Effects of long-term progesterone exposure on porcine uterine gene expression: progesterone alone does not induce secreted phosphoprotein 1 (osteopontin) in glandular epithelium

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Abstract

Pigs experience significant conceptus loss near mid-gestation, correlating with increasing glandular epithelial (GE) development and secretory activity. Secreted phosphoprotein 1 (SPP1, osteopontin) increases in GE between days 30 and 40 of pregnancy and is expressed in the GE of day 90 pseudopregnant pigs, suggesting that progesterone (P₄) from corpora lutea is responsible for induction of SPP1 in GE. In this study, pigs were ovariectomized and treated daily with P₄ to assess effects of 40 days of P₄ exposure on SPP1, P₄ receptor (PGR), uteroferrin (ACP5), and fibroblast growth factor 7 (FGF7) expression in porcine endometria. PGR mRNA decreased in pigs injected with P₄ compared with pigs injected with corn oil (CO), and PGRs were downregulated in the luminal epithelium (LE) and GE. ACP5 mRNA increased in pigs injected with P₄ compared with pigs injected with CO, and ACP5 was induced in the GE of P₄-treated pigs. FGF7 mRNA increased in pigs injected with P₄ compared with pigs injected with CO, and FGF7 was induced in the LE and GE of P₄-treated pigs. SPP1 mRNA was not different between pigs injected with P₄ compared with pigs injected with CO, and SPP1 was not present in the GE of P₄-treated pigs. Therefore, long-term P₄, in the absence of ovarian and/or conceptus factors, does not induce SPP1 expression in GE. We hypothesize that a servomechanism involving sequential effects of multiple hormones and cytokines, similar to those for sheep and humans, is required for GE differentiation and function, including the synthesis and secretion of SPP1.

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Introduction

Progesterone (P₄) is the hormone of pregnancy in all mammals (Spencer *et al.* 2004). Concentrations of P₄ in peripheral blood peak with maturation of the corpus luteum (CL) around day 15 and are maintained throughout pregnancy by ovarian and placental synthesis and secretion (Spencer *et al.* 2004). P₄ stimulates transformation of the endometrium into a secretory tissue to create an environment permissive to early embryonic development, implantation, placentation, and fetal/placental development (Spencer *et al.* 2004). Importantly, P₄ increases the expression of various uterine secretory proteins that contribute to histotroph, which supports conceptus development in pigs (Knight *et al.* 1974, Roberts & Bazer 1988). The majority of histotroph is thought to be secreted by the endometrial glandular epithelium (GE). Secreted phosphoprotein 1 (SPP1, commonly referred to as osteopontin) is an accepted major constituent of histotroph that is expressed in GE coincident with long-term secretion of P₄ by CLs of pigs (Garlow *et al.* 2002, White *et al.* 2005).

SPP1 is an extracellular matrix/adhesion molecule that is upregulated in the pregnant uterus and has been proposed to contribute to implantation and placentation in several mammalian species (Nomura *et al.* 1988, Johnson *et al.* 1999b, 2003a, 2003b, Apparao *et al.* 2001, 2003, Carson *et al.* 2002, Garlow *et al.* 2002, Kao *et al.* 2002, Girotti & Zingg 2003, Joyce *et al.* 2005a). In pigs, estrogens secreted by the elongating day 12 conceptus induce synthesis and secretion of SPP1 by endometrial luminal epithelium (LE; Garlow *et al.* 2002, White *et al.* 2005). SPP1 then binds the integrin heterodimer $\alpha v \beta 6$ to trophectoderm (Tr) and $\alpha v \beta 3$ to uterine LE to stimulate Tr cell and LE cell adhesion and migration, suggesting roles to promote Tr migration for conceptus elongation and attachment to endometrial LE for implantation in pigs (Erikson *et al.* 2009).

In addition to expression in LE during the peri-implantation period, total endometrial SPP1 mRNA increases 20-fold between days 25 and 85 of pregnancy due to induction of SPP1 expression in the GE, which is delayed until between days 30 and 35 of pregnancy

when placental growth and placentation are key events in pig pregnancy (Garlow *et al.* 2002). SPP1 is also expressed in the GE of day 90 pseudopregnant pigs, suggesting that maintenance of secretion of P₄ by CLs may be responsible for induction of SPP1 in GE (White *et al.* 2005). Indeed, P₄ induces expression of SPP1 in the GE of sheep and rabbits (Johnson *et al.* 2000, Apparao *et al.* 2003) and induces SPP1 synthesis by human Ishikawa cells (Apparao *et al.* 2001). We hypothesized that P₄ induces SPP1 expression in pig GE after day 30 of pregnancy.

However, estrogens, interferons (IFNs), and prolactin (PRL) have the potential to interact with P₄ to influence GE synthesis of SPP1. The first trimester of pregnancy in pigs is complex and incorporates the overlapping events of conceptus elongation, pregnancy recognition signaling, and endometrial remodeling for implantation and placentation. Pregnancy recognition is the result of conceptus secretion of estrogens on days 11 and 12 of pregnancy to redirect PGF secretion from the uterine vasculature to the uterine lumen. In addition to pregnancy recognition, conceptus estrogens modulate uterine gene expression that is essential for conceptus development and implantation (Geisert *et al.* 1982, Ross *et al.* 2007, Johnson *et al.* 2009). Pig Tr also secretes IFNs, both the type I IFN δ and the type II IFN γ during the peri-implantation period (La Bonnardire *et al.* 1991, Lefvre *et al.* 1998, Joyce *et al.* 2007). In contrast to sheep, in which the type I IFN τ is the signal for maternal recognition of pregnancy (Bazer *et al.* 2009), pig conceptus IFNs have yet to be shown to influence pregnancy recognition. However, paracrine effects for IFNs are to increase the expression of several known IFN-responsive genes in the endometrium (Johnson *et al.* 2009). Finally, other hormones, such as PRL, may work in conjunction with P₄ to regulate GE expression of SPP1 (Young *et al.* 1990). Ovariectomized ewes treated with P₄ and placental lactogen exhibit increased GE development and SPP1 mRNA expression over ovariectomized ewes treated with P₄ alone (Spencer *et al.* 1999). Furthermore, both pregnant and pseudopregnant pigs express SPP1 in GE and have similar concentrations of PRL, and endometrial PRL receptors (Kensinger *et al.* 1986, Young *et al.* 1990).

Therefore, in this study, gilts were ovariectomized and treated daily with P₄ for 40 days to assess the effects of P₄ on SPP1 expression in porcine endometrial GE in the absence of ovarian or conceptus factors. The expression of three additional genes was assessed in these tissues. P₄ receptors (PGRs) downregulate in endometrial epithelia in response to P₄ treatment and served as an index of P₄'s ability to negatively regulate GE gene expression in this study (Ka *et al.* 2007). There were no previous reports of PGR localization in the endometrium of day 40 pregnant pigs or in ovariectomized pigs treated long-term with P₄. Acid phosphate 5, tartrate resistant (ACP5, commonly referred

to as uteroferrin), is induced in pig GE during the late luteal phase in response to P₄ treatment and served as an index of P₄'s ability to positively regulate early pregnancy GE gene expression in this study (Chen *et al.* 1973, Knight *et al.* 1973, Schlosnagle *et al.* 1974, Renegar *et al.* 1982). Fibroblast growth factor 7 (FGF7, commonly referred to as keratinocyte growth factor) is similar to SPP1 in that it is induced in the GE of pigs between days 30 and 40 of pregnancy, and served as an index of P₄'s ability to positively regulate GE gene expression beyond the peri-implantation period (Ka *et al.* 2000). The hormonal regulation of FGF7 expression in pig GE was previously unknown.

Results

Figure 1 illustrates effects of long-term P₄ exposure to increase hypertrophy and hyperplasia of the uterine wall of ovariectomized pigs as compared with treatment with corn oil (CO).

Long-term P₄ supports downregulation of PGR in endometrial LE and GE

Slot blot hybridization revealed that steady-state levels of endometrial PGR mRNA decreased in pigs injected with P₄ compared with pigs injected with CO ($P < 0.01$; Fig. 2A). Results of *in situ* hybridization analysis revealed that PGR mRNA was expressed in the LE, GE, and stroma of CO-treated pigs, but expression was limited to the stromal cells of P₄-treated pigs (Fig. 2B). Therefore, an effect of long-term P₄ was the expected downregulation of PGR in endometrial LE and GE.

Long-term P₄ supports induction of ACP5 in endometrial GE

Slot blot hybridization revealed that steady-state levels of endometrial ACP5 mRNA increased in pigs injected with P₄ compared with pigs injected with CO ($P < 0.01$; Fig. 3A). ACP5 mRNA and protein were localized in the endometrial GE of P₄- but not CO-treated pigs (Fig. 3B and C). Immunoreactive proteins of ~37–38 kDa were detected in the uterine flushings of P₄-treated pigs (Fig. 3D). Therefore, a second effect of long-term P₄ was the expected induction of ACP5 synthesis and secretion from endometrial GE.

Long-term P₄ supports induction of FGF7 in endometrial LE and GE

Slot blot hybridization revealed that steady-state levels of endometrial FGF7 mRNA increased in pigs injected with P₄ compared with pigs injected with CO ($P < 0.01$; Fig. 4A). FGF7 mRNA was localized in both the endometrial LE and GE of P₄- but not CO-treated pigs (Fig. 4B).

Figure 1 Gross histological effects of progesterone on pig uteri. Representative uterine cross sections from corn oil (CO)- and progesterone (P_4)-treated gilts stained with Mayer hematoxylin and eosin. Note that on qualitative observation, it is evident that there is an increased area of the lumen as well as thickness of the endometrium (End) and myometrium (Myo) in response to long-term treatment with progesterone. Histotroph is also seen in the uterine lumen of pigs after long-term treatment with progesterone.

Therefore, additional effect of long-term P_4 was the expected induction of *FGF7* expression in endometrial LE, and the hypothesized induction of *FGF7* in GE by P_4 .

Long-term P_4 does not support induction of SPP1 in endometrial GE

Slot blot hybridization revealed that steady-state levels of endometrial *SPP1* mRNA were not different between pigs injected with P_4 compared with pigs injected with CO ($P > 0.01$; Fig. 5A). *In situ* hybridization indicated that *SPP1* mRNA was not present in endometrial GE of either P_4 - or CO-treated pigs (Fig. 5B). In agreement with mRNA data, little to no immunoreactive SPP1 protein was detected in uterine flushings from P_4 -treated pigs (Fig. 5C). Therefore, contrary to our hypothesis, long-term P_4 , in the absence of ovarian and/or conceptus factors, did not affect SPP1 expression in GE.

Discussion

It is generally accepted that endometrial LE and GE synthesize and secrete histotroph that facilitates conceptus elongation, implantation, placentation, and fetal growth and development. We examined the effects of daily exposure to P_4 for 40 days on the expression of four epithelial genes in ovariectomized pigs. In this study, long-term P_4 , in the absence of conceptus or ovarian factors, 1) was sufficient to support initial gene regulatory events that occur during the late luteal phase and the peri-implantation period of intact cyclic and pregnant pigs including downregulation of PGR in LE and GE, and induction of ACP5 in GE (Chen *et al.* 1973, Schlosnagle *et al.* 1974, Geisert *et al.* 1994); but 2) disregulated some delayed gene expression that occurs after day 30 of pregnancy including induction of SPP1 in GE (Garlow *et al.* 2002, White *et al.* 2005). Notably, *FGF7* has a similar pattern of GE expression to SPP1 during pig pregnancy, but diverged from SPP1 in its endocrine regulation in that it was induced in GE by long-term P_4 (Ka *et al.* 2000). Collectively, these results suggest that the hormonal milieu necessary for the production of histotroph varies for its individual components, and that genes that delay expression in GE until after the peri-implantation period may require specific servomechanisms of sequential exposure of the pregnant endometrium to ovarian, conceptus/placental, and/or uterine factors that may include P_4 , estrogens, IFNs, and PRL (Young *et al.* 1990, Spencer *et al.* 2004, Johnson *et al.* 2009).

In several species, downregulation of PGR in uterine GE is required for induction of secretory protein gene expression by P_4 in the same epithelia (Bazer *et al.* 2009). This also occurs in pigs. Initial temporal and spatial localization of PGR in pig endometria established that PGR decreases in LE and GE during the luteal phase (Geisert *et al.* 1994); subsequent ovariectomy and hormone replacement experiments also confirmed that P_4 downregulates PGR by day 15 (Ka *et al.* 2007). Temporal and spatial expression of PGR in pig endometrium beyond day 20 of pregnancy has not been reported. Results of this study confirm that long-term P_4 exposure continues to prevent PGR expression in LE and GE, which may allow for continued and increased expression of genes for secretory proteins such as ACP5 in GE (Schlosnagle *et al.* 1974, Renegar *et al.* 1982).

ACP5 is an established P_4 -induced secretory product of pig GE. In pigs, ACP5 increases in uterine secretions between days 12 and 16 in response to luteal phase levels of P_4 , and is produced in large amounts at mid-pregnancy when secretion can exceed 1 g/day. Endometrial secretion of ACP5 can be maintained in ovariectomized, P_4 -treated (daily) pigs for 60 days (Chen *et al.* 1973, Schlosnagle *et al.* 1974, Renegar *et al.* 1982). As a basic glycoprotein contains one molecule of iron and possesses tartrate-resistant acid phosphatase activity

Figure 2 Long-term progesterone supports downregulation of *PGR* in endometrial LE and GE. (A) Steady-state levels of mRNA for *PGR* in endometria of pigs treated with corn oil (CO) and progesterone (P₄). The mRNA levels, expressed as least square means of relative units of counts per minute with overall S.E.M., are normalized for differences in sample loading using 18S rRNA and represent 20 µg of total endometrial mRNA per sample. **P* < 0.05. (B) *In situ* hybridization analysis of *PGR* mRNA in cross sections of pig endometria. Corresponding bright-field and dark-field images from CO- and P₄-treated pigs are shown. A representative endometrial section from a P₄-treated pig hybridized with radiolabeled sense cRNA probe (Sense) served as a negative control. Width of each field is 940 µm. LE, luminal epithelium; GE, glandular epithelium; ST, stratum compactum stroma.

ACP5 functions in iron transport from mother to fetal-placental unit and has hematopoietic growth factor activity (Chen *et al.* 1973, Roberts & Bazer 1980, Renegar *et al.* 1982, Bazer *et al.* 1991). Results of this study confirm that long-term P₄ exposure alone can maintain high levels of ACP5 synthesis and secretion from GE throughout the first trimester of pig pregnancy.

The pig endometrium is unique in that *FGF7* is expressed by the LE and GE rather than by cells of mesenchymal (stromal) origin, as observed in endometria of other species (Cooke *et al.* 1998, Ka *et al.* 2000). *FGF7* is expressed in the endometrial epithelia of pigs; its receptor, *FGFR2*, is present in both the endometrial epithelia and conceptus Tr, and *FGF7* stimulates the Tr, but not endometrial epithelial cells, to undergo proliferation and differentiation. Therefore, *FGF7* is proposed to be a paracrine mediator of interactions between the uterus and the conceptus (Ka *et al.* 2000, 2001, Ka & Bazer 2005). *FGF7* mRNA is induced in LE between days 12 and 15 of the estrous cycle and pregnancy, but GE expression is delayed until days 30 and 40 of pregnancy (Ka *et al.* 2000). The induction and expression of *FGF7* in pig LE appears to be very complex. Ka *et al.* (2007) reported that *FGF7* is similar to ACP5 in that its induction requires that P₄ downregulates *PGR*, thereby eliminating *PGR*-dependent inhibition of expression of most P₄-regulated genes. They further postulated that endocrine effects of P₄ in inducing the expression of *FGF7* in endometrial LE were mediated by a paracrine-acting factor(s) (progestamedin) produced by the *PGR*-positive stromal cells. Finally, they showed that estrogen produced by pig conceptuses binds to *ESR1* in the LE to induce the maximal levels of *FGF7* expression observed on day 12 of pregnancy (Ka *et al.* 2007). The hormonal regulation of *FGF7* in pig GE had not been reported. Results of this study are novel in that long-term P₄ exposure, in the absence of estrogens, induced very high expression of *FGF7* in LE. This high expression contrasts with pregnancy, in which LE expression of *FGF7* is minimal after day 30 (Ka *et al.* 2000). Clearly, an unidentified ovarian or placental factor(s) and/or physical cellular responses to attachment with Tr for placentation are involved in reducing *FGF7* expression in LE. Long-term P₄ alone supported induction of *FGF7* in GE. It remains to be determined what physiological, cellular, and molecular interactions between cell types within the uterine endometrium are responsible for the delay in *FGF7* production by GE until after day 30 of pregnancy in the pig.

A cursory inspection of the temporal and spatial expression of *FGF7* and *SPP1* in the uteri of pigs led to the assumption that these two genes were regulated similarly during pregnancy. Both genes increase in the LE during the peri-implantation period, and then in the GE after day 30 of pregnancy (Ka *et al.* 2000, Garlow *et al.* 2002). However, previous reports by White *et al.* (2005) and Ka *et al.* (2007) showed that LE expression of these two genes is different. While *FGF7* requires the permissive actions of P₄ for induction, and estrogen to increase expression to maximal levels, *SPP1* is induced by conceptus estrogens in LE directly adjacent to the implanting conceptus where it potentially binds αvβ3 integrin at the apical surface of LE and αvβ6 on Tr to mediate attachment for implantation in pigs

Figure 3 Long-term progesterone supports induction of ACP5 in endometrial GE. (A) Steady-state levels of mRNA for ACP5 in endometria of pigs treated with corn oil (CO) and progesterone (P₄). The mRNA levels, expressed as least square means of relative units of counts per minute with overall S.E.M., are normalized for differences in sample loading using 18S rRNA and represent 20 µg of total endometrial mRNA per sample. **P* < 0.05. (B) *In situ* hybridization analysis of ACP5 mRNA in cross sections of pig endometria. Corresponding bright-field and dark-field images from CO- and P₄-treated pigs are shown. A representative endometrial section from a P₄-treated pig hybridized with radiolabeled sense cRNA probe (Sense) served as a negative control. (C) Immunohistochemical analysis of ACP5 in cross sections of pig endometria. Immunoreactive ACP5 protein was detected in the GE of P₄-treated pigs, but not in the GE of CO-treated pigs. Immunostaining in the GE of a day 40 pregnant pig serves as a reference for normal pregnancy expression of ACP5. Normal rabbit IgG was substituted for primary antibody as the control. Sections were not counterstained. Width of each field is 940 µm. LE, luminal epithelium; GE, glandular epithelium. (D) Western blot analysis (under reducing conditions) of uterine secretions from CO- and P₄-treated pigs. Normal rabbit IgG was substituted for primary antibody as the control. Two isoforms of ACP5 protein of ~37 and 38 kDa were detected in uterine secretions from P₄-treated pigs only.

(White *et al.* 2005, Ka *et al.* 2007, Erikson *et al.* 2009). It was therefore expected that long-term P₄ exposure, in the absence of estrogens, would not induce *SPP1* expression in LE, and this was true in this study. *SPP1* is also expressed in GE after day 30 of pregnancy (Johnson *et al.* 1999b). Direct hormonal regulation of *SPP1* in pig GE had not been reported; however, day 90 pseudopregnant pigs express *SPP1* in GE (White *et al.* 2005). The most intriguing results of this study are that long-term P₄ did not induce GE expression of *SPP1*. Indeed, the divergence in regulation of *SPP1* and *FGF7* by P₄ is surprising, and suggests that gene regulation in GE after day 30 of pregnancy is complex and gene specific. At the time that *SPP1* begins to be expressed in GE, the endometrial glands are undergoing extensive hyperplasia and hypertrophy. The GE remain simple, coiled, and tubular until day 30 of pregnancy, but shows numerous characteristics of high secretory activity by day 35. By mid-pregnancy, the lumens of uterine glands are greatly enlarged and filled with secretory products (Perry & Crombie 1982, Sinowatz & Friess 1983). During early pregnancy, the pig uterus secretes only moderate levels

of histotroph, but total uterine secretions increase dramatically after day 30 of pregnancy (Knight *et al.* 1974, Schlosnagle *et al.* 1974, Basha *et al.* 1980). Although progress has been made toward deciphering the mechanism(s) through which P₄ influences endometrial GE development in other species, these processes are poorly understood in pigs. In sheep, it is known that upregulation of *SPP1* in uterine GE coincides with the P₄-induced downregulation of *PGR* after days 11 and 12 of pregnancy (Johnson *et al.* 2000). Furthermore, maximal *SPP1* mRNA expression in GE is attained after sequential exposure to IFN τ , P₄, and placental lactogen in what has been termed a servomechanism (Spencer *et al.* 1999). In pigs, however, induction of *SPP1* in GE is delayed over 20 days after the initial downregulation of *PGR*, and a servomechanism similar to that for sheep and humans has not been defined (Spencer *et al.* 1999, Garlow *et al.* 2002, Burton *et al.* 2007).

Results of this study, and those of a previous study that examined *SPP1* expression in pseudopregnant pigs (White *et al.* 2005), are the first to clearly indicate that GE expression of *SPP1* in pigs may be the result of an

Figure 4 Long-term progesterone supports induction of *FGF7* in endometrial LE and GE. (A) Steady-state levels of mRNA for *FGF7* in endometria of pigs treated with corn oil (CO) and progesterone (P_4). The mRNA levels, expressed as least square means of relative units of counts per minute with overall S.E.M., are normalized for differences in sample loading using 18S rRNA and represent 20 μ g of total endometrial mRNA per sample. $*P < 0.05$. (B) *In situ* hybridization analysis of *FGF7* mRNA in cross sections of pig endometria. Corresponding bright-field and dark-field images from CO- and P_4 -treated pigs are shown. A representative endometrial section from a P_4 -treated pig hybridized with radiolabeled sense cRNA probe (Sense) served as a negative control. Width of each field is 940 μ m. LE, luminal epithelium; GE, glandular epithelium.

undefined hormonal servomechanism, and allow us to begin to predict the identity of some of the factors involved in this servomechanism for GE function in pigs. White *et al.* reported that *SPP1* is present in the GE of day 90 pseudopregnant pigs, generated by the injection of

estradiol benzoate on days 11–15 of the estrous cycle. From these data, it was reasonably concluded that maintenance of CL P_4 secretion was, at least in part, responsible for induction of *SPP1* in GE (White *et al.* 2005). However, results presented in this study clearly show that P_4 alone did not induce *SPP1* expression in pseudopregnant pig GE, and suggest that the treatment of pigs with estrogen (days 11–15) may be necessary for the later (days 30–40) expression of *SPP1*. Furthermore, because pseudopregnant pigs have intact ovaries, these ovaries might provide additional estrogens that modulate GE gene expression, and other unidentified factors that may stimulate PRL release for binding to PRL receptors in pig endometrium (Knight *et al.* 1973, Young *et al.* 1990). Therefore, *SPP1* induction in GE may be the result of complex temporal interactions between effects of conceptus estrogens, unidentified ovarian factors, and CL P_4 on the pig endometrium.

Pigs experience two periods of significant loss of conceptuses. The first occurs during the attachment phase of the peri-implantation period and involves 30% of conceptuses. The second involves 10–15% of the remaining conceptuses and occurs during early to mid-gestation (Pope 1994). This second period of conceptus loss correlates with increasing GE development, endometrial secretory activity, placental growth and differentiation, and fetal growth (Knight *et al.* 1974, 1977, Perry & Crombie 1982, Sinowatz & Friess 1983, Dwyer *et al.* 1994). Much is not understood about the mechanisms that underlie these waves of conceptus loss. *SPP1* in the pig offers an excellent model to study these mechanisms because of its regulated and prominent LE and GE expression during these two critical periods in a species that depends significantly on histotroph throughout pregnancy (Garlow *et al.* 2002, White *et al.* 2005, Erikson *et al.* 2009).

Materials and Methods

Animals, experimental design, and tissue collection

Sexually mature gilts of similar age, weight, and genetic background were observed daily for estrus (day 0) and exhibited at least two estrous cycles of normal duration (18–21 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 long-term P_4 treatment without effects of ovarian or conceptus factors on uterine epithelia, gilts were ovariectomized on day 12 of the estrous cycle and assigned randomly to receive daily injections (i.m.) of either 4 ml CO or 200 mg P_4 (200 mg in 4 ml CO) on days 12 through 39 post estrus ($n=4$ /treatment). All gilts were hysterectomized on day 40 post estrus. Uterine flushings were obtained by introducing and recovering 40 ml sterile Hanks balanced salt solution (Sigma Chemical Co.) from

Figure 5 Long-term progesterone does not support induction of *SPP1* in endometrial GE. (A) Steady-state levels of mRNA for *SPP1* in endometrium of pigs treated with corn oil (CO) and progesterone (P₄). The mRNA levels, expressed as least square means of relative units of counts per minute with overall s.e.m., are normalized for differences in sample loading using 18S rRNA and represent 20 µg of total endometrial mRNA per sample. (B) *In situ* hybridization analysis of *SPP1* mRNA in cross sections of pig endometria. Corresponding bright-field and dark-field images from CO- and P₄-treated pigs are shown. Sections from day 40 of pregnancy (D40P) are shown as examples of *SPP1* expression in GE. Representative endometrial sections from a P₄-treated and a D40P pig hybridized with radiolabeled sense cRNA probe (Sense) served as negative controls. Width of each field is 940 µm. GE, glandular epithelium. (C) Western blot analysis (under reducing conditions) of *SPP1* in uterine secretions from CO- and P₄-treated pigs. Immunoreactive proteins were detected using either polyclonal rabbit anti-hSPP1 (LF-123 and LF-124; Fisher *et al.* 1995) or rabbit IgG (rIgG) as a control (see Fig. 3D). Three forms of *SPP1* of ~25, 45, and 70 kDa were detected at either very low levels or were not detected in uterine secretions from both CO- and P₄-treated pigs.

the uterine lumen before hysterectomy. The uterine flushings were cleared of cellular debris by centrifugation (3000 *g* for 10 min at 4 °C) and frozen at –80 °C until analyzed. To confirm the effects of pregnancy and compare these with the effects of long-term P₄ treatment on expression of *ACP5* and *SPP1*, four gilts were bred when detected in estrus and 12 and 24 h thereafter. Gilts were then ovariectomized on

day 40 of pregnancy. From all gilts, several sections (thickness ~1–1.5 cm) from the middle of each uterine horn were placed in fresh 4% paraformaldehyde fixative for 24 h and then embedded in Paraplast Plus (Oxford Labware, St Louis, MO, USA). A sample of endometrium from each gilt was dissected from myometrium, frozen in liquid nitrogen, and stored at –80 °C for RNA and protein extraction.

Histology

Embedded tissues were sectioned (5 µm), deparaffinized, and stained with Mayer hematoxylin and eosin for general histomorphological evaluation.

Slot blot hybridization

Steady-state levels of mRNA in porcine endometria were assessed by slot blot hybridization as described previously (Johnson *et al.* 1999a). Radiolabeled antisense cRNA probes were generated by *in vitro* transcription using linearized plasmid template, RNA polymerases, and [α - 32 P]-UTP. Plasmids included a partial-length ovine *PGR* cDNA (Spencer *et al.* 1995), a partial-length porcine *FGF7* cDNA (Ka *et al.* 2000), and a full-length porcine *SPP1* cDNA (Wrana *et al.* 1989). A partial cDNA for porcine *ACP5* mRNA was amplified by RT-PCR using total RNA from day 40 pregnant porcine endometrial tissues using the specific; forward, 5'-ccagaaccaacaccaggact-3'; reverse, 5'-ggtagtaagggtgggaag-3'. A partial porcine *ACP5* cDNA was cloned into pCRII using a T/A Cloning kit (Invitrogen) and the sequence verified using an ABI PRISM Dye Terminator Cycle Sequencing kit and ABI PRISM automated DNA sequencer (Perkin-Elmer Applied Biosystems, Waltham, MA, USA). Denatured total endometrial RNA (20 µg) from each gilt was hybridized with radiolabeled cRNA probes. To correct for variation in total RNA loading, a duplicate RNA slot membrane was hybridized with radiolabeled antisense 18S rRNA (pT718S; Ambion, Austin, TX, USA). After washing, the blots were digested with RNase A and radioactivity associated with slots quantified using a Typhoon 8600 Multilmager (Molecular Dynamics, Piscataway, NJ, USA). The mRNA levels were expressed as least square means of relative units (RU) of counts per minute with overall S.E.M., and differences in sample loading were normalized using 18S rRNA.

In situ hybridization

Cell-specific expression of mRNAs in sections (5 µm) of the porcine uterine endometria (tissue from three paraffin blocks) was determined by radioactive *in situ* hybridization analysis as described previously (Johnson *et al.* 1999a). Sections from three independent paraffin blocks from each animal were analyzed. Radiolabeled antisense or sense cRNA probes were generated by *in vitro* transcription using linearized plasmid template, RNA polymerases, and [α - 35 S]-UTP. Deparaffinized, rehydrated, and deproteinized uterine tissue sections were hybridized with radiolabeled antisense or sense cRNA probes. After hybridization, washing, and RNase A digestion, slides were dipped in NTB-2 liquid photographic emulsion (Kodak) and exposed at 4 °C for 7–10 days. Slides were developed in Kodak D-19 developer, counterstained with Gill's hematoxylin (Fisher Scientific, Fairlawn, NJ, USA), and then dehydrated through a graded series of alcohol to xylene. Coverslips were then affixed with Permount (Fisher Scientific). Digital images of representative fields were recorded under bright-field or dark-field illumination and evaluated using an Axioplan 2 microscope (Carl Zeiss, Thornwood, NY, USA) 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.).

Western blot analyses

Protein concentrations in uterine flushings were determined using the Bradford protein assay (Bio-Rad) with BSA as the standard. Proteins were denatured and separated by 12% SDS-PAGE, and western blot analysis was performed as described previously (Johnson *et al.* 1999b) using ECL detection (SuperSignal West Pico, Pierce, Rockford, IL, USA) and X-OMAT AR X-ray film (Kodak). Immunoreactive ACP5 protein was detected using affinity purified rabbit anti-ACP5 (2.5 µg/ml; Ellenberger *et al.* 2008) and SPP1 protein was detected using a cocktail containing rabbit polyclonal antibodies against recombinant human SPP1 (anti-hSPP1; LF-123 and LF-124; 5 µg/ml (Fisher *et al.* 1995). Rabbit IgG (5 µg/ml; Sigma) was used as the primary antibody for controls. Multiple exposures of each western blot were performed to ensure linearity of chemiluminescent signals. Western blots were quantified by measuring the intensity of light emitted from correctly sized bands under u.v. light using a ChemiDoc EQ system and Quantity One software (Bio-Rad).

Immunohistochemical analysis

Immunocytochemical localization of immunoreactive ACP5 in the porcine uterus was performed as described previously (Joyce *et al.* 2005b) in uterine tissue cross sections using rabbit anti-porcine ACP5 (4.0 µg/ml; Ellenberger *et al.* 2008). Antigen retrieval for ACP5 was performed by boiling citrate buffer. Negative controls included substitution of the primary antibody with nonimmune rabbit IgG (Sigma Chemical Co.) at the same final concentration.

Statistical analysis

Data from slot blot hybridization analysis were subjected to least squares ANOVA using the general linear models procedures of the statistical analysis system and were corrected for differences in sample loading using the 18S rRNA data as a covariate. In all analyses, error terms used in tests of significance were identified according to the expectation of the mean squares for error. Slot blot data are presented as least squared means with overall S.E.M. in RU.

Declaration of interest

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

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