**Expression of Progesterone Receptor in the Porcine Uterus and Placenta throughout
Gestation: Correlation with Expression of Uteroferrin and Osteopontin**

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ABSTRACT

Progesterone (P4) stimulates production and secretion of histotroph, a mixture of hormones, growth factors, nutrients, and other substances required for growth and development of the conceptus (embryo/fetus and placental membranes). Progesterone acts through the progesterone receptor (PGR); however, there is a gap in our understanding of P4 during pregnancy because PGR have not been localized in the uteri and placentae of pigs beyond Day 18. Therefore, we determined endometrial expression of *PGR* mRNA and localized PGR protein in uterine/placental tissues throughout the estrous cycle and through Day 85 of pregnancy in pigs. Further, two components of histotroph, tartrate-resistant acid phosphatase 5 (ACP5; uteroferrin) and secreted phosphoprotein 1 (SPP1; osteopontin) proteins, were localized in relation to PGR during pregnancy. Endometrial expression of *PGR* mRNA was highest at Day 5 of the estrous cycle, decreased between Days 5 and 11 of both the estrous cycle and pregnancy, and then increased between Days 11 and 17 of the estrous cycle ($P < 0.01$), but decreased from Days 13 to 40 of pregnancy ($P < 0.01$). PGR protein localized to uterine stroma and myometrium throughout all days of the estrous cycle and pregnancy. PGR were expressed by uterine luminal epithelium (LE) between Days 5 and 11 of the estrous cycle and pregnancy, then PGR became undetectable in LE through Day 85 of pregnancy. During the estrous cycle PGR were down-regulated in LE between Days 11 and 15, but expression returned to LE on Day 17. All uterine glandular epithelial (GE) cells expressed PGR from Days 5 to 11 of the estrous cycle and pregnancy, but expression decreased in the superficial GE by Day 12. Expression of PGR in GE continued to decrease between Days 25 and 85 of pregnancy; however, a few glands near the myometrium and in close proximity to areolae maintained expression of PGR protein. ACP5 protein was detected in the GE from Days 12 to 85 of gestation and in areolae. SPP1 protein was detected in

uterine LE in apposition to inter-areolar, but not areolar areas of the chorioallantois on all days examined, and in uterine GE between Days 35 and 85 of gestation. Interestingly, uterine GE cells adjacent to areolae expressed PGR, but not ACP5 or SPP1, suggesting these are excretory ducts involved in the passage, but not secretion, of histotroph into the areolar lumen, and highlighting that P4 does not stimulate histotroph production in epithelial cells that express PGR.

KEYWORDS: progesterone receptor, pig, pregnancy, SPP1, ACP5, endometrium

1. INTRODUCTION

Progesterone (P4), the hormone of pregnancy, exerts its actions on tissues through the nuclear progesterone receptor (PGR), which is encoded by a single gene, *PGR*, that has three isoforms (A, B, and C) with differing lengths and activities [1,2]. Of the three PGR isoforms, B is the most transcriptionally active [3]. PGRA has more inhibitory effects due to an active transcription inhibitory domain, which is blocked in PGRB [4]. The third isoform, PGRC, is a truncated version of PGRA and PGRB that cannot bind DNA directly, but can still bind P4 and possibly sequester ligand or the other isoforms to decrease P4 signaling [5]. Both PGRA and PGRB have been studied extensively in multiple species to determine their roles in the uterus during pregnancy [1].

Previous studies have described PGR localization in pigs during the estrous cycle and early pregnancy, but not beyond Day 18 of pregnancy [6, 7]. Progesterone receptor B (PGRB) protein is expressed in the uterine luminal (LE), glandular epithelia (GE), stromal fibroblasts, and myometrium through Day 7 of the estrous cycle and pregnancy. Prolonged exposure to P4 downregulates PGRB in uterine LE by Day 10 and in uterine GE by Day 12 of both the estrous

cycle and pregnancy; however, PGRB continues to be expressed in the stromal cells and myometrium through Day 18 [6, 8]. Progesterone receptor A (PGRA) protein has been detected by western blot in endometria from Days 0 to 10 of the estrous cycle and pregnancy [6].

One of the major roles of P4 is to stimulate the production and secretion of histotroph, a complex mixture of hormones, growth factors, nutrients, and other substances required for growth and development of the conceptus (embryo/fetus and associated placental membranes) [9-11]. Two major protein components of histotroph are tartrate-resistant acid phosphatase 5 (ACP5; uteroferrin) and secreted phosphoprotein 1 (SPP1; osteopontin; OPN) [12, 13]. During the peri-implantation period of pregnancy, P4 induces ACP5 expression by uterine GE [14] and estrogen induces SPP1 expression by uterine LE [15]. A second induction of SPP1 occurs between Days 30 and 40 in the uterine GE [12]. Although this latter induction is hypothesized to be in response to P4, ovariectomized pigs exposed to P4 for 40 days do not express SPP1 in GE, suggesting that multiple factors may be required [10, 14, 16]. The consensus is that the role of P4 in producing histotroph is mediated via PGR; however, PGR are not expressed in uterine epithelia that secrete histotroph during the peri-implantation period [6]. It is clear that regulation of gene expression in the endometrium by P4 during the peri-implantation period is complex. Induction of genes in uterine epithelia may require that P4 down-regulate PGR, thereby eliminating PGR-dependent inhibition of expression of progesterone-regulated genes. However, another explanation is that P4 induction of expression of genes in uterine epithelia is mediated by a paracrine-acting factor(s) (progestamedin) produced by the PGR-positive stromal cells [17].

P4 continues to profoundly influence the uterus and placenta throughout pregnancy [18], and, as just described, the temporal and spatial expression of PGR is critical to understanding the complex mechanisms by which P4 exerts its effects. However, the temporal and spatial

expression of PGR in pig endometrium beyond Day 18 of pregnancy has not been reported. Therefore, in order to fill this critical gap in **our understanding** of the function of P4 during pregnancy, the aim of this study was to quantify total endometrial mRNA and localize PGR protein in cells of the uterus throughout the estrous cycle and pregnancy in pigs. In addition, ACP5 and SPP1 were localized in porcine uterine and placental tissues throughout gestation to gain physiological insight into the relationship between the pattern of PGR expression during mid- to late-pregnancy and the production of those particular components of histotroph.

2. MATERIALS AND METHODS

*2.1 Animals and **tissue collection***

Sexually mature, 8-month-old crossbred gilts were observed daily for estrus (Day 0) and exhibited at least two estrous cycles of normal duration (18 to 21 days) before being used in these studies. All experimental and surgical procedures were in compliance with the Guide for Care and Use of Agricultural Animals in Teaching and Research and approved by the Institutional Animal Care and Use Committee of Texas A&M University.

To evaluate the effects of pregnancy on temporal and cell-specific expression of PGR mRNA and PGR, ACP5, and SPP1 proteins, gilts were assigned randomly to either cyclic or pregnant status. Cyclic gilts were ovariectomized on either Day 5, 9, 11, 13, 15 or 17 of the estrous cycle. Gilts in the pregnant group were bred and ovariectomized on either Day 9, 11, 13, 15, 17, 20, 30, 40, 50, 60, or 85 of pregnancy (n = 3 or 4 gilts/day/status). The lumens of both uterine horns of each gilt were flushed separately with 20 **mL** physiological saline for those collected on Days 5 to 17 post-estrus. Pregnancy was confirmed in mated gilts by the presence of morphologically normal conceptuses in uterine flushings. Tissue sections (~1 cm

thick) from the middle of each uterine horn of all gilts were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2) and embedded in Paraplast-Plus (Oxford Laboratory, St. Louis, MO). Additionally, endometrium was physically dissected from the myometrium, snap-frozen in liquid nitrogen, and stored at -80°C for RNA extraction.

2.2 Progesterone *model*

Because P4 is the hormone of pregnancy and induces the glandular expression of some components of histotroph, including ACP5 [10, 11], we evaluated effects of long-term progesterone (P4) treatment, in the absence of other ovarian hormones, on endometrial expression of PGR mRNA and localization of PGR protein. Gilts were ovariectomized on Day 12 of the estrous cycle and assigned randomly to receive daily intramuscular injections of either corn oil (CO, 4 mL) or P4 (200 mg P4 in 4 mL CO) on Days 12 through 39 post-estrus (n = 3/treatment). All gilts were hysterectomized on Day 40 post-estrus and tissues collected as previously described.

2.3 RNA *extraction*, cDNA *synthesis*, and *primer design*

Total RNA was extracted from endometrial tissue samples using Trizol reagent (Life Technologies, Carlsbad, CA) according to manufacturer's recommendations. First strand cDNA was synthesized using Superscript III First Strand Kit (Life Technologies) according to manufacturer's instructions. First strand cDNA was diluted 10x for the qPCR reaction. Primers for qPCR were designed using NCBI Genbank sequences and Primer-BLAST (<http://www.ncbi.nlm.nih.gov/>). Primers were submitted to BLAST to test for specificity against the known porcine genome. The PGR forward 5'-GTGTCCTTACCTGTGGGAGC-3' and

reverse 5'-TTCTAAGGCGACAAGCTGGG-3' primers were developed from NCBI reference NM_001166488.1 and have a length of 138 nucleotides, and are designed to detect both the A and B isoforms of the *PGR*.

2.4 Quantitative PCR

The qPCR assays were performed using PerfeCta SYBR Green Mastermix (Quanta Biosciences, Gaithersburg, MD) in 10 μ L reactions with 2.5 mM of each specific primer, on a Roche 480 Lightcycler (Roche) with approximately 60 ng of cDNA per reaction. The PCR program began with 5 min at 95°C followed by 40 cycles of 95°C denaturation for 10 sec and 60°C annealing/extension for 30 sec. A melt curve was produced to verify a single gene-specific peak. A standard curve with a 2-fold serial dilution was run to determine the primer set efficiency. The primer correlation coefficient was 0.96 and efficiency was 98%. The geometric mean of the Ct values for *TATA-binding protein (TBP)*, *succinate dehydrogenase complex subunit A flavoprotein (SDHA)*, and *beta actin (ACTB)* was used to normalize data from endometrial tissue. The $2^{-\Delta\Delta C_t}$ Method was utilized to normalize data and those fold changes were subjected to statistical analyses.

2.5 Immunohistochemistry

PGR, SPP1, and ACP5 proteins were immunolocalized in porcine uterine and conceptus tissues as described previously [19]. A rabbit anti-human PGRA/PGRB polyclonal IgG (catalog no. RB-9017-P0; Thermo Fisher Scientific) was used at 5 μ g/mL with a boiling citrate buffer antigen retrieval. A rabbit anti-bovine SPP1 affinity-purified polyclonal IgG (kindly provided by

Dr. G. Killian) was used at 25 µg/mL with a boiling citrate buffer antigen retrieval [20]. A rabbit anti-porcine ACP5 polyclonal IgG was used at 5 µg/mL with a boiling citrate buffer antigen retrieval [10, 21]. Purified non-relevant rabbit IgG, at a concentration equal to that for the primary IgG, was used as the negative control. Immunoreactive protein was visualized in paraffin-embedded sections (5µm thick) using the Vectastain ABC Kit (catalog no. PK-4001; Vector Laboratories, Inc., Burlingame, CA) according to kit instructions with 3,3'-diaminobenzidine tetrahydrochloride (catalog no. D5637; Sigma-Aldrich, St. Louis, MO) as the color substrate. Sections immunostained for SPP1 and ACP5 were counterstained with Harris modified hematoxylin (Fisher Scientific), whereas sections immunostained for PGRA/PGRB were not counterstained. Coverslips were affixed using Permount mounting medium (Fisher Scientific). Digital images of representative fields of immune-stained tissue were examined and recorded under brightfield illumination using an Axioplan 2 microscope (Carl Zeiss, Thornwood, NY) interfaced with an Axioplan HR digital camera and Axiovision 4.3 software. Photographic plates were assembled using Adobe Photoshop (version 6.0, Adobe Systems Inc., San Jose, CA).

2.6 Statistical analyses

The qPCR data for normal tissues were analyzed for effects of day, pregnancy status, and their interaction for Days 9, 11, 13, 15 and 17 by 2-way ANOVA via the General Linear Models (GLM) procedures of the Statistical Analysis System (SAS Institute, Cary, NC) with the least significant difference (LSD) multiple testing method. The qPCR data were also subjected to a sliding time window linear regression analysis to determine if there was an effect of day of gestation on the expression of genes of interest throughout pregnancy (Days 9 to 85). The sliding time windows of the analysis were designed to detect effect of day on gene expression during

three biologically relevant periods of time: the beginning, the middle, and the end of the study period. Each window analyzed contained a minimum of three days. Some windows contained greater than three days as long as the regression model showed no decrease in performance in terms of the r^2 and p-value of the F-statistics.

3. RESULTS

3.1 Endometrial *expression of PGR*

Endometrial expression of *PGR* mRNA was highest at Day 5 of the estrous cycle, decreased between Days 5 and 11 of both the estrous cycle and pregnancy, and then increased between Days 11 and 17 of the estrous cycle (Figure 1; day by status $P < 0.01$). Endometrial expression of *PGR* mRNA decreased from Days 13 to 40 of pregnancy ($r^2 = 0.847$; $P < 0.01$).

The antibody used to detect PGR in the present study was generated against both PGRA and PGRB antigens, but henceforth will be referred to as PGR. PGR protein was localized to uterine LE, GE, stromal cells, and myometrium on Day 5 of the estrous cycle and expression was maintained in those cells through Day 11 (Figure 2). On Day 12, PGR protein was no longer detectable in uterine LE or superficial GE, but PGR protein was detected in the stroma, myometrium, and deep GE through Day 17 of the estrous cycle.

PGR protein was localized to uterine LE, GE, stromal cells, and myometrium on Day 9 of pregnancy. Expression of PGR was maintained in GE, stroma, and myometrium through Day 17 of pregnancy with deep GE appearing to have stronger immunostaining than superficial GE; however, expression in LE was no longer detected on Days 11 through 17 of gestation (Figure 3).

On Day 25 of pregnancy, PGR protein was detected in stromal cells, myometrium and in some, but not all uterine GE. Glandular epithelia in close proximity to areolae had stronger expression of PGR protein (Figure 4 and Figure 7). On Day 40 of pregnancy, expression of PGR protein was maintained in stromal cells and myometrium, but the proportion of cells in the GE with detectable PGR decreased between Day 25 and Day 40 (Figure 4). Expression of PGR protein continued to decrease in GE between Days 40 and 85, whereas expression of PGR was maintained in stromal cells and myometrium through Day 85 of gestation (Figure 4).

Endometria from gilts treated with P4 had lower expression of *PGR* mRNA than CO-treated gilts (Figure 5; $p < 0.05$). Expression of PGR protein was detected in the nuclei of stromal and myometrial cells, but was not detected in the LE of uteri from ovariectomized gilts administered exogenous P4 (Figure 5). In contrast, uteri from control gilts had detectable PGR in uterine LE, stromal cells, and myometrium. Neither treatment group of gilts had detectable PGR in uterine GE (Figure 5).

3.2 ACP5 protein localizes to uterine GE and areolae for the majority of pregnancy

ACP5 protein was detected in uterine GE on Day 12 of pregnancy, abundance of ACP5 in GE appeared to increase between Days 12 and 25, and expression in GE remained high between Day 25 and Day 85 (Figure 6). On Day 25, ACP5 protein localized to the lumen and chorionic epithelium (CE) of the developing areolae, and expression in the areolae continued through Day 85 of gestation. ACP5 protein was not detectable in endometria from cyclic gilts (data not shown).

3.3 SPPI protein localizes to uterine LE and GE for the majority of pregnancy

SPP1 protein was not detected in uterine LE on Day 12, but was detected in LE on Day 25 of pregnancy. Expression of SPP1 protein in uterine LE was maintained throughout gestation (Figure 6). Interestingly, SPP1 protein was only detectable in uterine LE in the inter-areolar region of the uterine-placental interface. There was no detectable SPP1 in uterine LE proximal to areolae (Figure 6). SPP1 protein was barely detectable in uterine GE on Day 35, but was highly expressed in uterine GE on Day 40 of pregnancy. SPP1 was also detectable in some cells of the allantois on Day 40 of pregnancy (Figure 6). Glandular epithelial and allantoic localization of SPP1 continued through Day 85. SPP1 was not detected in endometria from cycling gilts (data not shown).

3.4 PGR, but not ACP5 or SPP1, is expressed in uterine glands that immediately empty into areolae

PGR protein was expressed in uterine GE immediately adjacent to areolae, specifically cells of the GE that open directly into the areola. In contrast, PGR protein was not expressed in all other shallow GE (Figure 7). ACP5 protein was expressed by and detected within the lumens of the majority of uterine GE and all areolae; however, ACP5 was not expressed in the PGR-positive cells of uterine GE that open directly into the areolae. SPP1 protein was expressed by the majority of uterine GE, but not in PGR-positive cells near areolae (see comparison of serial sections in Figure 7). Therefore, GE cells in close proximity to areolae were PGR+ACP5-SPP1-, but GE less than 200 μ m away in the histological section were PGR-ACP5+SPP1+.

4. DISCUSSION

P4 is required for successful pregnancy, yet the localization of PGR, an essential mediator for effects of P4, has not previously been evaluated beyond Day 18 of pregnancy in the pig. This study establishes that: 1) PGR protein expression in uterine LE returns by Day 17 of the estrous cycle; 2) PGR protein expression is maintained in the deep GE throughout the estrous cycle and early pregnancy; 3) PGR protein is not expressed by the LE between Days 25 and 85 of gestation; 4) PGR protein expression in uterine GE continues to decrease as pregnancy progresses, with almost no expression in GE by Day 50 of pregnancy; 5) PGR protein expression is maintained in the uterine stroma and myometrium through Day 85 of gestation; and 6) PGR protein, but not ACP5 or SPP1 proteins, is expressed in the necks of uterine GE that empty into the uterine lumen proximal to areolae, suggesting that these regions of GE are excretory ducts that are not involved in the synthesis of histotroph, but serve as conduits for the passage of histotroph into the areolar lumen.

During the estrous cycle, uterine expression of *PGR* mRNA was high on Days 5 and 9, lower on Days 11 and 13, and again higher on Days 15 and 17. The localization for PGR protein suggests that these changes are due to loss of PGR from the uterine LE and shallow GE by Day 11, which returns to LE and shallow GE by Day 17 of the estrous cycle. The loss of PGR in uterine LE due to down-regulation by its ligand, P4, has been described, but this is the first study to demonstrate the return of PGR protein to uterine LE late in the estrous cycle, and this expression provides additional complimentary information to the previous report by Geisert et al., [6, 10, 22, 23]. The return of expression of PGR in uterine LE may be due to decreasing concentrations of P4 as a result of luteolysis, and increasing concentrations of estrogen with the onset of proestrus [24].

The pattern of PGR expression during early pregnancy is similar to that observed for the estrous cycle with the loss of detectable PGR from uterine LE around Day 11, and a decrease in detectable PGR in the superficial GE after Day 12, resulting in only a few sporadic cells in the shallow GE expressing PGR by Day 17. As expected, PGR remained detectable in stromal cells and myometrium through Day 17 of pregnancy. These results are similar to those reported by Geisert et al. [6] in terms of uterine LE, stromal cells, and myometrium; however, in the present study PGR was expressed in GE beyond Day 12, particularly in the deep GE, although not in every gland or every GE cell, and this expression provides additional complimentary information to the previous report by Geisert et al., [6]. This could be partly attributed to the use of an antibody against PGRA and PGRB in this study, whereas previous reports on cellular localization of PGR in porcine uteri utilized antibodies generated against PGRB alone [6]. Interestingly SPP1 and fibroblast growth factor 7 (FGF7) are two components of histotroph that are not expressed by the uterine GE during the peri-implantation period, but expression is delayed until after Day 30 when the vast majority of GE lack PGR expression [6, 25]

This study, for the first time, localized PGR in the uteri of pregnant gilts between Days 20 and 85 of gestation. On Day 25, some GE cells expressed PGR, but the proportion of cells expressing PGR decreased gradually until expression was undetectable between Days 50 and 85 of pregnancy. The loss of PGR in uterine GE from Days 25 to 50 corresponds to the stage in gestation when uterine GE is undergoing extensive hyperplasia and hypertrophy, which results in a dramatic increase in total uterine secretions after Day 30 of pregnancy [11, 26, 27]. The concurrent timing of PGR down-regulation in uterine GE and activation of secretion of histotroph by uterine GE suggests that PGR down-regulation may be necessary for maximal

production of histotroph. As expected, the uterine stromal cells and myometrium continued to express PGR between Days 25 and 85 of gestation.

Expression of PGR protein in uterine LE and GE was down-regulated in ovariectomized gilts exposed to long-term P4 treatment. However, surprisingly, there was no detectable PGR in uterine GE from control gilts that received i.m. injections of corn oil in place of P4. This suggests that downregulation of PGR protein in uterine GE may not be completely P4-dependent since PGR protein was not observed in endometria that had not been exposed to ovarian or exogenous steroid hormones. Conversely, estrogen may be required to upregulate and/or maintain PGR expression in uterine GE and the endometria of ovariectomized gilts in this study were not exposed to estrogens. Glandular expression of SPP1 is hypothesized to be regulated by P4 in pigs, but, using this same hormone replacement model, P4 alone did not induce SPP1 expression in uterine GE [10]. It is probable that these two unexpected results, lack of down-regulation of PGR and lack of up-regulation of SPP1 in uterine GE in response to P4, are physiologically linked. A key thing to note is the apparent discrepancy between our previous published results, using *in situ* hybridization on the same tissues as the present study, that GE maintained PGR mRNA expression in CO-treated gilts [10]. On careful examination of immunohistochemistry for PGR it is clear that there is significant PGR expression by several stromal cells immediately surrounding the GE, and these cells may be responsible for the localization of PGR mRNA to GE using *in situ* hybridization, which depends on the activation of silver grains in the photographic emulsion that lies above the tissue section, making it difficult to determine which of two intimately associated cells expresses the mRNA. These data highlight the informative value of examining both mRNA and protein for many genes.

ACP5 and SPP1 proteins have not previously been localized in porcine endometria throughout pregnancy. Immunohistochemistry was used previously to demonstrate that ACP5 is expressed by uterine GE of pigs at Day 60 of pregnancy, and is present in the areolae at Day 75 [13]. Results from the present study show that, in addition to expression of ACP5 in GE and areolae during mid-pregnancy, ACP5 is detected in uterine GE at Day 12 of pregnancy, in areolae by Day 25 when areolae are initially developing, and continues to be expressed in uterine GE and areolae through Day 85 of pregnancy. *In situ* hybridization analysis demonstrated that estrogens secreted by the conceptus induce expression of *SPP1* mRNA in discrete regions of uterine LE juxtaposed to the conceptus, and expression expands to the entire LE by Day 20, where expression is maintained through Day 85 [28]. In addition to expression in uterine LE total uterine *SPP1* mRNA increased 20-fold between Days 25 and 85 of gestation due to induction of SPP1 expression in uterine GE [28]. Results from the present study provide further details beyond those previously reported, in that SPP1 is not expressed by the entire uterine LE, but expression is absent in LE at the surface of chorioallantoic areolae. The physiological significance of this pattern of expression is not known, but SPP1 is proposed to attach uterine LE to chorion during implantation, and areolae are the only regions of the chorion that do not attach to uterine LE. It has been known for some time that *SPP1* mRNA localizes cells to the allantois of the placenta beginning between Days 20 and 25 of pregnancy in pigs, and the number of these cells increases as gestation progresses [15]. The present results are the first to localize SPP1 protein to the allantois (Figure 6). Although the cell type(s) that expresses SPP1 within the stroma of the chorioallantoic membranes is unknown, the protein is localized near the blood vessels and may be involved in placental angiogenesis.

Of interest is the observation that PGR was detected in uterine GE that are proximal to the openings of uterine glands into the areolae. The GE comprise the opening of the uterine glands have been hypothesized to have minimal secretory activity due to their cuboidal cytological form and small surface area [29, 30]. Additionally, the capillary network that supplies the necks of the glands is a sparse, wide mesh of large capillaries, but as the gland tubule is followed down into the uterine stroma, toward the myometrium, the capillary bed becomes more intricate with smaller vessels indicative of transport occurring between the blood vessels and uterine GE [29]. Further, we observed that two protein components of histotroph, ACP5 and SPP1, are not detected in uterine GE that express PGR, although immunoreactive secretory product for these proteins are present in the lumens of the necks of the uterine glands. We propose that GE in the necks of the uterine glands that directly open into the areolae are classic excretory ducts, similar to those for many other glands in the body, that do not produce histotroph or other **secretory** proteins, but serve only to transport histotroph/proteins into the lumen of the areolae.

The presence of PGR in the necks of glands that do not synthesize ACP5 and SPP1, contrasting with the lack of PGR in uterine GE that does synthesize ACP5 and SPP1, highlights the conundrum that P4 regulates the expression of genes in cells that do not express PGR, and that the presence of PGR in GE cells appears to prevent the ability of P4 to induce protein expression in those cells [31, 17]. Indeed, the presence of PGR appears to have an inhibitory effect on expression of ACP5 and SPP1. The paradox of having gene and protein upregulation by P4 in cells lacking PGR has been discussed extensively, but no definitive explanation has been put forward. The present results show how spatially precise the regulation of genes can be i.e.,

different expression of an individual gene between adjacent ductal and secretory cells of the GE, in complex epitheliomesenchymal organs such as the uterus and placenta.

5.1 Conclusions

These results are the first to show that PGR is maintained in uterine stromal cells and myometrium through Day 85 of pregnancy, but PGR expression is not detectable in epithelia beyond Day 50 of pregnancy, except in the necks of uterine GE that empty into areolae. PGR expression in the necks of uterine GE appears to inhibit the ability of GE to produce ACP5 and SPP1, which suggests that these regions are excretory ducts that serve as conduits for the passage of histotroph from uterine glands into the lumen of the areola.

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5. REFERENCES

- [1] Wetendorf M, DeMayo FJ. Progesterone receptor signaling in the initiation of pregnancy and preservation of a healthy uterus. *Int J Dev Biol.* 2014;58:95-106.
- [2] Mulac-Jericevic B, Conneely OM. Reproductive tissue selective actions of progesterone receptors. *Reproduction.* 2004;128:139-46.
- [3] Sartorius CA, Melville MY, Hovland AR, Tung L, Takimoto GS, Horwitz KB. A third transactivation function (AF3) of human progesterone receptors located in the unique N-terminal segment of the B-isoform. *Mol Endocrinol.* 1994;8:1347-60.
- [4] Giangrande PH, Pollio G, McDonnell DP. Mapping and characterization of the functional domains responsible for the differential activity of the A and B isoforms of the human progesterone receptor. *J Biol Chem.* 1997;272:32889-900.

- [5] Wei LL, Norris BM, Baker CJ. An N-terminally truncated third progesterone receptor protein, PR(C), forms heterodimers with PR(B) but interferes in PR(B)-DNA binding. *J Steroid Biochem Mol Biol.* 1997;62:287-97.
- [6] Geisert RD, Pratt TN, Bazer FW, Mayes JS, Watson GH. Immunocytochemical localization and changes in endometrial progesterin receptor protein during the porcine oestrous cycle and early pregnancy. *Reprod Fertil Dev.* 1994;6:749-60.
- [7] Sukjumlong S, Dalin A-M, Sahlin L, Persson E. Immunohistochemical studies on the progesterone receptor (PR) in the sow uterus during the oestrous cycle and in inseminated sows at oestrus and early pregnancy. *Reproduction.* 2005;129:349-59.
- [8] Ka H, Al-Ramadan S, Erikson DW, Johnson GA, Burghardt RC, Spencer TE, et al. Regulation of expression of fibroblast growth factor 7 in the pig uterus by progesterone and estradiol. *Biol Reprod.* 2007;77:172-80.
- [9] Roberts RM, Bazer FW. The functions of uterine secretions. *J Reprod Fertil.* 1988;82:875-92.
- [10] Bailey DW, Dunlap KA, Erikson DW, Patel AK, Bazer FW, Burghardt RC, et al. Effects of long-term progesterone exposure on porcine uterine gene expression: progesterone alone does not induce secreted phosphoprotein 1 (osteopontin) in glandular epithelium. *Reproduction.* 2010;140:595-604.
- [11] Knight JW, Bazer FW, Wallace HD. Effect of Progesterone Induced Increase in Uterine Secretory Activity on Development of the Porcine Conceptus. *J Anim Sci.* 1974;39:743-6.
- [12] Renegar RH, Bazer FW, Roberts RM. Placental transport and distribution of uteroferrin in the fetal pig. *Biol Reprod.* 1982;27:1247-60.
- [13] Garlow JE, Ka H, Johnson GA, Burghardt RC, Jaeger LA, Bazer FW. Analysis of Osteopontin at the Maternal-Placental Interface in Pigs. *Biol Reprod.* 2002;66:718-25.
- [14] Knight JW, Bazer FW, Wallace HD. Hormonal regulation of porcine uterine protein secretion. *J Anim Sci.* 1973;36:546-53.
- [15] White FJ, Ross JW, Joyce MM, Geisert RD, Burghardt RC, Johnson GA. Steroid regulation of cell specific secreted phosphoprotein 1 (osteopontin) expression in the pregnant porcine uterus. *Biol Reprod.* 2005;73:1294-1301.
- [16] Chen TT, Bazer FW, Cetorelli JJ, Pollard WE, Roberts RM. Purification and properties of a progesterone-induced basic glycoprotein from the uterine fluids of pigs. *J Biol Chem.* 1973;248:8560-6.
- [17] Spencer TE, Johnson GA, Burghardt RC, Bazer FW. Progesterone and placental hormone actions on the uterus: insights from domestic animals. *Biol Reprod.* 2004;71:2-10.
- [18] Bailey DW, Dunlap KA, Frank JW, Erikson DW, White BG, Bazer FW, et al. Effects of long-term progesterone on developmental and functional aspects of porcine uterine epithelia and vasculature: progesterone alone does not support development of uterine glands comparable to that of pregnancy. *Reproduction.* 2010;140:583-94.
- [19] Joyce MM, White FJ, Burghardt RC, Muniz JJ, Spencer TE, Bazer FW, et al. Interferon stimulated gene 15 conjugates to endometrial cytosolic proteins and is expressed at the uterine-placental interface throughout pregnancy in sheep. *Endocrinology.* 2005;146:675-84.
- [20] Gabler C, Chapman D, Killian G. Expression and presence of osteopontin and integrins in the bovine oviduct during the oestrous cycle. *Reproduction.* 2003;126:721-9.
- [21] Chen TT, Bazer FW, Gebhardt BM, Roberts RM. Uterine Secretion in Mammals: Synthesis and Placental Transport of a Purple Acid Phosphatase in Pigs. *Biol Reprod.* 1975;13:304-13.

438 [22] Mathew DJ, Sellner EM, Green JC, Okamura CS, Anderson LL, Lucy MC, et al. Uterine
 439 progesterone receptor expression, conceptus development, and ovarian function in pigs treated
 440 with RU 486 during early pregnancy. *Biol Reprod.* 2011;84:130-9.
 441 [23] Spencer TE, Burghardt RC, Johnson GA, Bazer FW. Conceptus signals for establishment
 442 and maintenance of pregnancy. *Anim Reprod Sci.* 2004;82-83:537-50.
 443 [24] Henricks DM, Guthrie HD, Handlin DL. Plasma estrogen, progesterone and luteinizing
 444 hormone levels during the estrous cycle in pigs. *Biol Reprod.* 1972;6:210-8.
 445 [25] Ka H, Spencer TE, Johnson GA, Bazer FW. Keratinocyte growth factor: expression by
 446 endometrial epithelia of the porcine uterus. *Biol Reprod.* 2000;62:1772-1778.
 447 [26] Perry JS, Crombie PR. Ultrastructure of the uterine glands of the pig. *J Anat.* 1982;134:339-
 448 50.
 449 [27] Basha SM, Bazer FW, Geisert RD, Roberts RM. Progesterone-induced uterine secretions in
 450 pigs. Recovery from pseudopregnant and unilaterally pregnant gilts. *J Anim Sci.* 1980;50:113-
 451 23.
 452 [28] Johnson GA, Burghardt RC Bazer FW. Osteopontin: A leading candidate adhesion
 453 molecule for implantation in pigs and sheep. *J Anim Sci & Biotechnol.* 2014;5:56.
 454 [29] Leiser R, Dantzer V. Initial vascularisation in the pig placenta: II. Demonstration of gland
 455 and areola-gland subunits by histology and corrosion casts. *Anat Rec.* 1994;238:326-34.
 456 [30] Friess AE, Sinowatz F, Skolek-Winnisch R, Trautner W. The placenta of the pig. II. The
 457 ultrastructure of the areolae. *Anat Embryol (Berl).* 1981;163:43-53.
 458 [31] Johnson GA, Spencer TE, Burghardt RC, Taylor KM, Gray CA, Bazer FW. Progesterone
 459 modulation of osteopontin gene expression in the ovine uterus. *Biol Reprod.* 2000;62:1315-21.

FIGURE LEGENDS

Figure 1. Expression of *PGR* mRNA in endometrial tissue during the estrous cycle and pregnancy.

Expression of *PGR* mRNA was greatest at Day 5, decreased until Day 11 during the estrous cycle, then increased again to Day 17 (day by status $P < 0.01$). *PGR* mRNA in pregnant endometria decreased from Days 13 to 40 ($r^2 = 0.847$, $P < 0.01$). Values are presented as means $\pm$ SEM.

Figure 2. Immunohistochemical localization of *PGR* in uteri of gilts during the estrous cycle.

PGR protein localized to uterine LE, GE, stroma, and myometrium on Days 5 to 11 of the estrous cycle. At Day 12, protein was not detected in uterine LE, but was still detected in a few cells of the superficial GE, deep GE, stroma, and myometrium. At Day 17, *PGR* protein was detectable in uterine LE. An IgG negative control panel is provided in Figure 3. Width of fields is 570 μ m. Asterisks (*) represent areas in magnified images in the panel below. White lines in the top panels represent regions of the uterine cross-section that were removed from the captured image so that all images conform to the same height. Care was taken so that relevant localization information was not lost during this process.

Figure 3. Immunohistochemical localization of *PGR* in uteri of gilts during early pregnancy.

PGR protein localized to uterine LE, GE, stroma, and myometrium at Day 9. By Day 11, *PGR* was not detected in uterine LE or the superficial GE, but was detected in deep GE, stromal cells, and myometrium through Day 17 of pregnancy. The D9 IgG panel is the negative control. Width of fields is 570 μ m. Asterisks (*) represent areas in magnified images in the panel below. White lines in the top panels represent regions of the uterine cross-section that were removed from the captured image so that all images conform to the same height. Care was taken so that relevant localization information was not lost during this process.

Figure 4. Immunohistochemical localization of *PGR* at the uterine-placental interface during mid- to late-pregnancy.

PGR localized to stromal cells and myometrium from Days 25 to 85 of pregnancy. Additionally, on Day 25, *PGR* localized to uterine GE, but not in every GE cell. On Day 40, the proportion of GE with detectable *PGR* decreased and this continued through Day 85 of pregnancy. The D50P IgG panel is the negative control. Width of fields is 870 μ m. White lines in panels represent regions of the uterine cross-section that were removed from the captured image so that all images conform to the same height. Legend: LE, luminal epithelium; GE, glandular epithelium; CE, chorion; ST, stroma; MYO, myometrium.

Figure 5. *PGR* mRNA and protein in endometria of P4-treated gilts.

(A) Endometrial expression of *PGR* mRNA was significantly less in the P4-treated than CO-treated gilts ($p < 0.05$). Values are presented as means $\pm$ SEM. (B) In uteri of P4-treated gilts, *PGR* was detected in stromal cells and myometrium, while in the CO-treated gilts, *PGR* was

detected in uterine LE, stromal cells, and myometrium. The IgG negative control panel is presented in Figure 3. Height of fields is 570 μm .

Figure 6. Immunohistochemical localization of ACP5 and SPP1 at the uterine-placental interface during pregnancy.

ACP5 protein was detected in uterine GE on Day 12 of pregnancy and in areolae on Day 25 with expression maintained through Day 85 of pregnancy in both tissues. SPP1 protein localized to uterine LE on Day 25, but was not detected in uterine LE proximal to areolae. SPP1 was detected in uterine GE on Day 35 and expression in LE and GE was maintained through Day 85 of pregnancy. The rIgG negative control panel is presented in Figure 7. Width of fields is 870 μm . Legend: LE, luminal epithelium; GE, glandular epithelium; CE, chorion.

Figure 7. Immunohistochemical localization of PGR, ACP5, and SPP1 in uterine GE proximal to areolae.

PGR is detected in uterine GE which open directly into the areola at Day 40 of pregnancy (*). GE not directly adjacent to the areola do not express PGR. ACP5 and SPP1 are not detected in uterine GE that contain PGR, but both proteins are observed in the lumen of the uterine glands and in GE distant from the areola. The rIgG panel is the negative control. Width of fields is 870 μm (10X) and 230 μm (40X). Values in parentheses refer to objective lens magnification used to capture images. Legend: GE, glandular epithelia.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7