL10 resulted in little to no angiogenic response after 6 days. This is in stark contrast to the angiogenic response seen with ELR+ chemokines alone (Strieter et al. 1995). The angiostatic/anti-angiogenic activity of these chemokines is mediated through the receptor CXCR3 (Lasagni et al. 2003; Kelsen et al. 2004). The ELR- CXC chemokines are also chemotactic towards several leukocytes, primarily mononuclear leukocytes such as T-cells, B-cells, macrophages, and NK cells. Chemotaxis of these cells is mediated through CXCR3, CXCR4, and CXCR5 signaling, depending on the chemokine (Griffith, Sokol, and Luster 2014). CXCL12 is known for its crucial role in regulating hematopoiesis and retention of immune cells in the bone marrow (Moser and Loetscher 2001). CXCL12- or CXCR4 (the receptor for CXCL12)-deficient mice die perinatally due to deficient B lymphopoiesis and myelopoiesis (Ma et al. 1998). Overall, the CXC chemokines serve many roles in the body that are important not only for immune system responses but also for tissue homeostasis. Maintaining a balance between innate and adaptive immune cell recruitment as well as between angiogenesis and angiostasis is important in maintaining homeostasis throughout the body.

The CC chemokine family is quite large in humans, consisting of about 27 individual chemokines. They can be group into 2 main gene clusters: the monocyte chemoattractant protein (MCP) cluster and the macrophage inflammatory protein (MIP) cluster (Widdison and Coffey 2011). The other members of the CC chemokine family that do not fall into these two clusters don't cluster with any other CC chemokine upon phylogenetic analysis. These chemokines likely represent an evolutionarily older group of chemokines because they are generally pretty conserved across species (Widdison and Coffey 2011). The MCP cluster consists of chemokines CCL2 (MCP-1), CCL7 (MCP-3), CCL8 (MCP-2), and -13 (MCP-4). CCL11 (eotaxin), despite not being a MCP, also falls into this cluster because it shares about 70% amino acid identity with the other MCP members (Luster and Rothenberg 1997). The characteristic feature of this group of CC chemokines is their ability to recruit monocytes and T-cells. All MCP-clustered CC chemokines (except CCL11) bind the receptor CCR2. When monocytes leave the bone marrow they can

further differentiate into anti- or pro-inflammatory monocytes that express CX_3CR1 and CCR2, respectively (Geissmann et al. 2003). CCL2, -7, -8, and -13 likely aid in the inflammatory process by recruiting $CCR2^+$ pro-inflammatory monocytes. CCL2 and CCL8 have been studied in the ovary and have been implicated in the process of luteinization and/or CL regression by recruiting pro-inflammatory monocytes to clear the cellular debris after follicular rupture or during luteolysis, respectively (Tsai et al. 1997; Haworth et al. 1998; Duffy et al. 2019). CCL11 is the one member of the MCP cluster that does not recruit monocytes or lymphocytes. CCL11, also known as eotaxin, is responsible for promoting residence of eosinophils and basophils in peripheral tissues and promoting their release from the bone marrow (Palframan et al. 1998; Mishra et al. 1999) The other cluster of CC chemokines is called the macrophage inflammatory protein (MIP) cluster. This cluster consists of CCL3, -4, -5, -6 (mouse only), -14, -15, -16, -18, and -23. This group of chemokines are primarily responsible for recruiting macrophages, NK cells, and T-cells to sites of inflammation. These chemokines serve many roles not just in inflammation but also in tissue homeostasis. For example, CCL3 and CCL5 are constitutively expressed in lymphoid tissues, however, CCL3, CCL4, and CCL5 are all implicated in fighting HIV infections (Cocchi et al. 1995; Baggiolini 1998).

It is clear that chemokines play important roles in various processes throughout the body. Because of the large number of them and their receptor promiscuity, there is still quite a bit that is unknown of chemokines. Differences between species only adds an additional layer of complexity to this already complex system of immunomodulators.

Inflammation, ovulation, and pregnancy

Role of the immune system during ovulation and luteinization

As previously described, the immune system is pivotal in protecting the body from the many threats posed by our environment. However, the immune system does far more than simply fight

infections. It is crucial for many homeostatic processes throughout the body. Leukocytes are constantly surveying the tissue environment and help to aid in removal of cellular debris during tissue remodeling and after apoptosis. Two processes that requires rapid modeling and cellular turnover are the processes of ovulation and luteinization. Ovulation can be defined as the rupture of the ovarian follicle for the release of a mature oocyte into the oviduct. During ovulation, the pre-ovulatory follicle wall undergoes proteolytic degradation that results in rupture and release of the oocyte. Immediately following follicular rupture, the follicle is infiltrated with new vasculature and the remaining follicular cells are reorganized to begin the process of luteinization (Espey 1967; Smith et al. 2002; Duffy et al. 2019).

In the pre-ovulatory ovarian follicle, the ovarian vasculature normally delivers circulating leukocytes, such as macrophages and neutrophils, to the ovarian tissue, some of which take up residency in the ovary. During follicular rupture, the secretions of cytokines and chemokines from the follicular cells and the resident immune cells induces an acute inflammatory response that, coupled with proteolytic activity, likely causes weakening of the follicular wall and rupture (Brännström and Enskog 2002; Duffy et al. 2019). There is a lot of evidence suggesting that immune infiltration of the ovarian follicle is not necessary for ovulation but rather are facilitators of ovulation. *In vitro,* rabbit and rat ovaries perfused with LH were still able to ovulate, but the efficiency was decreased (Brännström et al. 1988; Pall et al. 2000; Viana et al. 2011). While this does not rule out resident immune cells as contributors to ovulation, it may suggest that, without influx of circulating immune cells, full ovulatory efficiency cannot be reached. This is supported by data of immune supplementation of *in vitro* LH-perfused rat ovaries that showed increased ovulation rates (Hellberg et al. 1991). It's likely that the immune cells are performing multiple roles in the ovary during ovulation. Many leukocytes secrete proteases, such as MMPs, so it may be that the immune cells are facilitating matrix remodeling in the ovary (Webster and Crowe 2006). Immune cell protease substrates are not restricted to matrix proteins, however, but also include cytokines, chemokines, cell surface receptors, and adhesions molecules. This

would indicate their involvement not only in the matrix remodeling but in signal activation as well (Duffy et al. 2019). Additionally, monocytes, macrophages, and neutrophils (immune cells found in ovarian follicles) are all known to secrete angiogenic factors such as VEGF. Angiogenesis is a critical part of inflammation and ovulation so it's possible that the resident or invading immune cells are facilitating follicular angiogenesis (Gargett and Rogers 2001; Guimerà et al. 2009; Heissig et al. 2010).

There are several chemokines that are upregulated in ovarian tissues during ovulation. In the bovine ovary, CXCL8 levels are usually low. After the LH surge, CXCL8 production by the granulosa and theca cells of the ovary increases dramatically (Bukulmez and Arici 2000). As previously mentioned in this review, CXCL8 is not only a pro-angiogenic chemokine but it also is a potent neutrophil chemoattractant. Furthermore, neutralization of either CXCL8 or neutrophils with neutralizing antibodies reduces ovulation rate in rabbits (Ujioka et al. 1998). This clearly demonstrates the importance of the interplay between the follicular cells and the immune system. Other chemokines are upregulated in the ovulatory follicle including CCL20, CXCL12, and CCL2 (Kryczek et al. 2005; Dahm-Kähler et al. 2009; Al-Alem et al. 2015). Each of these chemokines recruits a different subset of leukocytes and CXCL12, in particular, is known to for being a pro-angiogenic chemokine. Taken together, these results demonstrate the intimate communication between the ovary and the immune system and the importance of the immune system in ovulation.

Influence of progesterone on immune system

During pregnancy, progesterone predominates as the main ovarian steroid hormone influencing the uterine environment. Progesterone is not only important for pregnancy maintenance but it is also a key regulator of immune function in the endometrium. Experiments placing skin allografts within the uterine lumen showed that treatment with progesterone can delay resorption of the allograft (Hansen et al. 1986). Progesterone can also reduce the number of endometrial lymphocytes in sheep. Analysis

of endometrial leukocyte populations in ovariectomized ewes treated with progesterone showed that treated ewes has significantly fewer CD45R+ lymphocytes at all time points (Gottshall and Hansen 1992). *In vitro* experiments have demonstrated that progesterone can directly inhibit activation and proliferation of ovine peripheral blood lymphocytes (L. D. Staples, Binns, and Heap 1983; Linton D. Staples et al. 1984; Monterroso and Hansen 1993). There is conflicting evidence as to whether progesterone can act as an immunosuppressant in nonuterine sites. Ewes experimentally infected with the parasitic worms *Haemonchus contortus* and treated with 25mg of progesterone daily exhibited an increase in load of adult and larval worms and a decreased proliferative response of peripheral blood lymphocytes to concanavalin A compared to the untreated controls (Fleming and Gamble 1993). One study did not observe any differences in peripheral lymphocyte proliferation between pregnant and nonpregnant ewes (Miyasaka and McCullagh 1982) while another study did record a reduced proliferative response during pregnancy (Rai-el-Balhaa et al. 1987). Whether progesterone has systemic immunosuppressive effects is unclear. However, progesterone does have local immunosuppressive/immunomodulatory effects in the uterus during pregnancy.

Role of immune system for establishment of pregnancy

Immune regulation in the uterus is crucial for the establishment and maintenance of pregnancy. Over 60 years ago, Sir Peter Medawar proposed that the presence of the maternal immune system at the implantation site was the key to understanding why the semi-allogenic fetus is not rejected by the maternal system (Medawar 1948 and 1952). This began the pursuit to understand the roles of the maternal immune system throughout pregnancy. Today, we still don't have a clear answer as to why the fetus and placenta are not rejected and destroyed by the maternal immune system. The common theory for many years was that pregnancy induced an immunosuppressed state in the mother and that was the reason the fetus was not rejected. This theory has gradually been disproven and we now know this to be incorrect. The popular theory today is that pregnancy does not suppress the maternal

immune system, instead it induces a more tolerogenic immune state (Mor et al. 2011). The true answer is most likely multifaceted and more than likely differs between species. In humans it has been proposed that placental trophoblasts may be able to induce differentiation of immune cells into a trophoblast-supporting phenotype (Mor et al. 2011). It has also been proposed that expression of classical and non-classical MHC molecules on the trophoblasts allows them to evade immunological attack (Papúchová et al. 2019; Xu et al. 2020).

In ruminants, little is known about how the maternal immune system responds to the attaching conceptus. It is thought that IFNτ may be responsible for priming the uterine microenvironment and maternal immune system for support of the developing placenta (Ott and Gifford 2010; Nagaoka et al. ; Oliveira et al. 2013; Oliveira and Hansen 2008; Kamat et al. 2016). As previously discussed, progesterone dominance during attachment and it's immunosuppressive actions in the uterus could also be another mechanism for immune tolerance. INFτ can upregulate the expression of chemokines CCL2 and CCL8 in the pregnant endometrium of cattle, both of which are known to be chemotactic for monocytes and macrophages. Additionally, an expansion of monocytes and dendritic cells in the bovine endometrial stroma has been recorded as early as day 13 of pregnancy (Mansouri-Attia et al. 2012). In mice, CCL2 has been suggested to stimulate Th2-type responses (Gu et al. 1997), so perhaps the increase in CCL2 and CCL8 expression during bovine pregnancy is a consequence of monocyte and macrophage invasion and is augmented by IFNτ to promote an anti-inflammatory Th2-type immune response. IFNτ has also been shown to upregulate endometrial expression of CXCL10, which is also chemotactic for macrophages (Nagaoka et al. 2003b). Endometrial CXCL10 has also been implicated in promoting trophoblast adhesion in cattle and sheep (Nagaoka et al. 2003a; Imakawa et al. 2006) This would further support the idea that interferon Tau is modulating the uterine immune environment to promote attachment and pregnancy. IFNτ may also modulate systemic immune cells in cattle. There is a pregnancy-associated increased in expression of interferon stimulated genes such as ISG15, Mx1 and

Mx2 in peripheral blood leukocytes as early as day 16 of pregnancy (Gifford et al. 2007b). Because this is during the window of MRP and INFτ secretion by the trophoblasts in cattle, it is believed that IFNτ may be acting systemically to modulate the maternal immune response. Additionally, the influx of macrophages and dendritic cells in the bovine endometrium is most likely in response to paternal antigens and MHC-I molecules on the pre- and peri-attachment conceptus (Doyle et al. 2009). It's possible that the cytokine and chemokine profiles created in the endometrium by IFNτ or other conceptus-derived products can drive differentiation of the invading macrophages towards the M2 activation pathway, which can decrease the activation of anti-conceptus immune responses (Oliveira et al. 2010).

In addition to macrophage infiltration, an increase in NK cell infiltration into the endometrium occurs during early pregnancy in cattle (Oliveira et al. 2013). In other species, NK cells make up a large population of the endometrial leukocyte population during implantation (Dietl et al. 2006; Hanna et al. 2006). In mice, NK cells have been shown to be crucial players in promoting fetal growth and development. The absence of maternal uterine NK cells during pregnancy in mice resulted in impaired fetal growth. Replacement of uterine NK cells from a normal individual was able to restore this phenotype (Fu et al. 2017). However, the role of uterine NK cells in cattle is still unclear. In mice and humans it is believed that uterine NK cells may be promoting vascular remodeling via the production of growth factors and angiogenic factors (Mor et al. 2011; Munoz-Suano, Hamilton, and Betz 2011). It's possible that NK cells in the bovine endometrium may be playing a similar role. However more research is needed to further explore this theory.

Overall, the immune system during early pregnancy is not suppressed as was once believed. Rather, the immune response appears to be modulated to perhaps promote a more protective environment for the conceptus. Research in the field of reproductive immunology is ongoing.

More insights into the maternal immune status during pregnancy are being gained with each study. The focus of the research of this thesis will focus on the potential roll of the PAGs as immunomodulators in the endometrium of early pregnancy. The focus will be primarily on the ability of the bovine PAGs to alter gene transcription of chemokines in the bovine endometrium.

CHAPTER II

EFFECTS OF BOVINE PREGNANCY-ASSOCIATED GLYCOPROTEINS ON GENE TRANSCRIPTION IN BOVINE ENDOMETRIAL EXPLANTS

ABSTRACT

Pregnancy-associated glycoproteins (PAGs) are a complex gene family, whose members are expressed by trophoblasts of ruminants and related species. In cattle, the PAGs accumulate at the trophoblast-uterine interface and many can enter the maternal circulation. However, very little is known about the role they play in pregnancy although preliminary results suggest that PAGs at the placenta-uterine interface play roles involving matrix turnover and immune modulation. This study was designed to provide further insight into the biological roles of bovine PAGs by measuring changes in endometrial transcript abundance for some matrix metalloproteinases (MMPs) and chemokines/cytokines. PAGs for these experiments were purified from mid-gestation bovine placental extracts by affinity chromatography. Heifers were synchronized and bred by artificial insemination with high fertility semen (n = 14) or dead semen (n = 5). Heifers were slaughtered at day 18 post-insemination and the reproductive tracts were obtained and flushed to determine if a conceptus was present. Endometrial explants were collected and split between 4 groups: pregnant with and without 15 μg/ml PAG (n = 10) and nonpregnant with and without 15 μg/ml PAG (n = 9). Endometrial explants were cultured with or without added PAGs for up to 96 hours at 37°C and 5% CO_2 and samples were harvested at 24 hour intervals for extraction of RNA and fixation. This study focuses on the 48 and 72 hour collection points. Transcript abundance for target genes was analyzed in the endometrial tissue by quantitative PCR. The normalization control transcript was peptidylprolyl isomerase A (PPIA). After 48 and 72 hours, significant increases in *CXCL1*, *CXCL2*, and *CXCL5* as well as *MMP1*, *MMP3* and *MMP13* were measured in the PAG-treated endometrium from both pregnant and non-pregnant animals ($P<0.05$). *CCL11* was upregulated at both time points in the pregnant endometrium but only after 72 hours in the nonpregnant endometrium. There were also significant decreases in message for *CCL2*,

CCL8 and *CCL16* in the PAG-treated groups from both pregnant and non-pregnant animals at each time point ($P<0.05$). Significant decreases in *CXCL10*, *CXCL12*, and *Regakine* message were seen only in PAG-treated endometrium from pregnant animals ($P<0.05$). Structural differences in the luminal and glandular epithelium were seen in the PAG-treated biopsies from both non-pregnant and pregnant heifers. These results suggest that PAGs are capable of inducing structural changes as well as changes in transcript abundance in bovine endometrial explants, which suggests that this model system might be useful to assess PAG function at the placenta-uterine interface.

INTRODUCTION

Establishment of pregnancy is a complex process that depends on a variety of factors taking place in a spatially and temporally synchronized fashion. This includes the establishment of endometrial receptivity, conceptus signaling, hormonal signaling, , maternal immune tolerance and much more. Over the years these factors, which contribute to the establishment of successful pregnancies, have been extensively studied. Although we now know a lot about these processes, there is still much that remains unclear. In cattle, one area that remains a major focus is placental development and function, especially in regard to the release of secretory products and the way in which they interact with maternal tissues. However, the roles that some of these secretory proteins play at the fetal-maternal interface, within the endometrium, or in the maternal system remain unclear. By studying the events of early pregnancy, we can potentially find ways to improve reproductive efficiency in livestock and gain a better mechanistic understanding of the roles of the placenta and its secretory products during pregnancy.

The role of the placenta during pregnancy is multifaceted. It acquires nutrients from maternal tissues, exchanges gasses, eliminates waste and provides protection for the growing fetus. Despite that mammalian placentas all serve the same basic functions, placental structure and extent of trophoblast

invasiveness varies among species (Roberts et al., 2016). Some of these distinctions are reflected by unique trophoblast forms and gene products that are restricted to certain orders or suborders of species or to certain placental types. An example of these aspects can be found within the ruminant placenta, which is distinct in several ways. One unique feature is the presence of a population of giant binucleated trophoblasts cells. These binucleated cells (BNC) are distinct in that they produce vast amounts of proteins that are packed into dense granules for secretion into maternal tissue (Wooding 1982 and 1992). A major component within these secretory granules is a family of proteins known as the pregnancy-associated glycoproteins (PAG; Wooding et al, 2005). The PAGs are products of a gene family of aspartic proteases produced exclusively by the trophoblasts of most even-toed ungulates and cetaceans within the Artiodactyla order (Xie et al, 1997; Hughes et al, 2000 and 2003).

Over the years the PAGs have been extensively studied. Their temporal and spatial Mrna expression patterns have been well characterized and their protein localization patterns at the interface and in the endometrium have been described as well. However, most of the research focus has been on the presence of PAGs within the maternal circulation during pregnancy. Their accumulation in maternal blood soon after the establishment of a functional chorioallantoic placenta has been used as the basis for pregnancy diagnosis (Zoli et al. 1992; Green et al. 2005). The gestational age at which PAGs become detectable and/or their circulating concentrations earlier in pregnancy can serve as useful markers for predicting embryonic loss (Pohler et al. 2013; 2016).

Despite these efforts, there is little understood about the specific functions being performed by the PAGs during pregnancy. However, several published reports have speculated about their potential roles. In ruminant ungulates, the PAGs can be divided into two distinct groups that have been defined as ‘ancient’ and ‘modern’ PAGs based on the times at which they arose during Artiodactyl evolution. The modern PAGs are expressed primarily in BNCs and these PAGs show distinct expression patterns. Most or all of the BNC-specific PAG can enter the maternal circulation. Some of these also accumulate in

the endometrial connective tissue adjacent to the cotyledonary villi. The ancient PAGs, most of which are produced by the mononucleated trophoblasts, tend to accumulate along the interface (Green et al, 2000; Wooding et al, 2005). Roles related to adhesion and protein turnover in the developing placentome have been proposed (Telugu et al, 2010; Wallace et al, 2019; Wooding et al, 2005). Previous research from our laboratory has demonstrated that PAGs can induce changes in transcript abundance of some matrix-metalloproteinases (MMP) in bovine endometrial explants (Wallace et al, 2019). Other research groups have suggested luteotropic roles for the PAGs. Bovine PAGs have been shown to increase secretion of PGE2 from luteal cells and endometrial tissue in vitro (Weems et al, 2003 and 2007); PGE2 has antiluteolytic and luteotrophic activity. Those PAGs that enter into the maternal circulation exhibit a large periparturient rise in circulating levels (Green et al, 2005), which is suggestive that the PAGs may be functioning to some degree outside of the uterine environment.

Among the speculated roles of the circulating PAGs is the ability to modulate the maternal immune system. The localization of some modern PAGs in the endometrial stroma of caruncular villi would place these proteins in a position to directly influence maternal immune responses (Wooding et al, 2005). Bovine PAG-1 was shown to reduce the proliferative activity of myeloid cells and granulocytes in vitro (Hoeben et al, 1999). Later, Klisch et al. suggested that the glycans on the surface of the bovine PAGs (boPAG) might be able to interact with selectins – adhesion molecules that function in lymphocyte migration – and thereby inhibit selectin-mediated cell adhesion of lymphocytes (Klisch et al. 2006). Furthermore, the PAGs are capable of interacting with uterine serpins – proteins capable of inhibiting proliferation of peripheral blood lymphocytes and natural killer cell activity in vitro (Mathialagan and Hansen, 1996; Peltier et al, 2000; Serrano- Pérez et al, 2016). The PAGs have also been implicated in modulation of immune cell chemotaxis by modulating the expression and release of chemokines – small chemotactic cytokines involved in immune cell recruitment and activation. Of particular interest are a group of chemokines in the C-X-C chemokine family known as the neutrophil activating chemokines. In

cattle, this group consists of CXCL1, -2, -3, -5, -7, and -8. Bovine pregnancy-specific protein B (PSPB; which is similar or identical to boPAG-1) was shown to induce the secretion of granulocyte chemotactic protein 2, now known as CXCL5 in cattle, in the endometrium in vitro (Austin et al, 1999). Previous research from our lab has demonstrated that PAGs can increase the Mrna abundance of CXCL5 in endometrial explants in vitro (Wallace et al, 2019).

Clearly, the role of the PAGs remains unclear and more research is needed to better define their function. This study was designed to gain more insight into the biological roles of bovine PAGs in the endometrium during pregnancy. Given the research that suggests the PAGs may play a role in immunomodulation, we hypothesized that the application of bovine PAGs to bovine endometrial explants would produce measurable changes in transcript abundance of immune-related proteins. Based on the putative roles of the PAGs mentioned previously, we investigated changes in chemokines known to be involved in immune cell recruitment and modulation. We also investigated the pathways associated with prostaglandin production because prostaglandins not only play an important role in pregnancy but in immune responses as well.

MATERIALS AND METHODS

Animals and Collection of Tissue

Angus cross bred heifers (n = 19) were synchronized by using a modified 5-Day CO-Synch + CIDR protocol. Heifers received an intravaginal CIDR insert (controlled Internal Drug Release; Eazi-Breed CIDR Insert; Zoetis Animal Health, Kalamazoo, Michigan) containing 1.38g progesterone from day -7 to day -2 with day 0 being the day of insemination. Heifers received 100 µg GnRH (Cystorelin; Merial, Duluth, Georgia) at the time of CIDR insertion. At the time of CIDR removal, heifers received an intramuscular injection of a prostaglandin F_2a analog (Estrumate; Merck, Summit, New Jersey; equivalent to 1mg of

cloprostenol) and were fitted with estrous detection patches (Estrotect; Rockway Inc., Spring Valley, Wisconsin). Heifers were artificially inseminated at a fixed-time (60 hours) after CIDR removal. At the time of breeding, heifers received another injection of 100µg GnRH and were inseminated with high fertility Jersey bull semen. Five heifers were assigned to the control group (dead semen). For the control heifers, semen from the same Jersey bull was left at room temperature for approximately 24 hr to immobilize the sperm. Lack of sperm motility was verified under a microscope before insemination of the control heifers. A second CIDR was inserted in all heifers at day 16 post-insemination to ensure progesterone levels remained elevated in both nonpregnant and pregnant animals at slaughter. On day 18 post-insemination, reproductive tracts were collected from each heifer immediately after slaughter.

Endometrial Explant Culture

Reproductive tracts from all heifers were collected and transported to the laboratory within 30 minutes after collection. At the abattoir, tracts were placed in a Ziploc bag that was then placed in a second Ziploc bag. In the lab, the outer Ziploc bag was removed and the inner bag rinsed with 10% bleach followed by sterile saline. The cervix and oviducts of each tract were clamped upon removal from the bag to prevent any outside materials from getting into the tract. Excess tissue was trimmed for ease of handling. The exterior of the tract was sprayed with 70% ethanol then rinsed with Dulbecco's PBS. Uterine horns were flushed with sterile Dulbecco's PBS and pregnancy was verified by the presence or absence of a conceptus in the flush. The conceptus was immediately snap frozen in liquid nitrogen and stored at -80^{o}C until use. Before opening the tract, the exterior was rinsed once more with 10% bleach followed by Dulbecco's PBS. The tract was placed on a clean tray and immediately transferred to a laminar flow safety cabinet where all subsequent steps were then carried out.

Explants, 5-6mm in diameter (n = 20; 50-60mg each), were collected from the horn ipsilateral to the corpus luteum. Due to the early stage of pregnancy and the heifers being nulliparous, caruncular tissue were nearly indistinguishable from intercaruncular tissue. Therefore, no effort was made to restrict explants exclusively to caruncular or intracaruncular tissue. Groups of explants were placed into culture medium with or without added bovine PAGs. Ultimately, four experimental treatment groups were created: 1) nonpregnant + no PAG (n =9), 2) nonpregnant + 15µg/Ml PAG (n = 9), 3) pregnant + no PAG (n = 10), and 4) pregnant + 15µg/Ml PAG (n = 10). The nonpregnant group contained the heifers bred to dead semen (n = 5) as well as the bred heifers that did not have a conceptus present at the time of collection (day 18; n = 4). Endometrial expression of interferon-stimulated genes (IFI6, ISG15, OAS-1) was analyzed in the four bred animals that were lacking a conceptus on day 18. No differences in the expression of these genes were seen when compared to the control nonpregnant group. This would suggest that they did not conceive or the embryo was lost prior to interferon tau production. These animals were then put into the control group.

Bovine PAGs for this experiment were purified from mid-gestation bovine cotyledonary extracts and evaluated to access quantity and quality (see Appendix I, Appendix II, and Appendix III). Endometrial explants from individual animals were cultured in 100 x 20mm cell culture plates (~720mg tissue/20ml of media) at 37^{o}C under 5% CO_2 with constant movement on a slowly rocking platform. The culture medium was made up of Dulbecco's Modified Eagle Medium with 4.5g/L D-glucose (Invitrogen, Grand Island, New York), 0.1% pure bovine serum albumin, 1% L-glutamine (Gibco, Grand Island, New York, 15 antibiotic-antimycotic (Gibco, Grand Island, New York), 10% heat treated horse serum (Gibco, Grand Island, New York), progesterone (10ng/Ml), and estradiol (5pg/Ml).

Four pieces of tissue were collected prior to incubation (0 hour) and after 24, 48, 72, and 96 hours of culture. The collected samples were stored at -20^{o}C in RNAlater^{TM} (Invitrogen; 3 replicates per heifer per experimental group) or in 10% Neutral Buffered Formalin at 4^{o}C (1 replicate per heifer per

experimental group) for RNA isolation and histology, respectively. At the 48-hour time point half of the spent media was removed and replenished. Aliquots of the media (2Ml) were collected at the 48- and 96-hour time points and stored at -80^{o}C.

RNA Isolation, Reverse Transcription, and Primer Validation

Total RNA was extracted from one endometrial tissue sample per heifer per experimental group using the Rneasy Mini Kit (Qiagen, Valencia, California) with On-Column Dnase Digestion according to the manufacturer's instructions. The following adjustment was made to the protocol: samples were minced by hand with two 10-gauge scalpels or homogenized using a GentleMACS Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany). The concentration and purity of RNA samples were determined using spectrophotometry at 260 and 280nm.

Complementary DNA was synthesized from 0.5µg total RNA by using the PrimeScript 1st Strand Cdna Synthesis Kit (Clontech Laboratories, Inc., Mountain View, California). For quality control and to check for genomic DNA contamination, water blanks and reactions without reverse transcriptase (-RT) were run alongside the normal endometrial RNA samples. On each 96-well plate, water blanks and -RT samples were run in triplicates with each real-time quantitative PCR (Qpcr) reaction.

All primer sets (Table 1) were validated using SYBR^{TM} Green reagents according to the manufacturer's instructions prior to use with the endometrial explant Cdna samples. Briefly, normal pregnant and nonpregnant endometrial Cdna pools were run in triplicates at a consistent concentration (1ng per reaction) while the forward and reverse primers were run in a checkerboard fashion with working concentrations ranging from 100Nm to 900Nm. To assess potential DNA contamination and primer dimerization, water blanks were also run in parallel with each primer set combination. The

optimal primer concentrations were ultimately picked based on the endometrial sample with the lowest Ct value and with the highest Ct value in the corresponding water blank.

Evaluation of transcript abundance

Gene expression analysis was performed by using real-time quantitative PCR with the SYBR™ Green detection assay. Specific primer sequences, the size of the PCR products, and their assay concentrations are shown in Table 1. Peptidylprolyl isomerase A (PPIA) was selected as the bovine reference gene for normalization (Y. Liu et al. 2015; Puech et al. 2015; Walker et al. 2009). All reactions were performed using 1.25ng of Cdna, 6.25Ul of 2x SYBR Green PCR Master Mix (Applied Biosystems), and primers at optimized concentrations. Final reaction volumes were made up to 12.5Ul with Rnase/Dnase-free H_2O. All reactions were carried out in triplicate in a QuantStudio 3 Real-Time PCR System (Applied Biosystems) under the following cycling conditions: 95°C for 10min; 40 cycles of 95°C for 15 seconds and 58°C for 1 minute. A dissociation curve was included in each Qpcr run to ensure specificity of the amplicons.

Histology

Fixed endometrial biopsies from each collection timepoint were dehydrated, embedded in paraffin, sectioned into 5µm thick sections and mounted on charged glass microscope slides. All antibodies and antigen retrieval methods used for immunohistochemical staining were previously optimized to work in bovine endometrial tissue.

A general hematoxylin and eosin staining procedure was performed. Briefly, mounted sections were deparaffinized in 3 changes of xylene for 5 minutes each. Sections were then gradually rehydrated

with decreasing concentrations of ethanol starting at 100% ethanol and ending in distilled water. Slides were immersed in Meyer Modified Hematoxylin for 6 minutes then washed in distilled water for 2 minutes. Slides were quickly rinsed in 95% ethanol then immersed in eosin Y solution (1% eosin Y, 76% ethanol) for 30 seconds or up to 1 minute. Sections were then dehydrated with 2 changes of 95% ethanol for 3 minutes each then 100% ethanol for 3 minutes. Sections were cleared with 2 changes of xylene for 3 minutes each then set out to air dry completely before mounting with Permount. Permount was allowed to set overnight at room temperature. All slides were examined under a light microscope

Statistical Analysis

Data were analyzed for homogeneity of variance by single factor ANOVA. Heterogeneity of variance was detected. Where homogeneity of variance was confirmed, a two sample *t-test* assuming equal variances with a two-tail distribution was used to analyze the log-transformed data. Where heterogeneity of variance was found, a two sample *t-test* assuming unequal variance with a two-tail distribution was used to analyze the log-transformed data. The graphs depict normalized, relative expression with accompanying standard errors. Significant differences were displayed as $P<0.05$, $P<0.01$, and $P<0.001$.

TABLE 1. List of oligonucleotide sequences, their product sizes, and concentrations used for RT-Qpcr

Oligonucleotide sequences 5' to 3'						
Gene	**Accession number**	**Forward Primer**	**Forward Primer Concentration**	**Reverse Primer**	**Reverse Primer Concentration**	**Product Size (bp)**
CCL2	NM_174006.2	CAG CCA GAT GCA ATT AAC TCC CA	300Mm	TGC TGG TGA CTC TTC TGT AGT TCA	300Mm	99
CCL8	NM_174007.1	GTG CTC GCT CAG CCA GAT TC	300Mm	GCT GTC CAG CTT CTT GAA GGG	100Mm	89
CCL11	NM_205773.2	ACA TGA AGG TCT CTG CAG TGC T	900Mm	TGG TTG GAA TAG AAG CTG GCT GA	300Mm	92
CCL16	XM_024980640.1	CCG GCA ATC GTC TTC ATC ACC	300Mm	GCG GAT GAA GTC TGG GAT CCT T	300Mm	99
CXCL1	NM_175700.2	AGA TGC TAA ACA AGG CTA GTG CC	300Mm	CTT TTA CTT CAC TTC CAC TGA GGC T	300Mm	89
CXCL2	NM_174299.3	GAT GCT AAA CTA GGC CAG CTC TAA	300Mm	TTT CTG TAG GGG CAG GGT CT	100Mm	89
CXCL5	NM_174300.2	CCA AAA CGG TCA GTG ATC TGC	300Mm	TCA AAG GAG CTT CTG GGT CC	300Mm	116
CXCL10	NM_001046551.2	AGT ACC TTC AGT TGC AGC ACC A	100Mm	AGA GAG AGG TAC ACC TTG ACT CAG A	300Mm	89
CXCL12	NM_001113174.1	TTC TTT GAG AGC CAT GTC GCC	300Mm	CAG CCT TGC CAC GAT CTG AA	300Mm	89
CXCL14	NM_001034410.2	GAA CGA GAA GCG CAG GGT CT	300Mm	CAA AGT CCT TTG CTT GTT TCC CAA C	100Mm	89
CXCR2	NM_001101285.1	AAG CCC AGA ATC ATG GCT GAA A	100Mm	TGT AAT TGC CAA AAT CTT CAT CGC T	300Mm	90
MMP1	NM_174112.1	ACA GGG ATG AGG TCC GGT T	300Mm	TTC ACC GTT CTC GGA AAG CC	300Mm	117
MMP3	NM_001206637.1	AGT TCC TGT ACG GGT CTC CC	300Mm	CTG CAT CGA AGG ACA AGG CA	300Mm	116
MMP7	NM_001075130.1	GGA GCG AAG CAA TCC CAC TG	300Mm	GGC CCA TCA AAG GGA TAT GGG	300Mm	108
MMP12	NM_001206640.1	ATC CTG GCC CAT GCT TTT GC	300Mm	ACA AGT TTG GGC CTT TGT GTC C	300Mm	99
MMP13	NM_174389.2	AAG ACA GAT TCT TCT GGC GGC	300Mm	AGG CGG CAT CAA TAC GGT TG	300Mm	101
MMP14	NM_174390.2	AGA CAC CAT GAA GGC CAT GAG	900Mm	ATG CTG CCA TTT GAG TCC CTG	900Mm	117
PLA2	NM_174646.3	ACC TGC AGC AGC GAA AAC AA	300Mm	TGT GCT CCT TGT TGT ATG GCA C	900Mm	99
PPIA	NM_178320.2	GCA TAC AGG TCC TGG CAT CT	300Mm	CAC GTG CTT GCC ATC CAA C	300Mm	108
PTGES	NM_174443.2	CAA AAT GTA CGT GGT GGC CGT	900Mm	GCC TCC ATG TCT CTG AGC GT	300Mm	90
PTGS1	NM_001105323.1	GCC CGC GCC AGT GAA C	300Mm	GTG CGG GTG CAG TCA CAT TG	300Mm	98
PTGS2	NM_174445.2	CCC ATG GGT GTG AAA GGG AGG	300Mm	GCC CTG GGG ATC AGG AAT GAA	300Mm	95
Regakine	NM_001034220.2	TCC TCG GTA ACA AGG AAA ATC CCA	300Mm	CTG GTC TGG AAG ATG ACC GCT	300Mm	94

RESULTS

Changes in transcript abundance of endometrial chemokines

Endometrial explants from nonpregnant and pregnant heifers were incubated for 48 and 72 hours in the presence or absence of 15 μg/ml bovine PAGs (boPAG) in the culture medium. The PAGs used in this culture system were the same as those used by Wallace and others (Wallace *et al* 2019). Briefly, the boPAG isolate consisted primarily of boPAG4, boPAG6, and boPAG9, which are all modern PAGs that are produced by the BNCs. To further explore the effect of the PAGs on chemokines in the bovine endometrium, we analyzed the collected explants for various chemokine transcripts using RT-Qpcr. The ability of this PAG preparation to alter transcript abundance was evaluated for 11 chemokine transcripts (Table 1). The abundance of each was determined relative to peptidylprolyl isomerase A (PPIA). The results are presented by status (pregnant or nonpregnant) with the results of the non-treated control samples next to those of the PAG-treated samples.

In the nonpregnant samples, transcript abundance of chemokines *CXCL1* ($P<0.01$), *CXCL2* ($P<0.01$) and *CXCL5* ($P<0.001$) were all significantly increased in response to boPAG after 48 and 72hr (Figure 6A). Conversely, transcripts for *CCL2* ($P<0.05$), *CCL8* ($P<0.001$) and *CCL16* ($P<0.05$) were decreased in response to boPAG at each timepoint. *CXCL14* and *CCL11* were increased in the nonpregnant samples only after 72hr ($P<0.05$) of culture with boPAG (Figure 6A and 6B). Some chemokines were not affected by treatment in the nonpregnant samples; *CXCL10*, *CXCL12* and *Regakine* were not significantly changed by treatment, although there was an overall trend toward a decrease in transcript abundance of these transcripts (Figure 6C).

In the pregnant samples, transcripts for *CXCL1, CXCL2* and *CXCL5* were significantly increased at both the 48 and 72hr timepoints ($P<0.001$; Figure 7A). Much like in the nonpregnant samples, *CCL2* ($P<0.05$) and *CCL8* ($P<0.001$) were decreased in response to boPAG at both timepoints. Unlike the

nonpregnant samples, where *CCL11* was increased only at 72hr, *CCL11* was increased at both timepoints in the pregnant samples ($P<0.05$). *CXCL14* was decreased in response to boPAG in the pregnant samples but it was only significant at the 72hr timepoint (Figure 7A and 7B). An effect of status was seen for *CXCL10* ($P<0.001$), *CXCL12* ($P<0.01$), and *Regakine* ($P<0.01$). All three transcripts were significantly decreased in response to boPAG in the pregnant samples but not the nonpregnant (Figure 7C).

To better illustrate the individual responses generated by treatment of bovine endometrium with boPAG, we also presented the data as a ratio of relative transcript abundance of the boPAG-treated explants vs. the respective non-treated controls. This was done for each transcript analyzed and the data are represented in Figures 9 and 10. These data also allowed us to more clearly see common trends of changes in transcript abundance between the nonpregnant and the pregnant samples. In both the nonpregnant (Figure 8) and pregnant (Figure 9) endometrial samples, *CXCL1*, *CXCL2* and *CXCL5* were all greatly increased by treatment with boPAG after 48 and 72hr while *CCL2*, *CCL8* and *CCL16* were all decreased by treatment with boPAG after 48 and 72hr in culture. *CCL11* was also increased by boPAG in both nonpregnant and pregnant samples but the fold-change was greater in the pregnant samples. While the overall trend of decreasing transcript abundance of *CXCL10*, *CXCL12*, and *Regakine* in response to boPAG was present in both pregnant and nonpregnant groups, the fold-change was much greater in the pregnant samples. The one difference between the pregnant and nonpregnant groups was the change in *CXCL14*. *CXCL14* was increased by boPAG in the nonpregnant samples at 72hr ($P<0.05$) but was decreased in the pregnant samples at both the 48 and 72hr time points ($P<0.05$).

Histology

To evaluate the effects of boPAG on a whole tissue level we fixed some of the collected endometrial explants after 24hr, 48hr, 72hr and 96hr of culture with or without added boPAG for

histological analysis. The histological effects of boPAG were similar between the pregnant and nonpregnant groups so the results stated here will be generalized for both groups. Treatment with boPAG seemed to have an effect on glandular morphology. After 24hr in culture, many of the endometrial glands has areas of very thin or missing epithelium. In the lumen of those glands was darkly stained cellular material. Some glands lacked epithelia entirely. The luminal epithelium also took on an irregular morphology in the boPAG treated tissues. There were areas where the luminal epithelium was very thin or even missing and in some cases the epithelium appeared vacuolated. In some cases the stroma appeared more diffuse than its untreated counterpart. The untreated control tissue after 24hr of culture showed some signs of glandular epithelial sloughing, as seen by darkly stained cells in the lumen, but the overall morphology of the glandular and luminal epithelia were normal (Figures 10A and 10B). By 48h, many of the boPAG-treated endometrial glands had entirely lost their epithelium. The luminal epithelium was still irregular with visible areas of detachment (Figures 10C and 10D). After 72hr in culture, it appeared that the some of the glandular epithelia as well as the luminal epithelia in the boPAG treated tissues were starting to grow back. The untreated controls at this time were starting to exhibit areas of thin or missing epithelium (Figures 10E and 10F). By 96hr in culture, the PAG-treated tissues were beginning to look much more normal. In the boPAG-treated tissues the glandular and luminal epithelia were returning to normal and the amount of cellular debris in the glandular lumen was decreased, though the luminal epithelium was still irregular. The untreated controls were now showing signs of glandular cell death and an irregular luminal epithelium (Figures 10G and 10H).

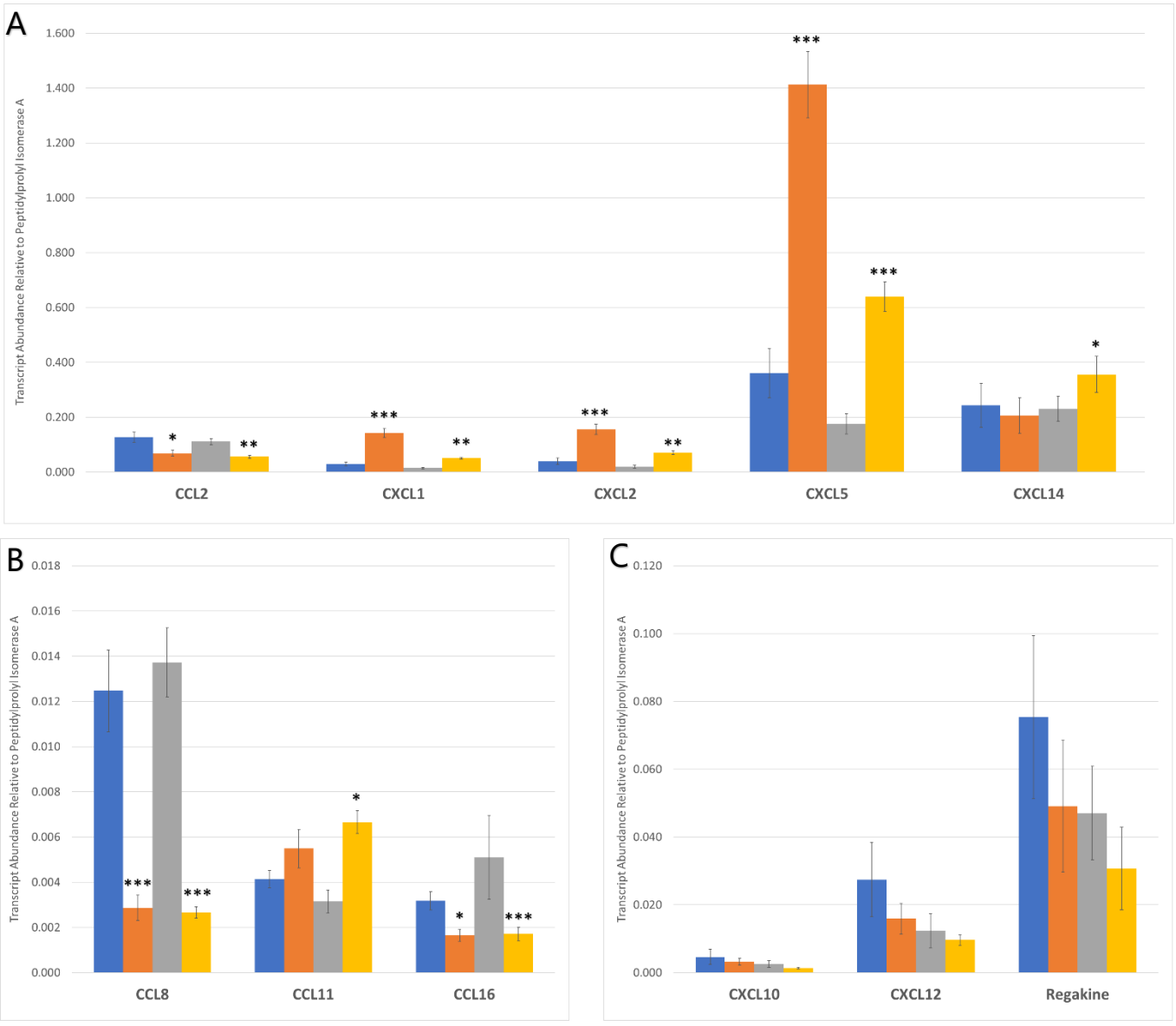


Figure 6. Relative transcript abundance of chemokines in nonpregnant endometrium after exposure to PAGs for 48 and 72hr

Target transcript abundance of several chemokines in endometrial explants from nonpregnant heifers exposed to PAGs for 48 (blue and orange bars) and 72 hours (grey and yellow bars). Data was quantified by RT-PCR and the results were normalized to PPIA. Data are presented as mean ±SEM. Blue bar: 48hr nontreated endometrium; Orange bar: 48hr PAG-treated endometrium; Grey bar: 72hr nontreated endometrium; Yellow bar: 72hr PAG-treated endometrium. *P<0.05; **P<0.01; ***P<0.001.

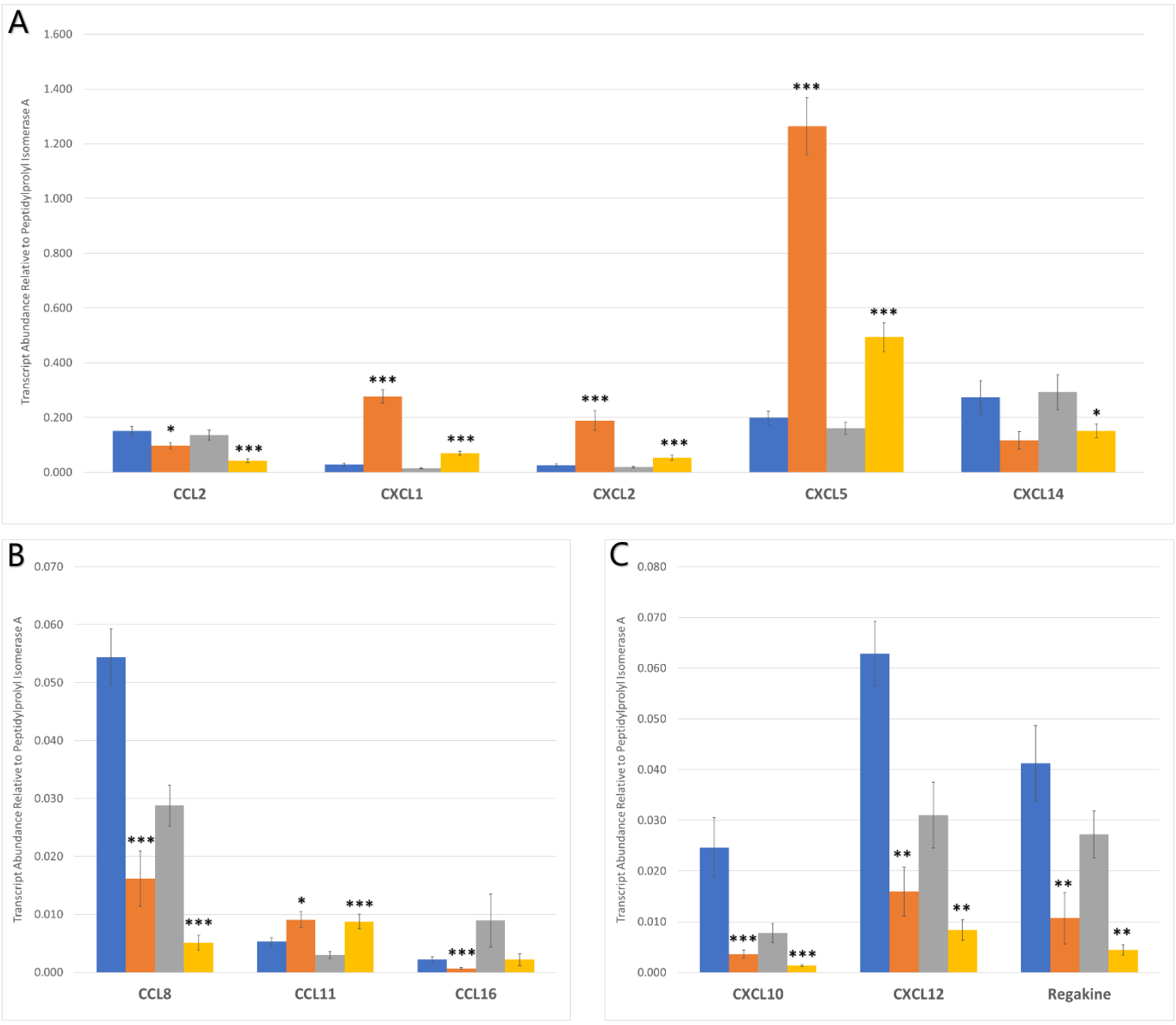


Figure 7. Relative transcript abundance of chemokines in pregnant endometrium after exposure to PAGs for 48 and 72hr

Target transcript abundance of several chemokines in endometrial explants from pregnant heifers exposed to PAGs for 48 (blue and orange bars) and 72 hours (grey and yellow bars). Data was quantified by RT-PCR and the results were normalized to PPIA. Data are presented as mean ±SEM. Blue bar: 48hr nontreated endometrium; Orange bar: 48hr PAG-treated endometrium; Grey bar: 72hr nontreated endometrium; Yellow bar: 72hr PAG-treated endometrium. *P<0.05; **P<0.01; ***P<0.001.

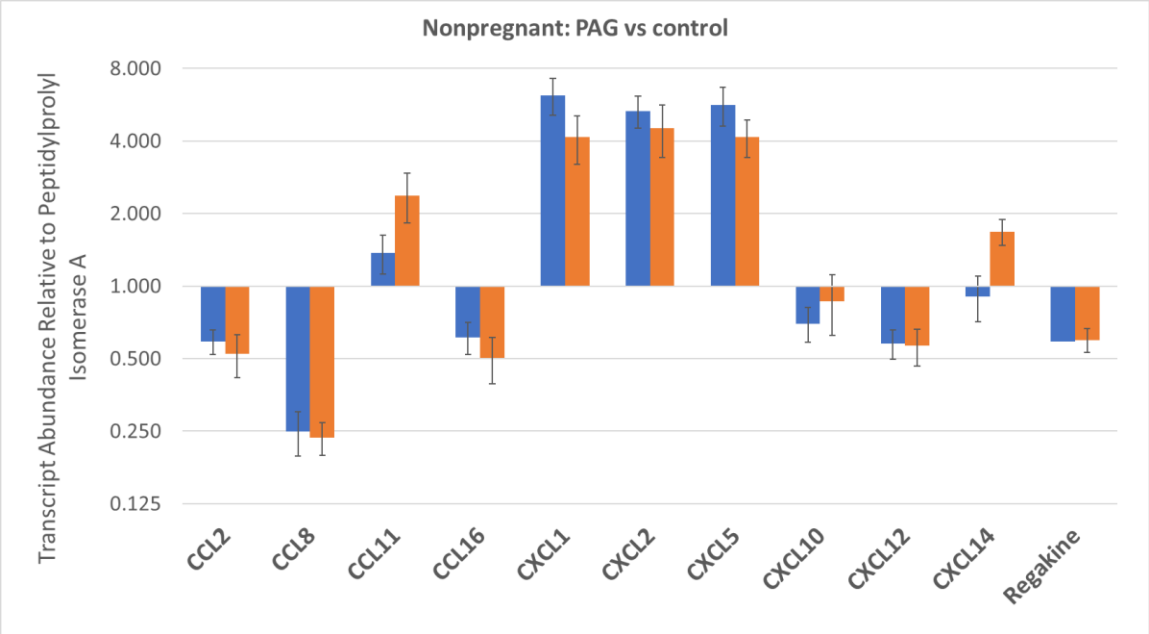


Figure 8. PAG-treated vs Nontreated transcript abundance of chemokines in nonpregnant endometrium after exposure to PAGs for 48 and 72hr

Target transcript abundance of several chemokines in endometrial explants from nonpregnant heifers exposed to PAGs for 48 (blue bars) and 72 hours (orange bars). Data are represented as a ratio of PAG-treated:Nontreated relative abundance Data was quantified by RT-PCR and the results were normalized to PPIA. Data are presented as mean ±SEM. Blue bar: 48hr PAG-treated vs nontreated control endometrium; Orange bar: 72hr PAG-treated vs nontreated control endometrium

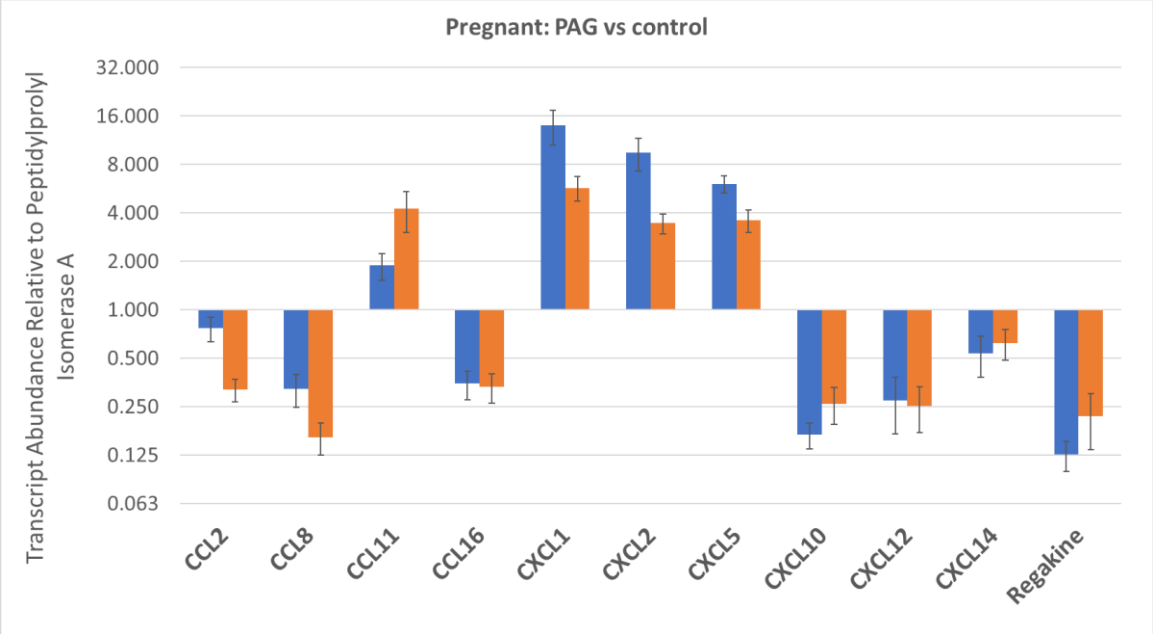


Figure 9. PAG-treated vs Nontreated transcript abundance of chemokines in pregnant endometrium after exposure to PAGs for 48 and 72hr

Target transcript abundance of several chemokines in endometrial explants from pregnant heifers exposed to PAGs for 48 (blue bars) and 72 hours (orange bars). Data are represented as a ratio of PAG-treated:Nontreated relative abundance Data was quantified by RT-PCR and the results were normalized to PPIA. Data are presented as mean ±SEM. Blue bar: 48hr PAG-treated vs nontreated control endometrium; Orange bar: 72hr PAG-treated vs nontreated control endometrium

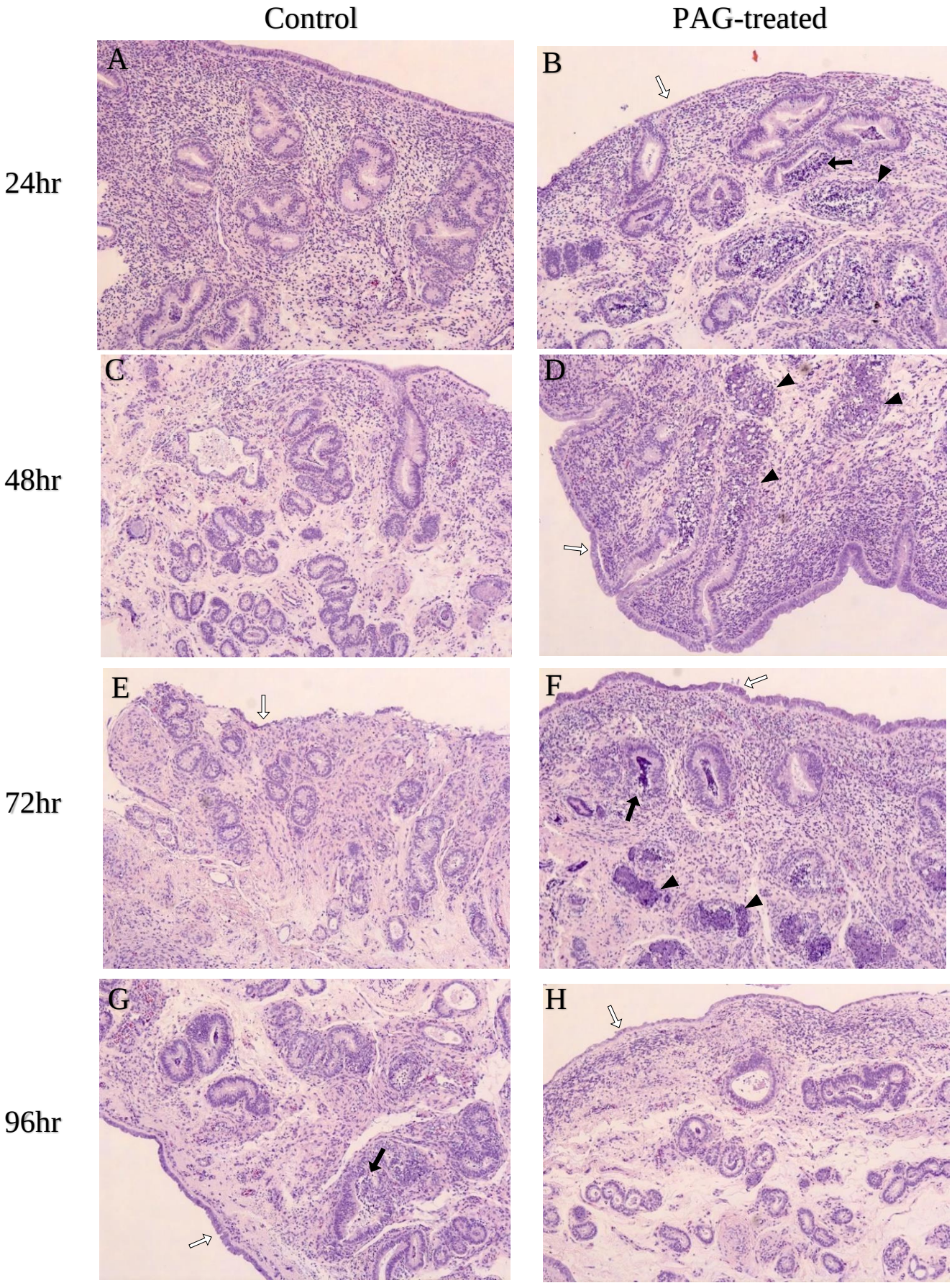

Control
PAG-treated
24hr
48hr
72hr
96hr
A
B
C
D
E
F
G
H

Figure 10. H&E staining of bovine endometrial explants after exposure to PAGs for 24, 48, 72, and 96 hours

Representative H&E staining of bovine endometrial explants cultured without (A, C, E, G) or with (B, D, F, H) bovine PAGs. Results were similar between nonpregnant and pregnant samples so a single representative is shown here. Samples in culture for 24hr (A and B); Samples cultured for 48hr (C and D); Samples cultured for 72hr (E and F); Samples cultured for 96h (G and H); Areas of irregular luminal epithelium were denoted with a white arrow (⇨); Areas of irregular glandular epithelium were denoted with a black arrow (➞); Areas of missing glandular epithelium were denoted with arrowheads (►).

DISCUSSION

The ruminant PAGs have been a subject of study since the early 1980's. Much of the published literature on the PAGs to date has focused on characterizing these proteins as tools for pregnancy diagnosis and pregnancy maintenance in ruminants (Green *et al*, 2005; Pohler *et al,* 2013 and 2016; Wallace *et al,* 2015; Xie *et al,* 1997b; Zoli *et al,* 1992). Additional research has been conducted to characterize the spatial and temporal expression patterns of ruminant PAGs (Green *et al,* 2000; Patel *et al*, 2004; Touzard *et al,* 2013; Wooding *et al,* 2005; Xie *et al,* 1997b). Some possible functions for the PAGs have been proposed, but even after years of study there is still little understood about how they are functioning during pregnancy. A suggested role for the PAGs is that they could be serving an immunomodulatory role during pregnancy (Austin *et al,* 1999; Dosogne *et al*, 1999; Hoeben *et al,* 1999 and 2000; Klisch *et al*, 2006; Mathialagan and Hansen, 1996; Serrano-Pérez *et al,* 2016; Wallace *et al*, 2015). Though mostly speculative, there is some evidence to support this theory. Recently, our lab demonstrated that bovine PAGs, when used to treat bovine endometrial explants for 24 and 96 hours, can alter expression of the endometrial chemokine CXCL5 (Wallace *et al,* 2019). Other research by Austin *et al*. (1999) showed similar results when endometrial explants and endometrial cells were treated with PSPB (a protein now known to be identical or similar to boPAG1). Whether this is a direct or indirect action of the PAGs remains unclear. However, what is clear is that the PAGs are, at least in part, likely performing their functional roles at the placenta-uterine interface. This led us to evaluate

this possibility further. The PAGs investigated in these experiments (a mixture consisting predominantly of boPAG4, -6 and -9) were ones produced by giant binucleated trophoblasts. Day 18 represented a stage in which the endometrium was likely capable of responding to these PAGs. Additionally, the tissue would not have been exposed to the particular PAGs used in these experiments prior to the in vitro culture because they are not expressed by the trophoblasts until after day 18 (R. M. Wallace et al. 2019).

Immune cells are present in the bovine endometrium throughout the estrous cycle and throughout pregnancy (Leung et al. 2000; Tekin and Hansen 2004; Velázquez et al. 2019). During pregnancy, immune regulation in the ruminant endometrium is important for fetal survival. Changes in the uterine immune cell population as well as immune cell function during pregnancy is driven by local signals from the conceptus and from hormonal changes that are mediated by either the placenta or the maternal system. Interferon tau (IFNT), a type 1 interferon secreted by the placental trophoblasts between days 15 and 21 of bovine pregnancy (Fuller W. Bazer et al. 2008; Ealy and Yang 2009; Ealy and Wooldridge 2017), is one such signal from the conceptus that is thought to be responsible for altering uterine immune cell function to create an environment conducive for embryonic growth and development (Gifford et al. 2007a; Glaucia Teixeira et al. 1997; Rashid et al. 2018; Talukder et al. 2017). Not only can IFNT stimulate classical interferon-stimulated genes in both the endometrium (M.-S. Kim, Min, and Imakawa 2013) and circulating immune cells (Gifford et al. 2007a), but it can also stimulate the release of interferon-inducible chemokines, such as CXCL10, in the endometrium (Imakawa et al. 2006). Progesterone is a key regulator of pregnancy and also uterine immune function (Lonergan and Forde 2015; Lonergan, Forde, and Spencer 2016; P. J Hansen 1998). Progesterone can inhibit proliferation of activated peripheral blood lymphocytes in vitro and reduce the number of uterine CD45R+ lymphocytes in vivo in sheep (Staples et al. 1983; Staples et al. 1984; Monterroso and Hansen 1993; Segerson et al. 1997). In sheep, progesterone has been shown to induce the release of a serine protease inhibitor known as uterine serpin, which has been shown to inhibit lymphocyte proliferation in vitro in sheep

(Peltier, Liu, and Hansen 2000) and natural-killer cell-mediated abortion in mice (W.-J. Liu and Hansen 1993). Both IFNτ and progesterone are thought to have either direct or indirect effects on uterine immune functions during early pregnancy, which is crucial for proper implantation and embryo development.

Chemokines are a family of small 8-10kDa cytokines noted for their roles in immune cell chemotaxis into sites of inflammation. They can be separated into four main groups based on the number and spacing of conserved N-terminal cysteine residues; C, CC, CXC, and CX_3C. The CXC chemokine family can be further subdivided into 2 groups based on the presence (ELR+) or absence (ELR-) of an 'ELR motif' (amino acid sequence Glu-Leu-Arg) immediately preceding the first N-terminal cysteine (Balestrieri et al. 2008). The ELR+ CXC chemokines are known as the neutrophil activating chemokines (NACs) because of their abilities to specifically recruit neutrophils into sites of inflammation (Rajarathnam et al. 2019). In cattle, the ELR+ CXC chemokines consist of CXCL1, CXCL2, CXCL3, CXCL5, CXCL7 and CXCL8/IL-8. The findings of the present study demonstrate that bovine PAGs are capable of upregulating endometrial expression of several NACs: *CXCL1, CXCL2*, and *CXCL5*. This may suggest a role for the PAGs in immune cell recruitment. Currently, endometrial neutrophil infiltration in early pregnancy is not well characterized in cattle. However, several studies have documented the deleterious effects of endometrial neutrophils in endometritis on reproductive performance (Lincke et al. 2007; LeBlanc et al. 2002; Gilbert et al. 2005; Kim and Kang 2003). Because the PAGs are such an abundant product of the placenta throughout pregnancy, it is unlikely that the PAGs would promote neutrophil recruitment for the purpose of potentially damaging inflammation. There is evidence of different subgroups of neutrophils that possess different functional qualities based on cytokine production, ability to activate macrophages, cell surface receptor expression, and cell surface antigen expression (Tsuda et al. 2004; Fridlender et al. 2009; Pillay et al. 2010). Perhaps these chemokines could be recruiting/activating neutrophils of a less inflammatory phenotype, or perhaps these chemokines are

not recruiting neutrophils at all and, rather, are performing some local function in the endometrium. This is speculation, of course. More research will need to be conducted to further explore the potential roles of these chemokines in the bovine endometrium.

Along with *CXCL1, CXCL2,* and *CXCL5* being upregulated, PAGs also induced an increase in *CCL11* expression. CCL11 is known to recruit eosinophils to sites of inflammation (Gouon-Evans and Pollard 2001). Together, all four of these chemokines recruit granulocytes. Little is known about the role of CCL11 in the reproductive tract. However, it's receptor CCR3 is present on the endometrial luminal epithelium, glandular epithelium, and on the trophectoderm (Sakumoto et al. 2017), so it is possible that CCL11 is functioning locally in the endometrium or at the interface. CCL11 has be shown to regulate extravillous trophoblast migration, invasion, and adhesion in human pregnancy (Chau et al. 2013). Perhaps CCL11 could be acting locally at the utero-placental interface to regulate conceptus attachment via its receptor, CCR3, on both epithelial surfaces.

CCL2, CCL8, CCL16, CXCL10, CXCL12, and CXCL14 are all chemotactic for monocytes and/or macrophages. In particular, CCL2, CCL8, and CXCL10 have been shown to be present in the ovine endometrium during pregnancy and upregulated by IFNτ (Asselin et al. 2001; Andoh et al. 2020; Nagaoka, Sakai, et al. 2003). It is interesting here that we would see the opposite response after treatment with PAGs in bovine tissue. However, expression levels of *CXCL10* were increased in the non-treated pregnant endometrium compared to the non-treated nonpregnant endometrium. This is in agreement with previous data that CXCL10 is upregulated in early pregnancy due to IFNτ stimulation (Nagaoka, Sakai, et al. 2003). Additionally, early bovine pregnancy is characterized by an expansion of endometrial macrophages and dendritic cells (Mansouri-Attia et al. 2012; Oliveira et al. 2010). Upregulation of these chemokines would fit that narrative as they all have macrophage chemoattractant properties. Perhaps the negative regulatory effect of the PAGs is normally canceled out *in vivo* due to the positive effects of IFNτ. However, individual functions of the PAGs cannot be ruled out. The

particular PAGs that were used in these experiments are not normally expressed this early in pregnancy. It is possible that the PAGs expressed during conceptus attachment have different individual functions than the later expressed PAGs. Furthermore, *CXCL10* and *CXCL12* expression was only significantly affected in the pregnant endometrium. Since progesterone levels in the nonpregnant and pregnant groups were made to be similar (R. M. Wallace et al. 2019), this might suggest that regulation of these chemokines by PAGs is dependent on other pregnancy-specific factors, such as IFNτ.

Unfortunately, little is known about the roles of CCL16 and CXCL14 in the endometrium. CCL16 has been shown to increase antigen presentation by macrophages and enhance T-cell cytotoxicity during inflammatory reactions (Cappello et al. 2004). All of which would be detrimental to pregnancy establishment. Downregulation of this chemokine would imply an immunoprotection function of the PAGs. CXCL14 is known to play critical roles in upregulation of MCH-I molecules on tumor cells as well as establishing immune surveillance in normal epithelial layers (Westrich et al. 2020). This would further support the idea that PAGs could be serving an immunoprotective role by indirectly decreasing the availability of harmful pro-inflammatory cells in early pregnancy.

As previously mentioned, the NACs are known to recruit neutrophils but in humans and mice they also characteristically promote angiogenesis by signaling through the receptor CXCR2 on neutrophils and endothelial cells (Addison et al. 2000; Strieter et al. 2006b). The ELR motif of these chemokines appears to be necessary for angiogenesis. Corneal micropocket assays using recombinant human CXCL1, CXCL5 and CXCL8 induced angiogenesis in rat corneas, without evidence of significant leukocyte infiltration. When the ELR motif is mutated to TVR (corresponding to the sequence of CXCL10, an anti-angiogenic ELR- chemokine) the angiogenic activity of CXCL8 is inhibited (Strieter et al. 1995). Results of the current paper showed that *CXCL1, CXCL2,* and *CXCL5* were all upregulated in the endometrium after exposure to bovine PAGs. This may suggest a role for the PAGs in the initiation of endometrial angiogenesis during early pregnancy. This is an interesting concept because the PAGs have

never been implicated in the promotion of angiogenesis before now. Furthermore, the PAGs were also able to downregulate expression of *CXCL10*, a known anti-angiogenic chemokine (Strieter et al. 1995). CCL2 has also been implicating in inhibition of angiogenesis in mice (Bonapace et al. 2014). This would further support the idea that PAGs may be promoting an angiogenic environment in the endometrium through chemokine signaling. Contrastingly, CXCL12 is a known promoter of angiogenesis (Salcedo and Oppenheim 2003; Quinn et al. 2014) and its expression was downregulated by PAGs in the pregnant endometrium. This doesn't quite fit the model of endometrial angiogenesis, however, CXCL12 is also important in the mobilization of monocytes and T-cells from the bone marrow and lymph nodes, respectively (Kryczek et al. 2005; Ding and Morrison 2013). Therefore, this would fit the model of immunoprotection of the attaching conceptus.

Regakine is a recently discovered CC chemokine that is present in high concentrations in bovine plasma and has been shown to synergize with CXCL8 to recruit neutrophils (Struyf et al. 2001; Gouwy et al. 2002). It is a very unique chemokine that shows less than 50% sequence identity to any known human or animal chemokine (Struyf et al. 2001) so it appears to be ruminant- (if not bovine-) specific (Widdison and Coffey 2011). It's function within the body is still not well characterized so it is difficult to imply its function in the endometrium. It currently has no known receptor so it's exact target cells or tissues are unclear as well. It could be possible that it has no receptor and its main function is to synergize with other chemokines, such as CXCL8, to enhance their chemotactic properties (Gouwy et al. 2004). If this is the case, then the downregulation of *Regakine* together with the upregulation of *CXCL5* (a chemokine also known to synergize with Regakine; Struyf et al. 2001; Gouwy et al. 2002) might imply that CXCL5 and its family members may not be recruiting neutrophils but performing some other local function, such as promotion of angiogenesis.

In summary, these data provide evidence that the binucleate cell-specific bovine PAGs are participating in local immunomodulation of the maternal immune system in the endometrium of cattle.

These results are supported by previously published data indicating that PAGs can induce expression of certain chemokines (e.g. *CXCL5*/*CXCL6*). However, some of these data conflict with our current knowledge of the immune populations in the endometrium of pregnant animals. Whether this is the result of the *in vitro* culture system or a true result of PAGs function remains to be determined. The prospect of the PAGs serving as immunomodulatory proteins and/or as pro-angiogenic factors is intriguing. Since many binucleate cell derived PAGs are delivered to the maternal endometrial stroma throughout pregnancy, this makes them good candidates for the functions proposed here. Further studies into the role(s) of the PAGs will surely provide more insight into the functions proposed in this paper.

APPENDIX I

The use of a monoclonal antibody to rapidly purify bovine pregnancy-associated glycoproteins.

Summary

Protein purification permits the isolation of target proteins in order to study their function, structure, and interactions with other proteins; however, purification of proteins, like PAGs, tends to be a laborious process. In the past, recombinant PAGs have been developed and numerous purification schemes have been utilized (S. Xie et al. 1997; Green et al. 2005b; Bériot et al. 2014); however, they consist of several steps and, in both cases, recovery yields are low relative to the amount of starting material. Therefore, a more efficient purification method was developed utilizing 87mmune-affinity chromatography with an anti-PAG monoclonal antibody.

Materials

1. Homogenization of Cotyledonary Extract

1. Cotyledons from mid-gestational bovine placenta
2. Homogenizer
3. 50,000 molecular weight dialysis tubing
4. Cotyledon Homogenization Buffer: 68.5 M*m* NaCl, 1.35 M*m* KCl, 5 M*m* $Na_2HPO_4*7H_2O$, Ph 7.4
5. Dialysis buffer A: 20 M*m* Tris, Ph 8.0
6. Buffer A: 20 M*m* Tris, 1.0 *M* NaCl, 1.0 M*m* EDTA, 0.2 M*m* PMSF, 0.02% NaN_3, *0*.1 M*m* 2-mercaptoethanol, Ph 8.0
7. Buffer B: 20 M*m* Tris, 0.8 *M* NaCl, 1.0 M*m* EDTA, 0.2 M*m* PMSF, 0.02% NaN_3, *0*.1 M*m* 2-mercaptoethanol, Ph 7.5
8. Buffer C: 20 M*m* Tris, 0.6 *M* NaCl, 1.0 M*m* EDTA, 0.2 M*m* PMSF, 0.02% NaN_3, *0*.1 M*m* 2-mercaptoethanol, Ph 7.5
9. Buffer D: 20 M*m* Tris, 0.4 *M* NaCl, 1.0 M*m* EDTA, 0.2 M*m* PMSF, 0.02% NaN_3, *0*.1 M*m* 2-mercaptoethanol, Ph 7.5
10. Buffer E: 20 M*m* Tris, 150 M*m* NaCl, 1.0 M*m* EDTA, 0.2 M*m* PMSF, 0.02% NaN_3, *0*.1 M*m* 2-mercaptoethanol, Ph 7.0
11. Screw top plastic centrifuge tubes

2. Ammonium Sulfate Precipitation of Cotyledonary Extract

1. Ammonium Sulfate
2. Screw top plastic centrifuge tubes
3. Large beakers
4. Re-suspension buffer: 137 M*m* NaCl, 2.7 M*m* KCl, 10 M*m* $Na_2HPO_4*7H_2O$, Ph 7.4
5. 50,000 molecular weight dialysis tubing
6. Dialysis buffer B: 137 M*m* NaCl, 2.7 M*m* KCl, 10 M*m* $Na_2HPO_4*7H_2O$, Ph 7.4
7. Dialysis buffer C: 0.1 *M* Tris, 0.15 *M* NaCL, Ph 7.6

3. Preparation of Affinity Chromatography Column

1. EZ-Link NHS-LC-LC biotin (Pierce 21343)
2. Agarose Avidin D (Vector Laboratories A-2010)
3. Anti-PAG monoclonal (L4)
4. Dialysis buffer B (see section 2.2)
5. Equilibration buffer: 137 M*m* NaCl, 2.7 M*m* KCl, 10 M*m* Na_2HPO_4*$7H_2O$, Ph 7.4

4. Affinity Chromatography Purification of PAGs

1. Wash buffer A: 0.1 *M* Tris, 0.15 *M* NaCl, Ph 7.6
2. Wash buffer B: 20 M*m* Tris, 1.0 *M* NaCl, 0.1% Tween-20, Ph 8.0
3. Elution buffer A: 0.1 *M* glycine, Ph 2.9
4. Elution buffer B: 0.5 *M* glycine, Ph 2.9
5. Neutralization buffer: 1.0 *M* Tris, Ph 9.5
6. Fraction collector and tubes

5. PAG Concentration

1. Polyethylene glycol
2. 1,000 molecular weight dialysis tubing
3. Media buffer: 1.8 M*m* $CaCl_2$, 0.000247 M*m* $Fe(NO_3)_3 \cdot 9H_2O$, 0.8 M*m* $MgSO_4$, 5.3 M*m* KCl, 44.5 M*m* $NaHCO_3$, 110.3 M*m* NaCl, 0.9 M*m* NaH_2PO_4-H_2O, Ph 7.6.

<u>Methods</u>

1. Homogenization of Cotyledonary Extract

1. Homogenize frozen cotyledons in cotyledon homogenization buffer.
2. Place cotyledon extract into 50,000 molecular weight dialysis tubing and dialyze in dialysis buffer A at 4°C overnight.
3. Dialyze cotyledon extract in Buffer A at 4°C for at least 12 hours. Repeat this dialysis using Buffers B-E in that order.
4. Transfer cotyledon extract to centrifuge bottle and centrifuge the solution at 8,000*g* for 10 minutes to remove insoluble debris, keep supernatant and discard pellet.
5. Save aliquot to quantify amount of PAG.

2. Ammonium Sulfate Precipitation of Cotyledonary Extract

1. Pour supernatant into beaker containing a stir bar and place on magnetic stirrer.
2. While sample is stirring, slowly add saturated ammonium sulfate to bring concentration to 40% saturation.
3. Once total volume of ammonium sulfate is added and thoroughly mixed, move beaker to 4°C overnight.
4. Transfer to centrifuge bottle and centrifuge the precipitate at 8,000*g* for 10 minutes.
5. Pour supernatant into a beaker containing a stir bar and then re-suspend pellet in re-suspension buffer and store at 4°C for later use.
6. Place beaker on magnetic stirrer. While sample is stirring, slowly add saturated ammonium sulfate to bring concentration to 80% saturation.
7. Once total volume of ammonium sulfate is added and thoroughly mixed, move beaker to 4°C overnight.
8. Transfer to centrifuge bottle and centrifuge the precipitate at 8,000*g* for 10 minutes.
9. Pour supernatant into fresh bottle being sure not to disturb the pellet and store at 4°C for later use.

10. Re-suspend the pellet in re-suspension buffer, this will be the 80% ammonium sulfate cotyledon extract.
11. Place cotyledon extract into 50,000 molecular weight dialysis tubing and dialyze in dialysis buffer B at 4°C for at least 12 hours. Repeat dialysis in dialysis buffer B at least 5-7 times to remove salts.
12. Transfer dialysis tubing to dialysis buffer C and dialyze at 4°C for at least 12 hours. Repeat dialysis 2-3 times.
13. Save aliquot to quantify amount of PAG.

3. Preparation of Affinity Chromatography Column

1. Bovine PAG antibody is biotinylated in dialysis buffer B with a 60-fold molar excess of EZ-Link NHS-LC-LC biotin at 4°C for 3 hours on a tube rotator.
2. Non-reacted biotinylation reagent is removed by repeated dialysis with dialysis buffer B.
3. To form the column matrix, 50 mg of biotinylated anti-PAG monoclonal (L4) is incubated with 50 Ml Agarose Avidin D at room temperature for 3 hours on a tube rotator.
4. Load avidin matrix into the column and wash with equilibration buffer to remove unbound biotinylated anti-PAG antibody.

4. Affinity Chromatography Purification of PAGs

1. Wash the column with 40 column volumes of wash buffer A.
2. Load the column with 200 ml of dialyzed 80% Ammonium Sulfate-precipitated material from section 3.2.
3. Wash the column with 10 column volumes of wash buffer A.
4. Wash the column with 5-10 column volumes of wash buffer B.
5. Wash the column with 25-30 column volumes of wash buffer A.
6. Elute column with 12 column volumes of elution buffer A and 7 column volumes of elution buffer B.
7. Neutralize the Ph of the eluted fractions with neutralization buffer immediately upon collection from the column.
8. Wash the column with 10-20 column volumes of wash buffer A and begin loading the column again if PAG is still present in cotyledonary extract.
9. Analyze the fractions by dot blot with anti-PAG sera to identify fractions containing PAG.
10. Combine PAG positive fractions into glass container.
11. Add E-64, EDTA, PMSF, Azide to purified PAG to prevent proteolysis.
12. Store at 4°C.
13. Save aliquot to quantify amount of PAG eluted off of column.

5. PAG Concentration

1. Place purified PAG into a glass container.
2. Put polyethylene glycol (PEG 15,000-20,000 molecular weight) into 1,000 molecular weight dialysis tubing and place into the glass container holding the PAG to withdraw water from the PAG solution via osmosis.
3. Concentrate at 4°C for approximately 30 hours, changing out the polyethylene glycol filled dialysis tubing 5 times.
4. Save aliquot to quantify final concentration of PAG.
5. Analyze the concentrated PAG by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), to determine purity via Sypro Red (Figure 11) staining
6. Dialyze concentrated PAG in media buffer for use in endometrial tissue culture.

4. Notes

1. In order to calculate the amount of ammonium sulfate, use the EnCor Biotechnology, Inc. Ammonium Sulfate Calculator. http://www.encorbio.com/protocols/AM-SO4.htm
2. When centrifuging the cotyledon mixture, it is important to keep in mind the centrifuge bottles must be balanced by mass, not volume.
3. It is often a good idea to repack the matrix between the first wash step and the loading step. With such a great amount of solution being run across the column, 'channels' can be created in the matrix. When this occurs, the flow dynamics of the column will become altered and the column will also suffer a decrease in apparent binding capacity.
4. Place enough of the neutralization buffer in each collection tube prior to the elution step to neutralize the solution as it is collected so that the purified proteins are exposed to the low Ph for as short a time as possible.
5. When concentrating the purified PAG, put enough polyethylene glycol into the 1,000 molecular weight dialysis tubing to ensure protein is being concentrated; however, do not put so much into the tube that it breaks during the concentration process.
6. An in-house PAG ELISA was used to quantify PAGs. The PAG ELISA seems to be the best method to determine protein concentration of purified PAG when compared to BCA, Lowry, and Bradford assays.

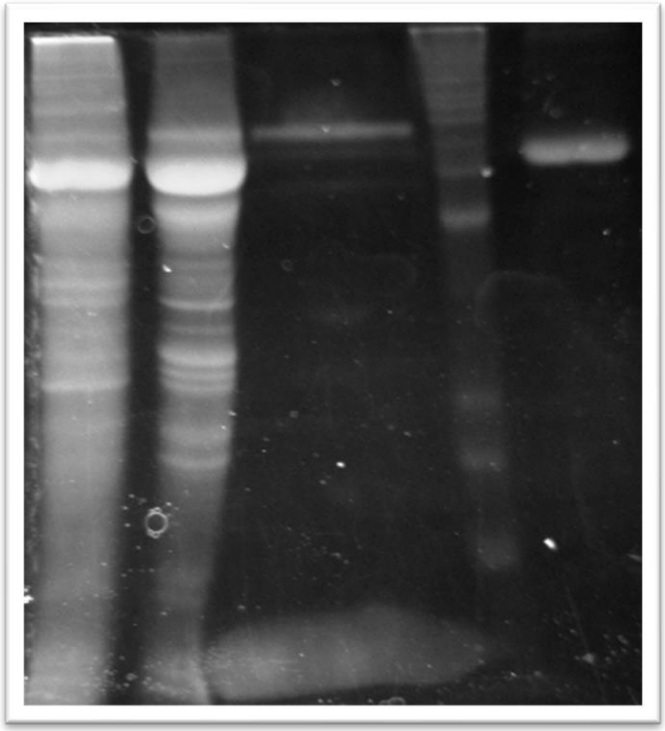


Figure 11. Analysis of the purification of PAGs from cotyledonary extract by SDS-PAGE and Sypro Red staining. *Lane 1:* beginning material (product of section 1); *Lane 2:* 80% ammonium sulfated cotyledonary extract (product of section 2); *Lane 3*: eluted, concentrated material (product of section 4 and 5); *Lane 4:* standard to compare protein sizes; *Lane 5:* bovine serum albumin

APPENDIX II

Sandwich ELISA for the detection of PAGs

Summary

Purification of the PAGs via affinity chromatography is a helpful tool in rapidly collecting a relatively pure pool of the target glycoprotein. However, depending on the size of the tissue, the number of tissues used for extraction, and the stage of pregnancy from which they were collected, the final yield can vary. Additionally, the final extract is never 100% pure PAGs. There is always additional protein that has not been removed, such as bovine serum albumin, which makes determining the PAG concentration via total protein content quite inaccurate. To better determine the yield PAG within the final placental extract an ELISA was performed to specifically measure the concentration of PAGs. The PAG ELISA was developed by Green et al. (2005). It uses anti-PAG monoclonal antibodies that recognize different placental binucleate cell-specific PAGs for a more accurate prediction of total PAG concentrations. This would then allow for a more accurate and consistent treatment of the cultured endometrial explants.

Materials

1. Plate preparations

1. 96-well, high binding ELISA plate
2. 0.1M sodium bicarbonate Ph 9.5

2. Assay buffers and wash buffers

1. Blocking buffer: 1.5% (w/v) nonfat dried milk dissolved in 0.1M sodium bicarbonate buffer Ph9.5
2. TBST: 20Mm Tris base, 150Mm NaCl, 0.05% (v/v) Tween-20, 0.001% w/v Thimerosal
3. Wash buffer: 150Mm NaCl, 0.05% (v/v) Tween-20, 0.001% (w/v) Thimerosal
4. AP buffer: 50Mm Tris base, 100Mm NaCl, 1 mg/Ml $MgCl_2$
5. Alkaline phosphate substrate: 1 mg/Ml p-Nitrophenyl phosphate (PNPP; Thermo Scientific)
6. Nonpregnant heifer serum

3. Antibodies and other reagents

1. Sheep-anti-Mouse Fc antibody (Jackson ImmunoResearch)
2. Anti-bovine PAG monoclonal antibodies A6, J2, and L4

3. PAG standard curve
4. Rabbit-anti-bovine PAG polyclonal antibody
5. Goat-anti-rabbit antibody conjugated to alkaline phosphatase (Jackson ImmunoResearch)

4. Hardware needed

1. Single or multi-channel pipettors
2. Pipette tips
3. Solution basins
4. Plate washer (optional)
5. Paper towels
6. Plate reader

Methods

1. Add 100ul of 10ug/ml of Sheep-anti-Mouse antibodies in 0.1M Sodium bicarbonate Ph9.5 to the wells of a 96-well ELISA plate. Leave at 4 degrees overnight.

2. Dump the solution and pat the plate on paper towels to remove excess solution from the wells. Add 200 µl per well of blocking buffer. Put on rocking platform (if available) for ≥ 1hr at room temperature.

3. Add 0.5ug/ml of each monoclonal antibody (J2, A6, and L4) to a solution of 70% (v/v) TBST + 30% (v/v) blocking solution (NFDM solution from previous step).

4. Dump the block from the plate and pat dry. Add 100ul per well of the monoclonal antibody solution. Incubate on a rocking platform for 1hr at room temperature.

5. Dump the plate and pat it dry. Add 50µl per well of TBST + blocking solutions (same as from step 3) to wells A through F to keep the wells moist. Add 100µl of Nonpregnant heifer serum (NPHS) to rows G and H. Add 100µl of each test sample to rows A through F with (at least) duplicates oriented horizontally. Add 50µl of a standard curve diluted in TBST + blocking solutions to rows G and H. The blank for the plate will be wells H11 & H12.

6. Put at 4 degrees on a rocking platform overnight. Alternatively, put the plate in an incubated shaker and shake at 750rpm and 37 degrees Celsius for 2 hours.

7. Dump the plate and pat dry. Wash the plate extensively with a plate washer or by using a spray bottle. Add 100µl of anti-Bovine PAG polyclonal antibody in TBST + block with 2% NPHS. Incubated at room temp for 1hr on a rocking platform.

8. Dump and pat out the blocking solution. Wash the plate extensively with a plate washer or by using a spray bottle. Add 100µl of a 1:2000 dilution of Goat-anti-Rabbit antibody conjugated to alkaline phosphatase in Alkaline Phosphatase (AP) buffer. Incubated at room temp for 30 min on a rocking platform.

9. Dump and pat out the blocking solution. Wash the plate extensively with a plate washer or by using a spray bottle.

10. Develop color by adding 100µl of 1mg/ml PNPP dissolved in AP buffer. Plates are typically ready to read in 5-15 mins.

11. Read the plate in a plate reader at a 405nm wavelength.

Notes

1. Just about any non-specific protein solution can be used as a blocking reagent.
2. The monoclonal antibodies used here are the same that were used by Green et al. (2005)
3. The PAG standard curve stock used for this assay started at 800 ng/Ml. From that a serial dilution was performed to obtain an 11-point curve with a 'blank'/negative control at the end.
4. The 'blank' wells consist solely of TBST + blocking buffers with no PAG from the standard curve or from serum samples
5. The anti-bovine PAG polyclonal antibody was made in-house by the laboratory of Jonathan Green
6. Horseradish peroxidase (HRP)-conjugated anti-rabbit antibody can be used in place of the AP-conjugated antibody. If an HRP-conjugated antibody is used, then TMB substrate should be used in place of PNPP and the colorimetric reaction should be stopped with 1M HCl after the desired time. Plate should be read at a 450nm wavelength.

APPENDIX III

SDS-polyacrylamide gel electrophoresis (PAGE) for detection and evaluation of bovine cotyledonary PAG extracts

Summary

Antibodies recognize and bind to specific epitopes on their target antigen that consist of either a particular amino acid sequence and/or a particular conformational structure. Either of these epitope types could be displayed on intact, properly folded proteins or misfolded or partially degraded proteins. That means that affinity chromatography of the PAGs does not ensure that the purified protein is intact. To verify that the purified protein was not degraded, the purified PAGs were assessed using a standard SDS-PAGE protocol. Intact PAG glycoproteins present as a single band between 62 and 65 kDa. Degraded protein would present as a smear or as multiple faint bands of varying sizes. This method also

serves as a semi-quantitative assay to roughly assess the amount of intact protein compared to the amount of degraded protein, if any.

Materials

1. Stacking gel

1. Acrylamide/Bis-acrylamide (Thermo Scientific)
2. 1.5M Tris-HCl Ph 8.8
3. 10% (w/v) SDS (Fisher)
4. TEMED (Fisher)
5. 10% (w/v) ammonium persulfate (AP) (Fisher)

Recipe for a 5Ml stacker gel:

H2O	2.975 ml
0.5 M Tris-HCl, Ph 6.8	1.25 ml
10% (w/v) SDS	0.05 ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	0.67 ml
10% (w/v) ammonium persulfate (AP)	0.05 ml
TEMED	0.005 ml

2. Loading buffer

1. 10% (w/v) SDS
2. Dithiothreitol or beta-mercapto-ethanol
3. Glycerol
4. Tris-HCl Ph 6.8
5. Bromophenolblue

Receipt for a 5x loading buffer stock:

10% w/v	SDS
10 Mm	Dithiothreitol, or beta-mercapto-ethanol
20 % v/v	Glycerol
0.2 M	Tris-HCl, Ph 6.8
0.05% w/v	Bromophenolblue

2. Resolving gel

1. Acrylamide/Bis-acrylamide (Thermo Scientific)
2. 1.5M Tris Ph 8.8
3. 10% (w/v) SDS (Fisher)
4. 10% ammonium persulfate (AP) (Fisher)
5. TEMED (Fisher)

Recipe for a 10Ml resolving gel:

Acrylamide Percentage	6%	8%	10%	12%	15%
H2O	5.2ml	4.6ml	3.8ml	3.2ml	2.2ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	2ml	2.6ml	3.4ml	4ml	5ml
1.5M Tris(Ph=8.8)	2.6ml	2.6ml	2.6ml	2.6ml	2.6ml
10% (w/v)SDS	0.1ml	0.1ml	0.1ml	0.1ml	0.1ml
10% (w/v) ammonium persulfate (AP)	100µl	100µl	100µl	100µl	100µl
TEMED	10µl	10µl	10µl	10µl	10µl

3. Running buffer

1. 25Mm Tris-HCl
2. 200Mm Glycine

Methods

1. Make the separating gel:

- Set the casting frames (clamp two glass plates in the casting frames) on the casting stands.
- Prepare the gel solution (as described above) in a separate small beaker.
- Swirl the solution gently but thoroughly.
- Pipet appropriate amount of separating gel solution (listed above) into the gap between the glass plates.
- To make the top of the separating gel be horizontal, fill in water (either isopropanol) into the gap until a overflow.
- Wait for 20-30min to let it gelate.

2. Make the stacking gel:

- Discard the water and you can see separating gel left.
- Pipet in stacking gel until a overflow.

- Insert the well-forming comb without trapping air under the teeth. Wait for 20-30min to let it gelate.
- Make sure a complete gelation of the stacking gel and take out the comb. Take the glass plates out of the casting frame and set them in the cell buffer dam. Pour the running buffer (electrophoresis buffer) into the inner chamber and keep pouring after overflow until the buffer surface reaches the required level in the outer chamber.

3. Prepare the samples:

- Mix your samples with sample buffer (loading buffer).
- Heat them in boiling water for 5-10 min.

4. Load prepared samples into wells and make sure not to overflow. Don't forget loading protein marker into the first lane. Then cover the top and connect the anodes.

5. Set an appropriate volt and run the electrophoresis when everything's done.

6. As for the total running time, stop SDS-PAGE running when the downmost sign of the protein marker (if no visible sign, inquire the manufacturer) almost reaches the foot line of the glass plate (Figure 11). Generally, about 1 hour for a 120V voltage and a 12% separating gel. For a separating gel possessing higher percentage of acrylamide, the time will be longer.

Notes

- Higher concentration of AP and TEMED lead to faster gelation. I often double the normal amount to speed up the gelation time.
- The SDS-PAGE gels run in the validation of the PAGs for these experiments was 12.5%
- Loading buffer should be diluted to a 1x concentration into the desired sample
- Protein molecular weight marker that was used in these experiments was BenchMark Prestained protein ladder from Invitrogen
- This protocol is borrowed from http://www.assay-protocol.com/molecular-biology/electrophoresis/denaturing-page.html

APPENDIX IV

Assessment of changes in gene transcription of MMPs and prostaglandin synthases by real time PCR in bovine endometrial explants exposed to PAGs

Summary

The PAGs have been predicted to plays roles in matrix turnover at the utero-placental interface and/or in the maternal stroma (Wooding et al. 2005; Wallace et al. 2019). Several of the PAGs have been shown to be active aspartic proteinases (B. P. V. L. Telugu and Green 2008; B. P. V. L. Telugu et al. 2010) and the PAGs have previously been shown to upregulated expression of several MMPs in bovine endometrial explants (R. M. Wallace et al. 2019). Previous research has also implicated the PAGs in luteotrophic mechanisms by upregulating production of PGE_2 in ovine endometrial explants (Weems et al. 2003). To assess these potential functions of the PAGs, real time PCR (Qpcr) was performed analyze the changes in transcripts for several MMPs as well as some prostaglandin synthesis-related genes in endometrial explants exposed to bovine PAGs.

Materials

1. Hardware

1. Single or multichannel pipettor
2. Pipette tips
3. 96-well PCR reaction plate (Applied Biosystems)
4. Optical adhesive film (Applied Biosystems)
5. Quant Studio 3 real-time PCR system (Applied Biosystems)

2. PCR Reagents

1. Primers (Table 1)
2. Power SYBR Green PCR master mix (Applied Biosystems)
3. Template Cdna
4. Nuclease-free water

Methods

1. To one well of the reaction plate add 1.25ng of DNA template, 6.25Ul of SYBR Green master mix, forward and reverse primers at optimized concentrations, and enough nuclease-free water for a final reaction volume of 12.5Ul.
2. Apply optical adhesive film over the reaction plate to seal each well.

3. Plate the plate in the Qpcr reader and run under the following cycling conditions: 95°C for 10min; 40 cycles of 95°C for 15 seconds and 58°C for 1 minute.
4. A dissociation curve was included in each Qpcr run to ensure specificity of the amplicons.

Notes/Results

1. Primer sequences and concentrations used in each assay are listed in Table 1
2. In nonpregnant and pregnant samples, *MMP1, MMP3,* and *MMP13* were upregulated after 48 and 72 hours (Figure 12 A and B)
3. In nonpregnant samples, *MMP12* was upregulated on after 48 hours and *MMP14* was upregulated only after 72 hours (Figure 12A)
4. In pregnant samples, *MMP12* was downregulated only after 72 hours and there was no change in *MMP14* abundance (Figure 12B)
5. In nonpregnant samples, *PTGES, PTGS1,* and *PTGS2* were upregulated only after 48 hours (Figure 13)
6. In pregnant samples, *PTGES* and *PTGS1* were downregulated after 48 hours and *PTGS2* was upregulated after 48 and 72 hours (Figure 14)

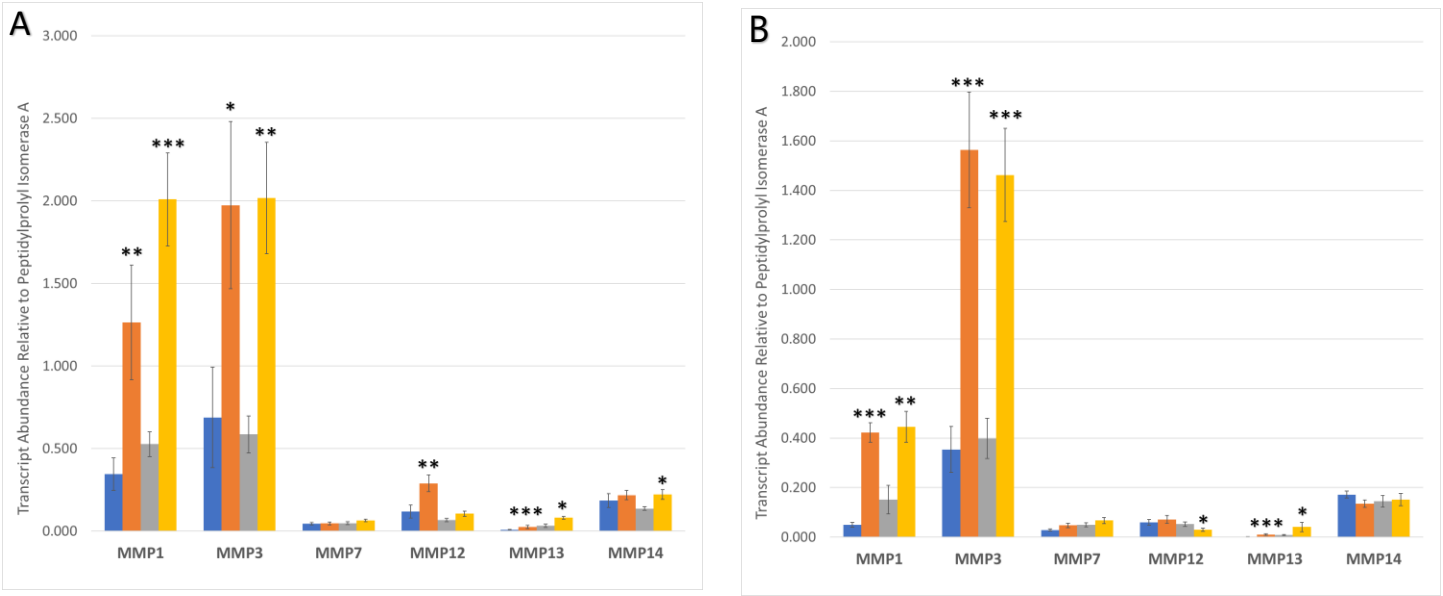
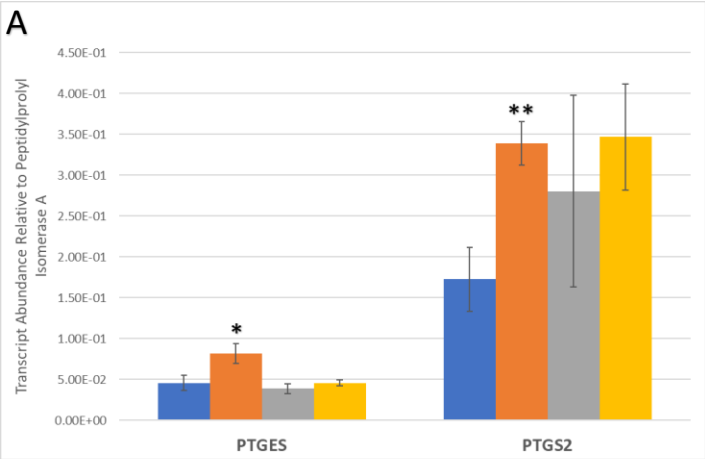
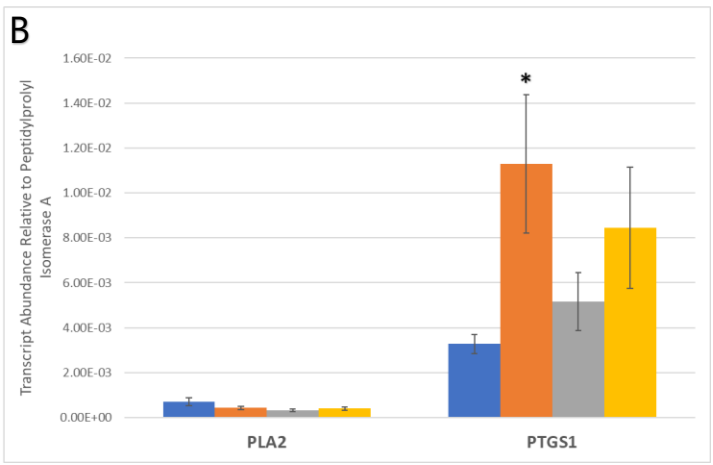
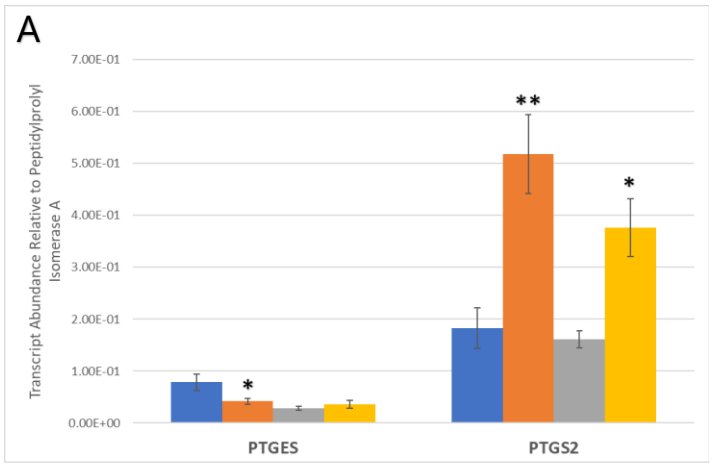


Figure 12. Relative transcript abundance of MMPs in nonpregnant and pregnant endometrium after exposure to PAGs for 48 and 72hr

Target transcript abundance of MMP1, MMP3, MMP7, MMP12, MMP13, and MMP14 in endometrial explants from nonpregnant (A) and pregnant (B) heifers exposed to PAGs for 48 (blue and orange bars) and 72 hours (grey and yellow bars). Data was quantified by RT-PCR and the results were normalized to PPIA. Data are presented as mean ±SEM. Blue bar: 48hr nontreated endometrium; Orange bar: 48hr PAG-treated endometrium; Grey bar: 72hr nontreated endometrium; Yellow bar: 72hr PAG-treated endometrium. *P<0.05; **P<0.01; ***P<0.001.







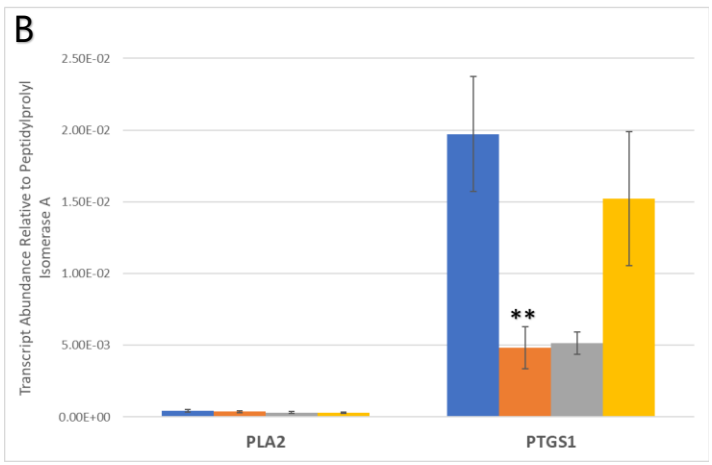


Figure 13. Relative transcript abundance of prostaglandin synthesis-related genes in nonpregnant endometrium after exposure to PAGs for 48 and 72hr

Target transcript abundance of several prostaglandin synthesis-related genes in endometrial explants from nonpregnant heifers exposed to PAGs for 48 (blue and orange bars) and 72 hours (grey and yellow bars). Data was quantified by RT-PCR and the results were normalized to PPIA. Data are presented as mean ±SEM. Blue bar: 48hr nontreated endometrium; Orange bar: 48hr PAG-treated endometrium; Grey bar: 72hr nontreated endometrium; Yellow bar: 72hr PAG-treated endometrium. *P<0.05; **P<0.01; ***P<0.001.

Figure 14. Relative transcript abundance of prostaglandin synthesis-related genes in pregnant endometrium after exposure to PAGs for 48 and 72hr

Target transcript abundance of several prostaglandin synthesis-related genes in endometrial explants from pregnant heifers exposed to PAGs for 48 (blue and orange bars) and 72 hours (grey and yellow bars). Data was quantified by RT-PCR and the results were normalized to PPIA. Data are presented as mean ±SEM. Blue bar: 48hr nontreated endometrium; Orange bar: 48hr PAG-treated endometrium; Grey bar: 72hr nontreated endometrium; Yellow bar: 72hr PAG-treated endometrium. *P<0.05; **P<0.01; ***P<0.001.

APPENDIX V

Caspase-3 and PCNA immunofluorescent (IF) assessment of apoptosis in endometrial explants exposed to PAGs

Summary

Explant culture system have their advantages over cell culture system. The main advantage is that the responses seen are more representative of the *in vivo* tissue. However, there are some disadvantages when it comes to maintaining a tissue outside of the body. There is no more blood supply, so unless the culture medium is changed often, the supply of oxygen and nutrients to the tissue is limited, especially to the cells at the center of the explant. This can be avoided by making the explant tissues small, but apoptosis is bound to occur and can negatively affect the results of the assay. As a result, tissue explant systems are not usually maintained for long because cells begin to die. Because the current study utilized an explant culture system that maintained the tissues outside of the body for up to four days, it was important to assess the degree of apoptosis that may have occurred. To assess this, immunofluorescence (IF) was performed for the presence of an apoptosis marker, activated Caspase-3, and a proliferation marker, PCNA, with fixed endometrial explants either treated or not treated with PAG for 24 and 96 hours.

Materials

1. Formalin-fixed, paraffin-embedded endometrial explants
2. Antigen retrieval: 10mM Tris base, 1mM EDTA, 0.05% (v/v) Tween-20 pH 9.0 (Tris-EDTA)
3. Serum block buffer: 2% (v/v) horse serum, 1% (w/v) bovine serum albumin, 0.1% (v/v)Triton X-100, 0.05% (v/v) Tween-20, PBS
4. Primary antibody: Anti-activated Caspase-3 antibody (Abcam)
5. Primary antibody: Anti-PCNA antibody (Abcam)
6. Secondary antibody: Anti-mouse IgG antibody conjugated to Alexa Fluor 488 (Jackson ImmunoResearch)
7. Secondary antibody: Anti-rabbit IgG antibody conjugated to Alexa Fluor 594 (Jackson ImmunoResearch)
8. 4’,6-diamidino-2-phenylindole (DAPI)
9. ProLong Diamond Antifade Mountant (Invitrogen)
10. Ethanol
11. Xylene

12. Phosphate buffered saline (PBS)
13. Phosphate buffered saline with Tween-20 (PBST)

Methods

1. Preparing the slides

1. Paraffin-embedded tissues were sectioned 5µm thick and adhered to charged glass slides
2. 2-3 sections were adhered to each slide

2. Staining the tissues

1. Tissue sections were deparaffinized in 3 changes of xylene, 5 minutes each
2. Tissue section were than gradually rehydrated by submerging in decreasing concentrations of ethanol starting at 100% ethanol and ending in deionized water
3. Tissues were washed in PBST for 3 minutes
4. Heat-mediated antigen retrieval was performed by submerging tissue sections in a staining dish containing Tris-EDTA buffer that had pre-heated in a steamer to 95-100^{o}C. Tissues were incubated for 20 minutes in the hot Tris-EDTA in the steamer.
5. With the tissue sections still submerged, the Tris-EDTA staining dish was taken out of the steamer and allowed to cool at room temperature for about 20 minutes.
6. Non-specific binding was blocked by covering the slides with serum blocking buffer until each of the tissues were covered. Tissues were incubated with the blocking buffer for 30 minutes at room temperature in a moisture chamber.
7. Blocking buffer was dumped off of the slides and they were then washed briefly by submerging in PBST.
8. Slides were carefully patted dry and a hydrophobic barrier was drawn around each tissue section using a PAP pen.
9. Appropriate primary antibodies were then applied to each tissue section individually at manufacturer recommended concentrations and were allowed to incubate at 37^{o}C for 1 hour in a moisture chamber.
10. Antibody was removed from slides and the slides were washed in 2 changes of PBST, 2-3 minutes each.
11. Appropriate fluorescent secondary antibodies were applied to each tissue section individually at manufacturers recommended concentrations and allowed to incubate for 30 minutes at room temperature in a moisture chamber in the dark.
12. Secondary antibodies were removed from slides and the slides were washed in 2 changes of PBST, 2-3 minutes each.
13. Nuclei were counterstained by applying DAPI (up to 10µg/mL) to each tissue individually and incubating for 5-7 minutes at room temperature in a moisture chamber in the dark.
14. Slides were then briefly washed in PBST and quickly dehydrated through 100% ethanol.
15. Coverslips were mounted over the slides with Prolong Diamond Antifade and the mountant was allowed to cure overnight in a dry place in the dark.
16. The next morning the slides were examined under a fluorescent microscope.

Notes/Results

1. Figure 15 shows results from a single representative animal
2. There were no differences between pregnant and nonpregnant samples
3. There were no differences between control (Figure 15A-C and G-I) and PAG-treated (Figure 15D-F and J-L) samples
4. Presence of activated caspase-3 and PCNA were low in the 24 hour samples of all animals and mainly in the subepithelial stroma
5. By 96 hours, activated caspase-3 was more abundance as was PCNA
6. Activated caspase-3 seemed to be more prevalent in the stroma, indicating an increase in apoptosis
7. In the 96hr samples PCNA was also more abundant and was localized in the luminal and glandular epithelia

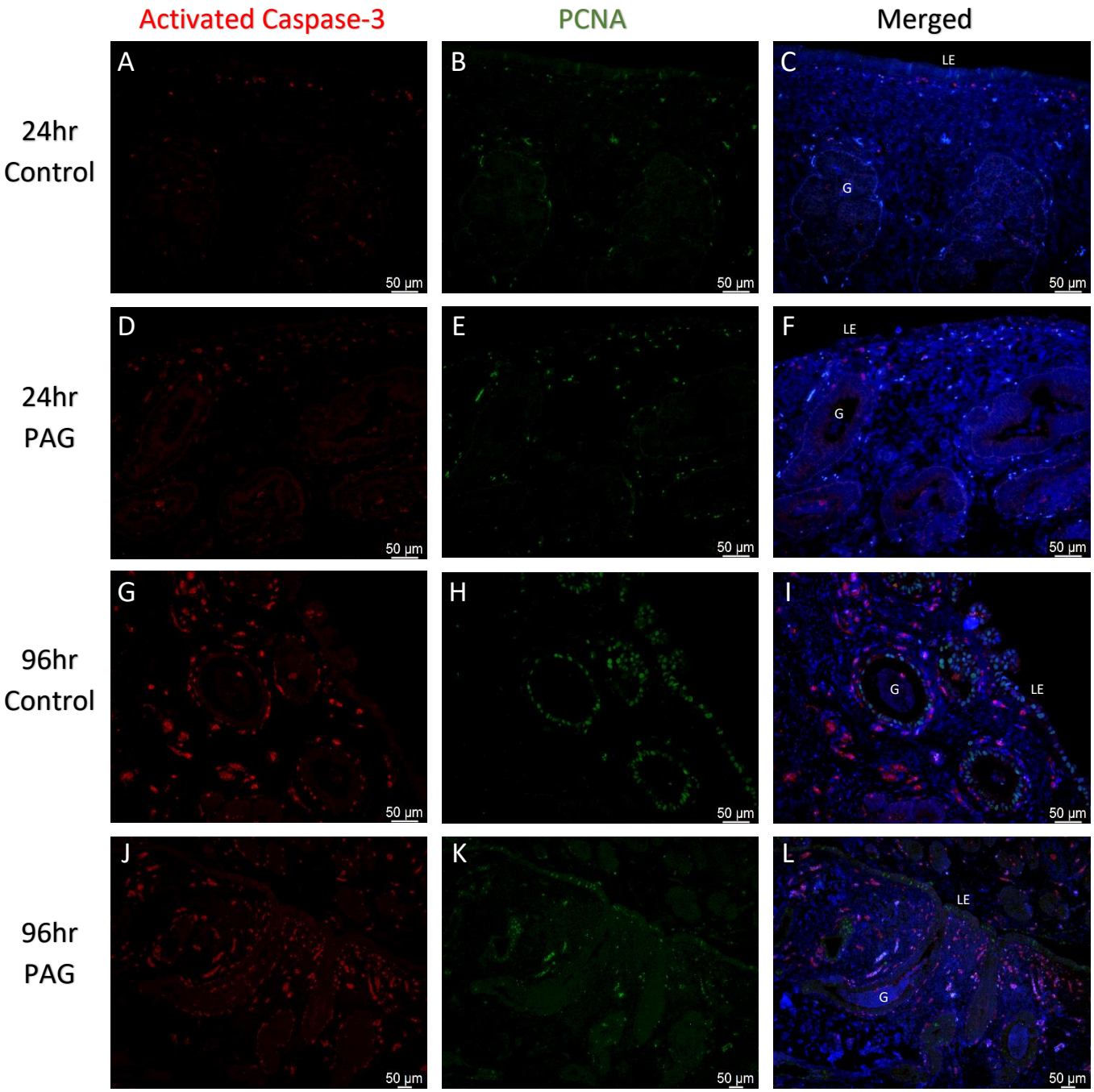


Figure 15 Immunofluorescent staining for activated caspase-3 and PCNA in endometrial explants exposed to PAGs for 24 and 96 hours

Bovine endometrial explants cultured for 24 hours (A-F) and 96 hours (G-L) either in the absence (A-C and G-I) or in the presence (D-F and J-L) of bovine PAGs. Tissues were stained for activated caspase-3 (red; left column) to assess the degree of cellular apoptosis. Tissues were also stained for PCNA (green; center column) to assess the amount of proliferation. Nuclei were counterstained with DAPI (blue). Results were similar between treatment and pregnancy status so results from a single animal is shown here as a representative. G, gland; LE, luminal epithelium

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