REPRODUCTION
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Transient estrogen exposure from birth affects uterine expression of developmental markers in neonatal gilts with lasting consequences in pregnant adults

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Abstract

Disruption of estrogen-sensitive, estrogen receptor (ER)-dependent events during porcine uterine development between birth (postnatal day = PND 0) and PND 14 affects patterns of uterine morphoregulatory gene expression in the neonate with lasting consequences for reproductive success. Uterine capacity for conceptus support is reduced in pregnant adult gilts exposed to estradiol valerate (EV) for 14 days from birth. Objectives here were to determine effects of EV exposure from birth through PND 13 on neonatal uterine and adult endometrial markers of growth, patterning, and remodeling. Targets included the relaxin receptor (RXFP1), estrogen receptor-α (ESR1) and vascular endothelial growth factor (VEGFA), morphoregulatory markers HOXA10 and WNT7A, and the matrix metalloproteinases (MMP)2 and MMP9. Gilts were treated daily with EV (50 μg/kg body weight per day, i.m.) or corn oil vehicle from birth through PND 13. Uteri were obtained from neonates on PND 14 and from adults on pregnancy day 12 (PxD 12). In neonates, EV exposure from birth increased uterine *RXFP1* gene expression, and both ESR1 and VEGFA proteins. At PxD 12, endometrial *RXFP1* mRNA remained elevated, while ESR1 protein was reduced. Early EV treatment decreased neonatal uterine *WNT7A*, but increased *HOXA10* expression. *WNT7A* expression was reduced in EV-treated adults. Transient EV exposure increased *MMP9* transcripts at PND 14, whereas both latent and active MMP9 activity was increased due to early EV treatment in adults on PxD 12. Results support the hypothesis that transient, estrogen-induced disruption of porcine uterine development from birth alters early programming events that lead to functional consequences in the adult.

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Introduction

Development of the uterus involves a series of morphogenetic and cytodifferentiative events that establish the framework for tissue function in adulthood. In the pig (*Sus scrofa domesticus*), uterine glands are absent at birth (postnatal day (PND) 0) and the uterus is estrogen receptor-α (ESR1) negative (Tarleton *et al*. 1998, Yan *et al*. 2006*a*). However, during the first 2 weeks of life, uterine glands differentiate and ESR1 expression is evident in both nascent glandular epithelium and endometrial stroma (Tarleton *et al*. 1999). These events are ovary independent and estrogen-sensitive (Bartol *et al*. 1993). Disruption of estrogen-sensitive uterine developmental events by exposure of neonatal gilts to estrogen from birth can have both short- and long-term consequences.

Administration of estrogens to neonatal gilts from birth affects uterine growth and endometrial development acutely at both structural and biochemical levels. Short-term exposure to estrogens from birth showed that uterine expression of the relaxin receptor (RXFP1), ESR1, and vascular endothelial growth factor (VEGFA) increased as early as PND 2 (Yan *et al*. 2008). Treatment with estradiol valerate (EV) for 2 weeks from birth increased uterine wet weight and advanced endometrial development by PND 14 as reflected by increased glandularity and premature development of endometrial folds (Tarleton *et al*. 1999). These effects were associated with changes in endometrial expression patterns for morphoregulatory genes in the *WNT/HOXA* family (Bartol *et al*. 2006).

In contrast, exposure of neonatal gilts to EV for 2 weeks from birth had anti-uterotropic effects in pregnant adult gilts. Neonatally estrogen-exposed adult gilts had smaller uteri at day 12 post estrus/mating. This effect was most pronounced in pregnant, as compared to cyclic, animals (Tarleton *et al*. 2003). Consistently, adult pregnant gilts that were exposed to EV neonatally

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displayed reduced uterine capacity on pregnancy day 45 (PxD 45) as reflected by reduced embryo survival (Bartol *et al*. 1993). Furthermore, early estrogen exposure did not affect ovulation rate or prevent conception, suggesting that the reduction in uterine capacity was the result of direct EV effects on neonatal uterine programming (Bartol *et al*. 1993, Tarleton *et al*. 2003).

Given that estrogen-induced disruption of neonatal uterine development has long-term consequences for uterine function and reproductive performance in the pig, it is reasonable to expect that associated changes in uterine expression of growth, patterning, and remodeling genes during neonatal life could mark and even mediate important developmental programming events. In this regard, both RXFP1 and ESR1 signaling systems have been implicated in an estrogen-sensitive, feed-forward system regulating uterine growth and endometrial development in the neonatal pig (Bartol *et al*. 2009). In addition, endometrial expression of patterning genes including *WNT7A* and *HOXA10* is recognized to be developmentally regulated and estrogen sensitive (Bartol *et al*. 2006). Tissue remodeling matrix metalloproteinases (MMPs), specifically the gelatinases MMP2 and MMP9, have yet to be evaluated in terms of their potential involvement in neonatal porcine uterine developmental programming. However, it is known that these enzymes are responsible for the coordinated breakdown of the extracellular matrix (ECM) and basement membrane remodeling important for tissue expansion and that these MMPs increase during relaxin-induced uterine growth in prepubertal gilts (Lenhart *et al*. 2001).

In studies by Tarleton *et al*. (2003), EV was used as a tool with which to disrupt estrogen-sensitive developmental events in the neonate in order to identify long-term outcomes of such developmental disruption on uterine morphology, biochemistry, and functionality in cyclic adult and early pregnant gilts, in which effects were most pronounced. Objectives of the current study were to extend these observations by determining: 1) short-term effects of EV, administered daily from birth through PND 13, on molecular markers of uterine growth, patterning, and remodeling at PND 14; and 2) long-term effects of this neonatal estrogen exposure strategy on these markers of endometrial development in adults at PxD 12.

Results

Neonatal uterine and adult endometrial mRNA expression

Data for the relative expression of uterine developmental genes in the neonate at PND 14 and at PxD 12 as a result of neonatal EV treatment are presented in Figs 1 and 2 respectively. Neonatal exposure to EV for 2 weeks from birth increased ($P<0.05$) uterine wet

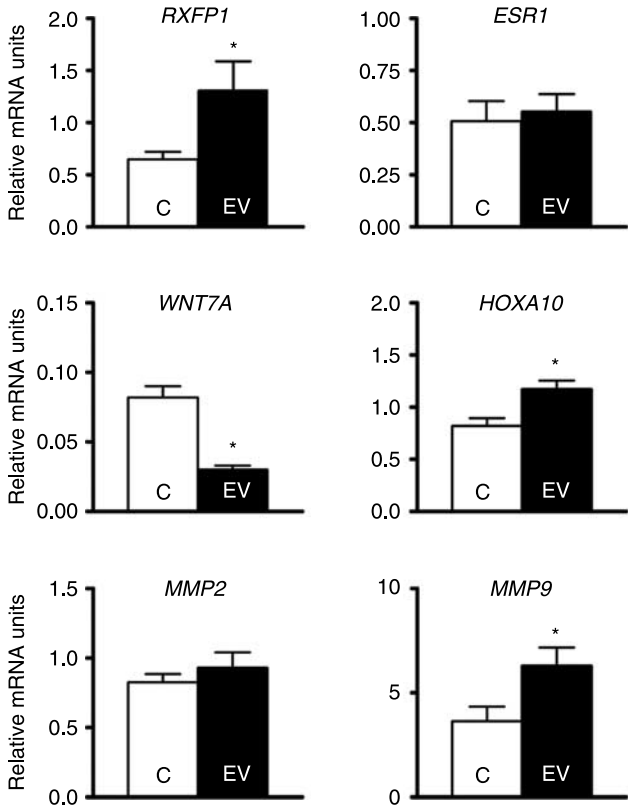

Figure 1 Effects of EV, administered from PND 0 to PND 13, on porcine uterine gene expression at PND 14. Transcripts for *RXFP1*, *ESR1*, *WNT7A*, *HOXA10*, *MMP2*, and *MMP9* were quantified by qPCR and normalized to the expression of the housekeeping gene *PPIA*. Data are expressed as LSM±S.E.M. $N=4$–6 animals per group. *$P<0.05$.

weight (EV: 1.40±0.16 g) compared to controls (C: 0.53±0.07 g) on PND 14, similar to previously reported data (Tarleton *et al*. 1999). Two weeks of EV treatment from birth decreased ($P<0.05$) porcine uterine *WNT7A* mRNA and increased ($P<0.05$) *RXFP1*, *HOXA10*, and *MMP9* transcripts at PND 14. However, there were no EV-induced changes in uterine *ESR1* or *MMP2* gene expression at PND 14. In adults at PxD 12, transient neonatal EV exposure decreased ($P<0.05$) endometrial *WNT7A* mRNA and increased ($P<0.05$) endometrial *RXFP1* mRNA. Contrary to uterine PND 14 results, at PxD 12 there was a decrease ($P<0.05$) in endometrial *MMP9* transcripts in gilts transiently exposed to EV for 2 weeks from birth. There were no EV-induced changes in endometrial *ESR1* or *HOXA10* gene expression when measured at PxD 12.

Neonatal uterine and adult endometrial ESR1 and VEGFA protein expression

Results of immunoblot analysis of neonatal uterine and adult endometrial proteins are shown in Figs 3 and 4 respectively. EV treatment of neonatal gilts during the first 2 weeks of life increased ($P<0.05$) the relative

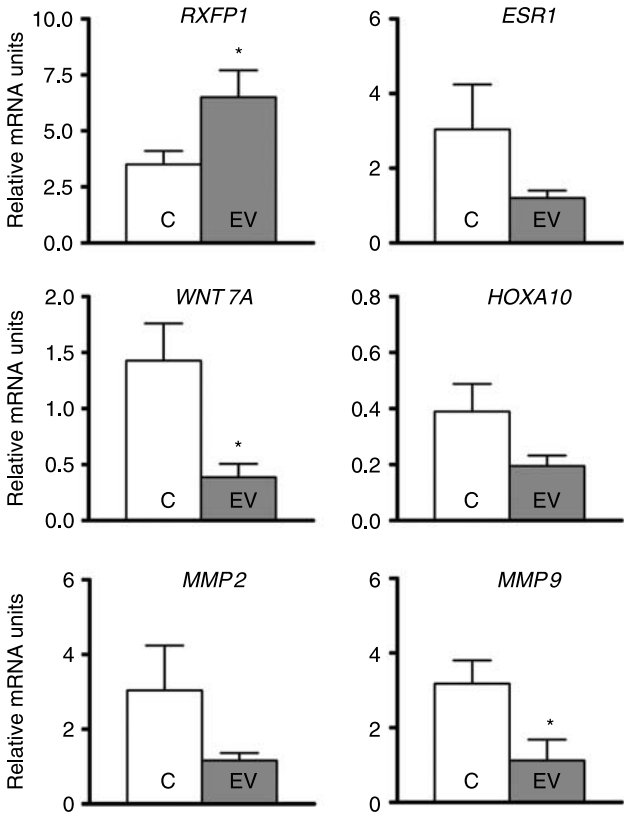

Figure 2 Effects of EV, administered from PND 0 to PND 13, on porcine endometrial levels of mRNA at PxD 12. Transcripts for *RXFP1*, *ESR1*, *WNT7A*, *HOXA10*, *MMP2*, and *MMP9* were determined by qPCR and normalized to the expression of the housekeeping gene *PPIA*. Data are expressed as LSM±S.E.M. $N=4$–6 animals per group. *$P<0.05$.

abundance of a single 51 kDa uterine protein band, corresponding to ESR1 (Fig. 3A), and a 46 kDa band, corresponding to dimeric VEGFA protein (Fig. 3B) at PND 14. At PxD 12, transient exposure to EV for 2 weeks from birth decreased the relative abundance of endometrial ESR1 protein (Fig. 4A; $P<0.05$); however, there were no changes in VEGFA protein levels (Fig. 4B).

Neonatal uterine and adult endometrial MMP2 and MMP9 gelatinase activity

Gelatin zymography was used to identify the type and measure the abundance of gelatinases in neonatal uterine and adult endometrial tissues. Representative zymograms illustrating the gelatinolytic activity of neonatal uterine and adult endometrial protein extracts are shown (Fig. 5). Zymographic analysis of PND 14 uterine proteins revealed the presence of lysis bands at 72 and 66 kDa (Fig. 5A), the reported sizes of pro-MMP2 and MMP2 respectively (Crabbe *et al.* 1993). However, densitometric analysis of these uterine MMP2 lysis bands indicated no effect of EV treatment for 2 weeks from birth in comparison to controls (data not shown). Zymographic analysis of PxD 12 adult endometrial proteins

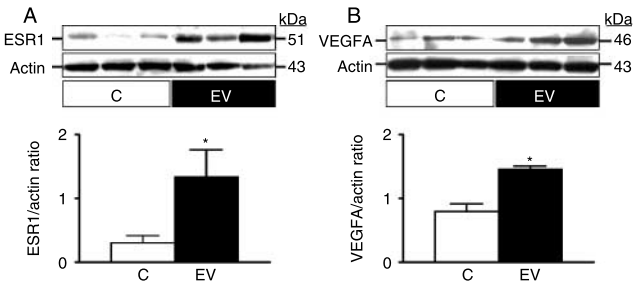

Figure 3 Effects of EV, administered from PND 0 to PND 13, on porcine uterine ESR1 (A) and VEGFA (B) proteins at PND 14. Representative immunoblots for each treatment group are shown. Both 51 and 46 kDa immunoreactive bands for ESR1 and VEGFA respectively are indicated (A and B). An immunoreactive band for actin was detected at 43 kDa and included as a loading reference. Densitometric data for the relative expression of ESR1 and VEGFA are expressed as LSM±S.E.M. $N=4$–6 animals per group. *$P<0.05$.

revealed the presence of lysis bands at 92 and 84 kDa (Fig. 5B), the reported sizes of pro-MMP9 and MMP9 respectively (O'Connell *et al.* 1994). In addition, bands for pro-MMP2 and MMP2 were also detected (Fig. 5B). Densitometric analyses illustrate that neonatal EV treatment resulted in a decline in both endometrial pro-MMP9 and MMP9 gelatinase activity at PxD 12 (Fig. 5C; $P<0.05$). There were no differences in endometrial pro-MMP2 or MMP2 gelatinase activity at PxD 12 as a result of transient EV exposure for 2 weeks from birth.

Discussion

Studies in the pig show that there are dynamic changes in the morphology and molecular profile of the porcine uterus during the first 2 weeks of life (Bartol *et al.* 2006), and that exposure to estrogens during this period can compromise adult uterine function (Tarleton *et al.* 2003). Although developmentally disruptive effects of estrogen

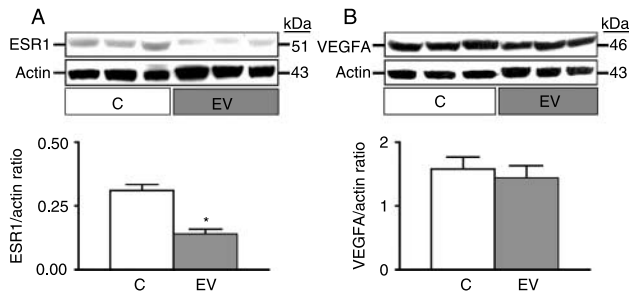

Figure 4 Effects of EV, administered from PND 0 to PND 13, on porcine uterine ESR1 (A) and VEGFA (B) proteins at PxD 12. Representative immunoblots for each treatment group are shown. Both 51 and 46 kDa immunoreactive bands for ESR1 and VEGFA respectively are indicated (A and B). An immunoreactive band for actin was detected at 43 kDa and included as a loading reference. Densitometric data for the relative expression of ESR1 and VEGFA are expressed as LSM±S.E.M. $N=4$–6 animals per group. *$P<0.05$.

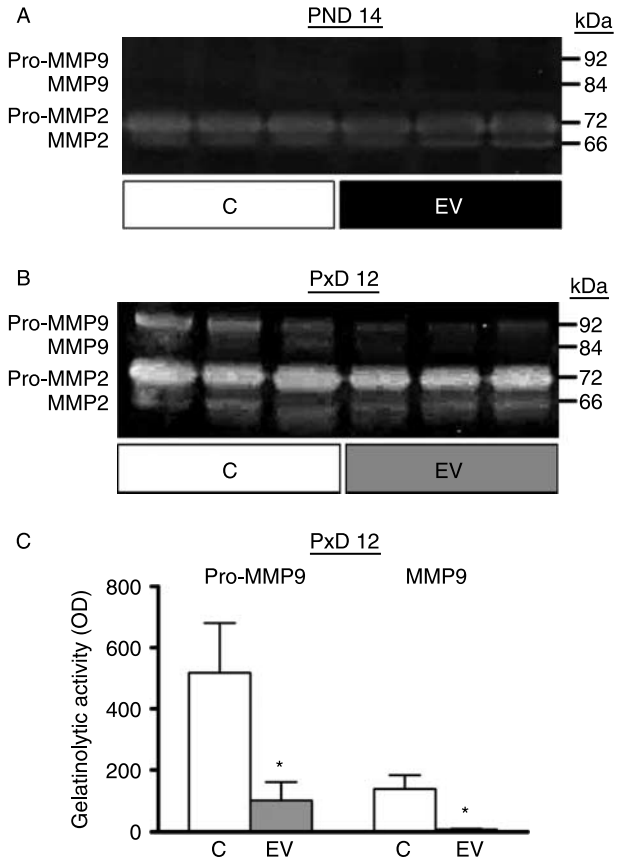

Figure 5 Effects of EV, administered from PND 0 to PND 13, on porcine uterine MMP9 and MMP2 gelatinase activity at PND 14 (A) and PxD 12 (B). Representative zymograms for each age and treatment group are shown. In the zymograms, clear zones against the dark background indicate gelatinolytic activity for pro-MMP9 (92 kDa), MMP9 (84 kDa), pro-MMP2 (72 kDa), and MMP2 (66 kDa). (C) Endometrial MMP9 gelatinolytic activity at PxD 12 was quantified by densitometry and graphed in OD units (LSM±S.E.M.; $N=4$–6 animals per group). *$P<0.05$.

exposure on the uterus have been documented, identifying the programming pathways that are altered by such exposures is an ongoing process. This study was designed to evaluate both short- and long-term programming effects of transient neonatal estrogen exposure on developmental markers of porcine uterine growth, patterning, and remodeling. Results show clearly that disruption of estrogen-sensitive early neonatal uterine developmental events affects the developmental trajectory and, ultimately, function of adult endometrium during the periattachment period of early pregnancy.

Porcine uterine ESR1 expression develops in an age-dependent manner during the first 2 weeks of life (Yan *et al*. 2006*a*) and can be activated by exogenous estrogens as early as PND 2 (Yan *et al*. 2008). In the current study, transient neonatal EV exposure from birth increased uterine ESR1 protein at PND 14. These data support the histological evidence that estrogens increased ESR1-positive uterine gland development during the first 2 weeks of life (Tarleton *et al*. 1999). The increase in uterine ESR1 protein occurred concurrently with an increase in uterine VEGFA protein, a marker of estrogen action in immature rat (Hyder *et al*. 1996) and neonatal porcine (Yan *et al*. 2008) uterine tissues, as well as an indicator of tissue angiogenesis and growth. Moreover, present data indicate that, while short-term effects of neonatal estrogen exposure include increased ESR1 expression, long-term effects include a reduction in endometrial ESR1 protein. These relationships are complementary to data reported for uterine wet weight under identical treatment conditions indicating that while short-term effects of neonatal estrogen treatment were uterotropic, long-term effects, as assessed on PxD 12, were anti-uterotropic (Tarleton *et al*. 2003). Since endometrial ESR1 is required for both recognition of pregnancy in pigs (Bazer & Thatcher 1977, Marengo *et al*. 1986) and the secretion of luminal proteins necessary for conceptus development (Flint *et al*. 1978, Roberts *et al*. 1993, Tarleton *et al*. 2003), decreases in uterine ESR1 expression induced by disruption of estrogen-sensitive developmental programming events can likely compromise endometrial capacity for support of the conceptus, as reflected by reduced embryonic survival (Bartol *et al*. 1993).

Relaxin, a milk-borne hormone, has been found in rodent, porcine, and human milk (Eddie *et al*. 1989, Steinetz *et al*. 1996, Yan *et al*. 2006*b*). Bioactive relaxin is present in porcine milk (Frankshun *et al*. 2009) and is detectable in the systemic circulation of newborn pigs only if they are allowed to nurse (Yan *et al*. 2006*b*, Frankshun *et al*. 2009). Relaxin increased uterine growth in neonatal gilts (Yan *et al*. 2006*a*) and stimulated expression of porcine uterine markers of growth and remodeling (Lenhart *et al*. 2001, Yan *et al*. 2008). Actions of relaxin are mediated via its receptor, RXFP1, a transmembrane protein belonging to the leucine-rich G protein receptor family (Hsu *et al*. 2002). Results of the present study, indicating that exposure of gilts to EV for 2 weeks from birth increased uterine *RXFP1* expression at PND 14, support previous studies that showed estrogens increased porcine uterine relaxin binding (Mercado-Simmen *et al*. 1982) as well as *RXFP1* gene expression in the porcine reproductive tract (Yan *et al*. 2008) and in a human uterine fibroblast cell line (Maseelall *et al*. 2009). Importantly, data also show that the estrogen-induced increase in uterine *RXFP1* expression observed on PND 14 is similar to that observed in adult endometrium obtained from neonatally EV-exposed gilts on PxD 12. These relationships could reflect neonatally programmed dysregulation of mechanisms governing dynamic adult endometrial RXFP1 expression at a time in early pregnancy when local estrogen signaling associated with maternal recognition of pregnancy in the pig is high (Tarleton *et al*. 2003). Data support the idea that uterine *RXFP1* expression can be used as a marker of transient, neonatal estrogen exposure in both neonatal and adult periods. In human pregnancy, increased decidual relaxin

(Bogic *et al*. 1997) and RXFP1 expression were linked to negative outcomes such as preterm delivery (Lowndes *et al*. 2006). Whether elevated endometrial *RXFP1* in PxD 12 gilts treated neonatally with EV is associated with dysregulation of relaxin-mediated periattachment events and reduced uterine capacity is unknown.

Evidence from *WNT7A* and *HOXA10* null mice demonstrated that these genes are not only responsible for organizationally critical uterine patterning and differentiation events (Benson *et al*. 1996, Miller & Sassoon 1998), but that *WNT7A* is necessary to maintain the expression of uterine stromal patterning genes, including *WNT4*, *WNT5A*, and *HOXA10* (Miller & Sassoon 1998, Kitajewski & Sassoon 2000). *WNT7A* expression is sensitive to estrogens, as murine models show that *WNT7A* expression in the uterus declines as estrus approaches and systemic estradiol increases (Kitajewski & Sassoon 2000). This effect can be reproduced by exogenous exposure to estrogenic compounds (Miller *et al*. 1998). Thus, the estrogen-regulated decline in *WNT7A* expression is postulated to be essential to insure up-regulation of patterning genes in the WNT/HOXA axis, including *HOXA10*, expression of which increases in response to estrogens (Taylor *et al*. 1998, Block *et al*. 2000, Taylor 2000). Exposure of fetal mice to estrogens resulted in uterine structural and functional irregularities that closely mimicked those observed in both *WNT7A* and *HOXA10* knockout mice (Satokata *et al*. 1995, Miller & Sassoon 1998), including homeosis of the reproductive tract and sterility (Miller *et al*. 1998, Block *et al*. 2000). Data reported here for uterine *WNT7A* and *HOXA10* expression at PND 14 confirm and extend previous observations involving qualitative *in situ* hybridization analysis of the neonatal porcine uterus (Bartol *et al*. 2006). In that report, both *WNT7A* and *HOXA10* mRNA developed in a time-dependent manner from birth to PND 14, and treatment with EV for 14 days from birth reduced *WNT7A* expression in luminal epithelium and increased *HOXA10* expression in endometrial stroma on PND 14. Again, estrogen-induced disruption of the neonatal developmental program documented here was also marked by reduced endometrial *WNT7A* expression on PxD 12 in neonatally estrogen-exposed pregnant adults. Present data indicating that disruption of the neonatal porcine uterine WNT/HOXA expression axis has long-term implications for endometrial function in the pig should not be surprising in light of similar data reported for the mouse.

While studies show that exposure of the prepubertal pig to relaxin increased uterine secretion of gelatinases, MMP2 and MMP9 (Lenhart *et al*. 2001), relatively little is known about the expression and activity of gelatinases in the neonatal porcine uterus. Even less is known about the functional consequences of dysregulated neonatal uterine MMP expression in the pig. MMPs are a family of proteinases that are responsible for degrading the ECM to facilitate tissue growth and remodeling (Hulboy *et al*. 1997, Visse & Nagase 2003). Gelatinolytic degradation of the basement membrane and the ECM allows for tissue expansion (Hulboy *et al*. 1997, Masson *et al*. 2005) and release of growth factors present in the ECM (Vukicevic *et al*. 1992). Both MMP2 and MMP9 have been detected in reproductive tissues and implicated as major players in uterine remodeling in several mammalian species, including mice (Hu *et al*. 2004), pigs (Lenhart *et al*. 2001), and humans (Martelli *et al*. 1993). Moreover, there is evidence that MMP2 and MMP9 are regulated, in part, by estrogens. In rat endometrial tumor cells, estrogen exposure induced *MMP2* transcripts (Tushaus *et al*. 2003) and, in mouse uterus, MMP9 activity increased with estrogen exposure (Zhang *et al*. 2007). Here, data indicate that neonatal EV treatment increased uterine *MMP9* expression at PND 14. Conversely, in the adult endometrium at PxD 12, neonatal EV treatment reduced *MMP9* expression, with corresponding decreases observed for both latent and active MMP9 protein activity. The expression of MMP9 transcripts and related protein activity at neonatal and adult time points complements changes associated with uterine weight as a result of transient neonatal EV exposure (Tarleton *et al*. 1999, 2003). Since tissue growth relies on the coordinated activity of MMPs, a reduction in MMP9 activity at PxD 12 may have contributed to the reduction in uterine size in adult gilts exposed neonatally to EV. These data provide evidence that estrogen-sensitive developmental programming of porcine uterine tissues between birth and PND 14 may also involve MMP9 as an important regulatory element.

Given that neonatal EV treatment did not affect ovulation rates or the ability of gilts to cycle or conceive (Bartol *et al*. 1993, Tarleton *et al*. 2003), it can be inferred that embryonic losses associated with such neonatal estrogen exposure were due to the direct effects of EV on uterine programming events. Collectively, data reinforce the idea that estrogen-sensitive markers of neonatal porcine uterine growth, patterning, and remodeling can be altered during the first 2 weeks of neonatal life with consequences in adults. These markers of uterine development should be included as elements of the organizational palette of factors that define the porcine uterine developmental program and determine the developmental trajectory of uterine tissues, including the endometrium. Temporospatial uterine expression patterns observed for such genes shortly after birth can define the potential for developmental success and determine the functional capacity of adult uterine tissues (Bartol *et al*. 2006). Identification of the complete array and role of factors that define the porcine uterine developmental program and determine the developmental trajectory of these tissues will provide an important insight into mechanisms regulating reproductive efficiency and performance.

Materials and Methods

Materials

TRI Reagent was obtained from Sigma–Aldrich. RNeasy Mini kits and RNase-Free DNase Sets were obtained from Qiagen Inc. SuperScript III First-Strand Synthesis System for RT-PCR was from Invitrogen. SYBR Green PCR Master Mix was purchased from Applied Biosystems (Foster City, CA, USA). Primers were synthesized by Sigma Genosys. Detergent-compatible protein assay kits (DC Protein Assay) were purchased from Bio-Rad Laboratories. Mouse anti-human ESR1 (Ab-15) mAb was from NeoMarkers, Inc. (Fremont, CA, USA). Rabbit anti-human VEGFA (A-20-G) and goat anti-human actin (sc-1615) polyclonal antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). HRP-conjugated anti-mouse and anti-goat secondary antibodies were purchased from Zymed (Carlsbad, CA, USA). Nitrocellulose membranes were obtained from Bio-Rad Laboratories. The Renaissance Western Blot Chemiluminescence Reagent Plus kits were acquired from Perkin Elmer Life Sciences (Waltham, MA, USA). XOmatic films were purchased from American Imaging (South Plainfield, NJ, USA). Zymogram gels and buffers were purchased from Invitrogen. EV and other chemicals were purchased from Sigma–Aldrich and Invitrogen, unless otherwise specified.

Experimental design

At birth, crossbred gilts (*Sus scrofa domesticus*) were assigned randomly to one of two neonatal treatment groups ($n=4$–6 gilts/group). Care was taken to ensure that treatments were balanced for potential effects of litter and sows were nursing litters of similar size. Neonatal treatments, administered as daily injections from birth (PND 0) through PND 13, were either corn oil vehicle (C; 50 μl/kg body weight (BW) per day, i.m.) or EV (50 μg/kg BW per day, i.m.). The dosage, timing, and route of EV administration were based on previous studies (Tarleton *et al*. 2003). In study 1, neonatal uterine tissues from gilts were collected on PND 14 from both EV- and vehicle-treated gilts. In study 2, neonatally treated gilts were allowed to reach puberty as evidenced by display of at least two consecutive estrous cycles of normal length. At the second estrus, gilts from each treatment group were bred by natural service at first estrus and again 24 h later. Endometrial tissues were collected from the adults at PxD 12.

Tissue collection, RNA isolation, and cDNA generation

In study 1, neonatal uterine tissues were removed, trimmed of fat and associated tissues and ligaments, and wet weights were recorded. In study 2, pregnant gilts were hysterectomized on day 12 post mating. Each uterine horn was flushed to confirm the presence of conceptus tissues and pregnancy as previously described (Tarleton *et al*. 2003). Uteri were opened along their mesometrial border, and the endometrium was harvested using a scalpel and forceps (Tarleton *et al*. 2003). Uterine tissues from PND 14 and endometrium from PxD 12 gilts were frozen using liquid nitrogen and stored at −80 °C. Animals in study 1 were obtained from the Swine Unit of the New Jersey Agricultural Experiment Station, Rutgers University. Animals in study 2 were obtained from the Auburn University Swine Research and Education Center. The tissues used in study 2 were the same endometrial tissues used in the 2003 Tarleton study. All procedures involving animals were reviewed and approved by relevant Institutional Animal Care and Use Committees and were conducted in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (1999; Federation of Animal Science Society, Savoy, IL, USA).

Total RNA was isolated from 30 to 50 mg tissue for each sample using TRI Reagent and the RNeasy Mini kit. Traces of DNA were removed using the RNase-Free DNase Set. RNA concentration and purity were evaluated by spectrophotometry. RNA integrity was checked by agarose gel electrophoresis and ethidium bromide staining to visualize sharp, clear 28S and 18S rRNA bands. RT was performed with 500 ng total RNA per sample using the PTC-200 Peltier Thermal Cycler (Bio-Rad Laboratories Inc.) and SuperScript III First-Strand Synthesis System for RT-PCR. All procedures were carried out following the manufacturers' instructions.

Real-time RT-PCR

Real-time RT-PCR (qPCR) was performed using an Applied Biosystems Gene Amp 7000 Sequence Detection System (Applied Biosystems) with the SYBR Green method following the universal thermal cycling parameters indicated by the manufacturer. Primers for qPCR were designed using Primer Express Software (Applied Biosystems) and synthesized by Sigma Genosys. All primer sequences were directed to the porcine genome (Table 1). To ensure specific amplification, controls including water only, no primers, and no template were included in the assay. The quality of the primers was

Table 1 Porcine primer accession numbers, sequences, and amplicon sizes for targeted uterine developmental genes.

Gene	Accession number	Forward primer	Reverse primer	Amplicon (bp)
WNT7A	CA997684	CACCACCAAGACCTGCTGG	TCCTTGAGCACGTAGCCCA	63
HOXA10	AF281156	CGGCCGGAAGAAGCA	AGAAACTCCTTCTCCAGCTCCA	63
ESR1	AF035775	AGGGAGAGGAGTTTGTGTG	TCTCCAGCAGCAGGTCATAG	306
RXFP1	CA994862	GCATCACTTTGAGGCAGAGACA	CCTCGGCAAAGACATTGCAT	69
MMP2	NM214192	GAGCACCATCGAGACCATGA	TTGTAATTGGCCACGTCGG	60
MMP9	DQ132879	TGGATCCAAAACTACTCGGAAGAC	CGGACAAAGGCGTCG	59
PPIA	AU058466	TTATAAAGGTTCCTGCTTTCACAGAA	TGCCATTATGGCGTGTGAAG	77

evaluated by amplifying serial dilutions of the cDNA template. In addition, dissociation curves for each set of primers were checked to ensure no amplicon-independent amplification (i.e. primer–dimer). PCR amplification products were analyzed by agarose gel electrophoresis to further confirm the absence of non-specific amplification. Data were analyzed using the relative standard curve method for quantitation of gene expression as described by Applied Biosystems (ABI User Bulletin 2, 2001). Standard curves were generated for each gene using two-fold dilutions of cDNA from PND 14 uterus (study 1) or PxD 12 endometrium (study 2; Larionov *et al.* 2005).Target gene expression was normalized to the expression of porcine cyclophilin *(PPIA)*, and data from qPCR analyses are presented as relative mRNA units.

Protein extraction and evaluation of ESR1 and VEGFA expression

Uterine and endometrial tissue proteins were quantified using procedures described previously (Yan *et al.* 2008). Tissues (20–50 mg) were homogenized in 200 µl lysis buffer (1% Triton X-100, 10% glycerol, 150 mM Tris–HCl, 300 mM NaCl, and 1 mM $MgCl_2$, pH 7.5). Samples were then sonicated and centrifuged (12 000 ***g***, 4 °C) for 30 min, and the protein supernatant was removed and stored at −20 °C. Protein concentration was measured using the DC Protein Assay kit (Bio-Rad). To document ESR1 and VEGFA protein expression, uterine proteins (30 µg) were resolved on 12.0% total monomer, Bis–Tris–HCl-buffered polyacrylamide gels under reducing conditions and transferred onto nitrocellulose membranes. After blocking in 10.0% non-fat dry milk in Tris-buffered saline containing Tween-20 (TBST; 25 mM Tris (pH 7.5), 0.14 mM NaCl, 3 mM KCl, and 0.05% Tween-20), membranes were probed with either mouse anti-human ESR1 antibody (1:100) or rabbit anti-human VEGFA antibody (1:1000) overnight at 4 °C. After washing with TBST, blots were incubated with either HRP-conjugated anti-mouse secondary antibody (1:1000) or anti-rabbit secondary antibody (1:1000) for 1 h at room temperature. Bound antibodies were detected by ECL. Protein loading was monitored using actin as a reference. Chemiluminescence signals were quantified densitometrically from film using Scion Image for Windows (Scion Corporation, Frederick, MD, USA).

Gelatin zymography

Zymography was performed as described previously (Ho *et al.* 2007). Briefly, samples (30 µg protein) were mixed with equal amounts of SDS sample buffer and loaded under non-denaturing, non-reducing conditions using precast polyacrylamide zymogram gels supplemented with 1% gelatin as the proteinase substrate. Following electrophoresis, gels were washed in renaturing buffer for 30 min to remove the SDS and then incubated in developing buffer overnight at 37 °C. Gels were stained with 0.1% Coomassie brilliant blue R 250. Human MMP2 and MMP9 enzyme standards were used as positive controls. Gelatinolytic activity of uterine and endometrial MMP2 and MMP9 was quantified densitometrically using Scion Image for Windows.

Statistical analyses

All data were subjected to analyses of variance. For relative gene and protein expression data (generated by densitometry of immunoblots), statistical models accounted for variation due to the main effects of treatment. Neonatal treatment effects on uterine (PND 14) and endometrial (PxD 12) responses were evaluated on a within-day basis only. Error terms were identified based upon the expectations of the mean squares for error, and data were expressed as least-square means with S.E.M.

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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