FACTORS THAT DEFINE THE DEVELOPMENTAL PROGRAM AND TRAJECTORY OF THE PORCINE UTERUS

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ABSTRACT OF THE DISSERTATION

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AND TRAJECTORY OF THE PORCINE UTERUS

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The early days of life represent an important period for neonatal uterine development in the pig. The porcine uterus develops *in utero* and continues postnatally. Events during neonatal life that establish uterine histoarchitecture contribute to the developmental program of the uterus and set the trajectory for adult uterine phenotype. Uterine programming is sensitive to hormonal perturbations. Exposure to bioactive factors during neonatal life influences uterine maturation with long-term consequences for reproductive performance. The goal of this research was to study factors in the neonatal environment that alter uterine developmental gene expression. These include mediators of growth [estrogen receptor alpha (ESR1), vascular endothelial growth factor (VEGFA) and relaxin receptor (RXFP1)], patterning (WNT7A and HOXA10) and remodeling [matrix metalloproteinase (MMP)9 and 2]. Results indicated that exposure to estradiol valerate (EV) from birth (postnatal day [PND] 0) to PND 14 increased uterine ESR1 and VEGFA protein, along with *HOXA10*, *RXFP1* and *MMP9* transcripts and decreased *WNT7A* transcripts, in PND 14 neonates. Endometrial gene expression changes also occured in adulthood on day 12 of pregnancy as a consequence of neonatal

EV exposure. This included decreases in *WNT7A* and *MMP9* transcripts, ESR1 and VEGFA protein and reduced MMP9 activity. Next, exposing gilts perinatally to the estrogenic mycotoxin zearalenone (ZEA), decreased uterine *ESR1*, *WNT7A* and *RXFP1* mRNA levels on PND 21 compared to unexposed gilts. Extending the concept that postnatal uterine development in gilts can be influenced by maternally-derived signals, studies showed that nursing from birth supported uterine ESR1, VEGFA and MMP9 protein expression at PND 2 and PND 14 in comparison to milk replacer-fed gilts. Treatment with relaxin, an uterotrophic milk-borne hormone, did not restore the uterine phenotype of replacer-fed animals to that of nursing gilts at PND 2, although it did predictably affect *RXFP1* transcript levels. Results support the idea that nursing during a critical two-day period from birth supports the neonatal porcine uterine developmental program. Identification of mediators that regulate neonatal uterine programming, the time frame for this regulation and understanding how those mediators are influenced by environmental/lactocrine factors provide critical insights into the mechanisms that govern neonatal tissue development.

DEDICATION

I dedicate this to my parents, Charles and Sherri Chen,

for giving me a life filled with love, meaning and purpose.

To my wife Suruchi, your love is the greatest thing in my world,

while your family's love is the coolest.

To my sister Jane, for taking care of me as much, if not more,

than I ever took care of you, and giving me a wonderful

brother in your husband.

And finally, to my awesome, loud, extended, one of a kind family (the Chens, Khandelwals and Li's, all associated grandparents, uncles, aunts, cousins, nephews, nieces, future children et al), you guys are the best.

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CHAPTER I

REVIEW OF LITERATURE

A. The purpose and scope of the literature review

The goal of this research is to study factors present in the environment of neonatal gilts that affect development of the uterus during the early days of life. These factors can consist of exogenous hormones, including estrogens, estrogenic mycotoxins and milk-borne bioactive molecules that can affect, and thus define, the uterine developmental program. Alterations to this developmental program, defined as a series of events that occur during a critical period of neonatal development and directs the course of tissue development, have the potential to affect the mature tissue phenotype (Oster & Alberch 1982, Langley-Evans 2006). The purpose of this literature review is to introduce factors in the neonatal environment that can affect the developmental program and trajectory of the porcine uterus and discuss their mechanisms of action. In addition, the early events associated with the normal development program of the porcine uterus will be reviewed. These events are exemplified by the expression patterns of genes responsible for porcine uterine growth, histogenesis and connective tissue remodeling during neonatal life. The expression patterns of these genes are indicative of normal development, while changes in the expression of these genes due to exogenous perturbations are linked to negative consequences for uterine function. Evidence for this will also be presented here, as well as a rationale for using the expression patterns of these genes as markers of normal or aberrant uterine development in the neonatal pig.

B. Development of the porcine uterus

In the pig, as in other mammals, development of the uterus begins prenatally but is completed postnatally (Bartol *et al.* 1993, Gray *et al.* 2001, Bartol *et al.* 2006). Prenatally the urogenital tract, comprised of urinary and reproductive tracts, develops

from mesoderm and both male (Wolffian ducts) and female (Mullerian ducts) duct systems are present. In normal females, lacking testosterone produced by the male gonads, the Wolffian ducts degenerate and the Mullerian ducts differentiate and develop into anterior elements of the female reproductive tract, including the uterus (Swain & Lovell-Badge 1999, Capel 2000). At birth, differentiation between the oviducts, uterus, vagina, and cervix is complete. However, these reproductive organs are not fully developed and development continues postnatally. Postnatal establishment of uterine morphology occurs in multiple species, including mouse (Taylor *et al.* 1997), pig (Bartol *et al.* 1993) and human (Gray *et al.* 2001).

Postnatal development of the porcine uterus involves important histomorphological and molecular changes. At birth, the porcine uterus is devoid of endometrial glands. However, adenogenesis occurs in an age-dependent manner as glandular epithelium (GE) differentiates and buds from luminal epithelium (LE), followed by invagination, tubular coiling and branching morphogenesis of nascent glands from the shallow through the deep endometrial stroma to form secretory structures (Spencer *et al.* 1993, Tarleton *et al.* 1999). In the pig, GE proliferation occurs during the first 2 days of life and is accompanied by age-dependent increases in proliferating cell nuclear antigen (Masters *et al.* 2007), a mediator of polymerase-induced DNA synthesis and a marker of cellular proliferation. There are increases in uterine adenogenesis and uterine size from birth through day 60 which are largely independent of a requirement for ovarian support (Bartol *et al.* 1993). In addition to these postnatal morphological changes in the uterus, there are dynamic molecular changes during the first two weeks of neonatal life. Uterine expression of developmental patterning genes in the WNT/HOXA

family increases in an age-dependent manner (Bartol *et al.* 2006). In addition, matrix metalloproteinases, proteinases that facilitate connective tissue remodeling necessary for normal growth and development (Visse & Nagase 2003) and detectable in neonatal mice uteri (Hu *et al.* 2004), could also play a role for porcine uterine development . Collectively, these data illustrate that dynamic events at the cellular level occur in the porcine uterus during the early days of life and could be vital for uterine developmental success.

C. Uterine-mediated events associated with the periattachment period

The uterus plays an important role in establishing and maintaining pregnancy. Conceptus signaling that initiates maternal recognition of pregnancy includes secretion of hormones that target endometrial tissues to initiate further downstream signaling that ensures maintenance of ovarian luteal sources of progesterone necessary to maintain pregnancy (Hearn *et al.* 1991, Roberts *et al.* 1996). In addition, successful signaling to the maternal endometrium and subsequent placentation in eutherian mammals requires the establishment of an interface between the conceptus and the mother, and is largely dependent on the receptivity of the maternal uterus. Studies in mice and rats showed that embryos flushed out of unreceptive uteri and transferred into uteri of receptive mothers yielded successful pregnancies (McLaren A 1955, Dickmann 1960). Thus, communication between the conceptus and the endometrium is a pivotal step in establishing and maintaining pregnancy.

Uniquely in the pig, signaling between estrogens secreted by the conceptus and the uterus triggers maternal recognition of pregnancy (Marengo *et al.* 1986). At day 12 of pregnancy, porcine conceptuses secrete estrogen into the uterine lumen, which

promotes two major events. First, conceptus-secreted estrogens induce exocrine secretion of prostaglandin $F_{2\alpha}$, the luteolytic agent in pigs, to ensure survival of corpora lutea (Bazer & Thatcher 1977). Second, the endometrium responds to conceptus estrogen signaling by secreting histotroph into the lumen which supports developing embryos (Knight *et al.* 1973, Geisert *et al.* 1982). Endometrially secreted histotroph is required for pregnancy to be established and maintained. This idea is supported by studies in ewes illustrating that progestin-induced knockout of uterine glands during neonatal life resulted in inhibition of glandular development at two weeks of age (Bartol *et al.* 1988) and adulthood (Bartol *et al.* 1999), resulting in sterility (Allison Gray *et al.* 2000). Uterine flushings from these ewes are also missing osteopontin, an integrin present in histotroph that mediates cell-cell adhesion processes and acts as an initiator of the implantation cascade critical for conceptus-endometrial attachment (Burghardt *et al.* 2002, Johnson *et al.* 2003). In pigs, contrary to the invasive nature of human and rodent endometrial implantation, attachment of conceptuses to the endometrium is diffuse, forming an epitheliochorial placenta (Bazer *et al.* 2009). For this type of maternal-fetal interface, the histotroph not only provides nourishment and immunological support, as it does for all mammals, but is necessary to support pregnancy (Gray *et al.* 2001, Spencer & Bazer 2004).

D. Estrogen receptor α (ERα or ESR1) is required for growth and development of the porcine uterus

The uterus is a major target for estrogen-mediated growth, and the estrogen receptor (ER) is present in the uterus of mammals (Benassayag *et al.* 1999, Tarleton *et al.* 1999, Weihua *et al.* 2000). There are two major subtypes of ERs, ERα (ESR1) and ERβ

(ESR2). ERα is the predominant ER subtype expressed in the uterus (Couse *et al.* 1997). Uteri of ERα knockout mice are hypoplastic and unresponsive to normally uterotrophic effects of estrogen (Lubahn *et al.* 1993, Enmark *et al.* 1997). In 1996, ERβ was discovered by Kuiper and colleagues as another ER subtype coded by a separate gene and having distinct structural differences and tissue distribution compared to those observed for ERα (Kuiper *et al.* 1996, Enmark *et al.* 1997, Krege *et al.* 1998). ERβ expression was demonstrated in rodent and human uterine tissues (Benassayag *et al.* 1999, Weihua *et al.* 2000). However, in the postpubertal pig, uterine ERβ transcripts are low in comparison to ERα (Cardenas & Pope 2004). In the neonatal porcine uterus, ERβ protein was undetectable (Yan *et al.* 2006a), whereas ERα protein was expressed as early as PND 2 (Yan *et al.* 2008) and both ERα transcripts and protein expression increased through PND 14 (Tarleton *et al.* 1999, Yan *et al.* 2006a).

In situ hybridization and immunoblotting studies in porcine uteri showed that ERα expression is absent at birth (Tarleton *et al.* 1998, Yan *et al.* 2006a), localized primarily in the GE by two weeks of age, and increased in signal strength until 120 days of age, when ER was localized in GE, LE and stroma (Tarleton *et al.* 1998). Although, it was shown that the age-dependent growth of the uterus in ovariectomized gilts is similar to intact gilts from birth to PND 60, indicating that uterine growth during this period is largely independent of systemic ovarian support (Bartol *et al.* 1993), this growth is dependent on uterine ERα signaling, as administration of the anti-estrogen ICI 182, 780 (ICI) from birth reduced gland formation and endometrial thickness compared to controls at PND 14. Pretreatment of gilts with ICI also abolished the uterotrophic effects of exogenous estrogen (Tarleton *et al.* 1999). Porcine uterine ERα also plays a pivotal role

during pregnancy. Stromal ERα is absent during pregnancy, whereas surface and glandular ERα is expressed from day 0 to 12 of pregnancy, and declines after day 15 (Geisert *et al.* 1993). This supports the idea that at day 12 of pregnancy in pigs, conceptus-secreted estrogens target luminal and glandular ERα to initiate secretion of pregnancy proteins into the uterine lumen in order to sustain developing embryos, initiate recognition of pregnancy (Bazer & Thatcher 1977) and ensure reproductive success (Tarleton *et al.* 2003). Therefore, the presence and function of uterine ERα is critical for appropriate, age-dependent development of the porcine uterus during the early weeks of life and is required to support the initiation and maintenance of pregnancy.

E. Effects of neonatal estrogen exposure and the presence of environmental estrogens

There is extensive work indicating that aberrant exposure of the developing uterus to hormones or compounds that mimic the actions of hormones such as estrogens can result in severe uterine abnormalities later in life. For example, in mice, neonatal exposure to bisphenol A, a chemical with estrogenic activity, resulted in uterine adenomyosis, hyperplasia and presence of polyps at adulthood (Newbold *et al.* 2007). In the pig, transient exposure to estrogens during the first two weeks of life altered uterine expression of genes at pregnancy (Tarleton *et al.* 2003) and ultimately resulted in reduced embryo survival at term (Bartol *et al.* 1993). Women exposed to the synthetic estrogen diethylstilbestrol (DES) *in utero* experienced severe pregnancy-related problems, including premature births, spontaneous losses, and were less likely to have full-term live births (Kaufman *et al.* 2000). These studies illustrate how early exposure to estrogenic compounds can compromise the development of the uterus and negatively influence

fertility later in life.

The potential for exposure to estrogenic endocrine-disrupting agents is a real. Some commercial pesticides used on animal and human food crops possess estrogenic activity (Soto *et al.* 1994, Andersen *et al.* 2007). Exposure to estrogenic compounds not limited to industrial chemicals alone, but may arise from naturally occurring estrogenic compounds in the environment such as phytoestrogenic flavonoids and mycotoxins that can infest commercial foods and animal feeds (McLachlan & Korach 1995, Humfrey 1998). Due to the number, variety, and presence of these compounds in the environment the risks for multiple exposures are high. Therefore, it is important to be able to identify these compounds, their actions on target organs and species, and to characterize specific cellular and molecular consequences of exposure to these agents.

F. Vascular endothelial growth factor mediates tissue angiogenesis and growth

The development of tissue vasculature involves two major processes: the *de novo* formation of blood vessels from hematopoietic cells (vasculogenesis), which takes place primarily in the developing embryo after conception; and the formation of blood vessels from existing vasculature (angiogenesis) (Cebe-Suarez *et al.* 2006, Olsson *et al.* 2006). Both processes are important for normal tissue development and function, and are regulated to an extent by vascular endothelial growth factor (VEGF). VEGF belongs to a sub-group of secreted proteins that belong to the platelet-derived growth factor family of cystine-knot growth factors (Senger *et al.* 1983, Cebe-Suarez *et al.* 2006, Olsson *et al.* 2006). The earliest discovered and most well known member of this sub-family of growth factors is VEGFA (Senger *et al.* 1983), which has been implicated in the development and function of a variety of tissues including cardiac (Li *et al.* 1997),

reproductive (Daikoku *et al.* 2003) and neural (Stone *et al.* 1995, Maurer *et al.* 2003). Binding of VEGFA to its receptors, VEGFR-1 or VEGFR-2 (Fuh *et al.* 1998, Christinger *et al.* 2004, Olsson *et al.* 2006) results in increased vascular permeability and edema in endothelial cells and tissues which stimulates both vasculogenesis and angiogenesis

Aberrant expression of VEGF is linked to pregnancy disorders that negatively influence fertility, such as placental insufficiency (Regnault *et al.* 2002). Moreover, data indicate that VEGFA expression may be a necessary component of endometrial development during pregnancy (Das *et al.* 1997, Sugino *et al.* 2002). Thus, factors that can alter the expression levels of uterine/endometrial VEGFA during periods of uterine development have the potential to affect uterine/endometrial vascularization and potentially, disrupt uterine function.

G. Expression of VEGFA in the porcine uterus and regulation by ESR1

VEGF is expressed in the endometrium of both pregnant and non-pregnant pigs (Ziecik 2002) and was suggested to play a role in facilitating blastocyst attachment (Ziecik 2002, Vonnahme & Ford 2004). Data also showed that porcine blastocysts secrete VEGFA (Vonnahme & Ford 2004) and that receptor transcripts for VEGFR-1 and VEGFR-2 are present in the endometrium of pregnancy. Consequently, VEGFA signaling between conceptus and endometrium may be an integral part of establishing conceptus periattachment in pigs.

Expression of VEGFA is affected by estrogen exposure and estrogens up-regulate uterine VEGFA expression in rats (Cullinan-Bove & Koos 1993) and sheep (Reynolds *et al.* 1998). In the rat uterus (Hyder *et al.* 2000) and in human endometrial stromal cells (Huang *et al.* 1998), expression of VEGFA requires ESR1 expression. Thus, factors that

can alter the developmental expression of porcine uterine ESR1 have the potential to affect VEGFA-mediated regulation of uterine angiogenesis.

H. WNT/HOXA genes are involved in uterine developmental patterning

Genes in the WNT/HOXA family are responsible for development of primary body axis and structural differentiation in the kingdom Metazoa, which includes all multicellular organisms ranging from insects to mammals. (Ryan & Baxevanis 2007). Phylogenetic work implicates genes from this axis as the ancestral patterning system for Metazoans (Lee *et al.* 2006). Studies involving mice indicated that products of these genes, including WNT gene family members (WNT4, -5A, and -7A) and homeobox HOXA gene family members (HOXA9, -10, -11, -12), act as principle mediators of organizationally critical patterning events and mediators of female reproductive tract differentiation (Kitajewski & Sassoon 2000, Taylor 2000, Heikkila *et al.* 2001). Studies using both WNT7A- and HOXA10-null mice showed that these two genes are responsible for events that are necessary for uterine differentiation and subsequently, adult uterine function and fertility (Satokata *et al.* 1995, Parr & McMahon 1998). Studies in developing ewes and gilts also show that both ovine and porcine WNT7A (Bartol *et al.* 2006, Hayashi & Spencer 2006) and porcine HOXA10 (Bartol *et al.* 2006) are expressed in early neonatal life and that their expression is sensitive to estrogenic exposure and age. Hence, these mediators of uterine differentiation may play a role in development of the neonatal uterus in large domestic animals, including the pig.

In the murine reproductive tract, WNT7A is involved with anterior-posterior development of the uterus, as well as formation of uterine glands. Loss of function studies in mice showed that loss of WNT7A results in formation of oviducts lacking a

distinct separation from the anterior uterus and that the uterus itself shared structural similarities with the vagina (Miller & Sassoon 1998). WNT7A deficient mice do not have uterine glands, have thinner uterine walls and are sterile (Parr & McMahon 1998). Interestingly, both WNT7A and HOXA10 null mice share a similar reproductive phenotype. In HOXA10 null mice, there is also homeosis, or lack of defined morphological borders, between the uterus and the oviduct compared to the wild type uterus, which shows distinct morphological borders between the oviduct and the uterus (Benson *et al.* 1996). There are also structural abnormalities within the uterine wall that prevent normal implantation events, leading to sterility (Satokata *et al.* 1995).

Since phenotypic outcome and reproductive failures of WNT7A and HOXA10 knockout mice closely resemble each other, these two genes may work in concert to facilitate appropriate uterine development. In mice, WNT7A is expressed mainly in the LE. Expression decreases as estrus approaches and circulating levels of estrogens increase (Miller & Sassoon 1998, Kitajewski & Sassoon 2000). Expression of WNT7A is also necessary to maintain the expression of uterine stromal patterning genes, such as WNT4, WNT5A and HOXA10 (Kitajewski & Sassoon 2000). These data suggest a hormonally regulated loop whereby WNT7A responds to circulating estrogens after puberty and during estrus to support the expression of subsequent downstream WNT/HOXA genes, resulting in uterine morphogenesis. In the porcine uterus, expression of luminal WNT7A and stromal HOXA10 increases with postnatal age, and similar to the mouse WNT7A expression decreases in response to exogenous estrogen exposure, suggesting a potential role for WNT7A and HOXA10 signaling in porcine uterine patterning and adenogensis (Bartol *et al.* 2006).

I. WNT7A and HOXA10 gene expression is sensitive to exogenous estrogens

Aberrant uterine exposure to exogenous estrogens can result in decreases in uterine WNT7A expression in a manner similar to that observed for the uterus during estrus. Exposure to DES reduced WNT7A expression in the newborn murine uterus (Miller *et al.* 1998). In addition, DES exposure resulted in structural abnormalities in both human and mouse uterine tissues that closely mimicked uterine abnormalities observed in WNT7A-deficient mice (Miller *et al.* 1998). Predictably, studies in human endometrial cells revealed that exposure to estradiol increased HOXA10 gene expression (Taylor *et al.* 1998). These studies support research in the pig indicating similar patterns of gene expression. Exposure of gilts to estrogens for 14 days from birth decreased WNT7A expression and increased HOXA10 expression at PND 14 (Bartol *et al.* 2006). The endometrium of these animals also exhibited reduced histotroph secretion and uteroferrin mRNA expression (Tarleton *et al.* 2003) and decreased reproductive capacity (Bartol *et al.* 1993). Since estrogen-mediated reductions in reproductive capacity in pigs was marked by aberrant expression patterns of WNT7A and HOXA10, it is likely that exposure to exogenous estrogens precociously stimulate WNT/HOX signaling which subsequently alters the developmental program and trajectory of uterine maturation.

J. MMPs facilitate growth and development through tissue remodeling

Matrix metalloproteinases (MMPs) are a family of proteins secreted by tissues into the extracellular environment where they digest components of the extracellular matrix (ECM) and basement membrane (BM) to facilitate tissue growth, development, and morphogenesis (Visse & Nagase 2003). The 24 major vertebrate MMPs are organized into six categories, including: (1) collagenases that digest interstitial collagen;

(2) gelatinases that digest collagen, gelatins, and laminin; (3) stromelysins that can activate other types of MMPs from latent to active forms; (4) matrilysins that can interact with cell-surface signaling molecules; (5) membrane MMPs, found as either transmembrane molecules or membrane-anchored molecules; and (6) the immune-cell/tooth enamel related group (Birkedal-Hansen *et al.* 1993, Visse & Nagase 2003).

Digestive capabilities of MMPs are facilitated by a catalytic site that holds a reactive zinc atom, histidine residues and a water molecule. Adjacent to this site is a substrate-specific pocket. This pocket is the determining factor for binding specificity of each MMP (Welch *et al.* 1996, Bode *et al.* 1999). While the exact mechanism of MMP-mediated ECM and BM breakdown remains to be defined, it is generally accepted that the zinc reacts with the carbonyl group of the substrate and water to facilitate hydrolysis of substrate peptide bonds (Kester & Matthews 1977, Browner *et al.* 1995, Manzetti *et al.* 2003).

MMPs are secreted in an inactive ‘pro-‘ form (Nagase 1998). Cellular processing by proteolytic agents and other MMPs is necessary to expose the catalytic zinc site, thus activating MMPs enzymatically. In vertebrates there are four major tissue inhibitors of MMPs (TIMPs). The TIMPs fit into the active site of MMPs in a manner similar to that recognized for typical ECM or BM substrates, thereby preventing the active site of MMPs from interaction with target substrates (Brew *et al.* 2000). All four TIMPs bind tightly to and can inactivate almost all of the MMPs (Murphy & Willenbrock 1995). However, recent studies showed that certain TIMPs display binding preferences for specific MMPs. For example, TIMP1 binds and inactivates MMP2 preferentially, whereas TIMP2 shows binding preference for MMP9 (Goldberg *et al.* 1992, Nagase

1998). It is this balance of MMP secretion and TIMP-mediated suppression that allows for regulatory homeostasis. MMP2 and MMP9 are implicated as the major players in uterine remodeling in several mammalian species, including mice, pigs, and humans (Martelli *et al.* 1993, Lenhart *et al.* 2001, Hu *et al.* 2004).

K. MMP2- and MMP9-induced uterine remodeling and regulation by estrogens

MMP2 and MMP9 are important in mammalian uterine development. Both MMP2 and MMP9 protein and gelatinase activity were detected in the developing neonatal mouse uterus (Hu *et al.* 2004). Human endometrial cells secrete MMPs (Martelli *et al.* 1993) and the target substrates for MMP2 and MMP9 were identified in human decidual cells (Iwahashi *et al.* 1996). MMP9 knockout studies revealed that MMP9 expression is required for normal adult uterine function, since MMP9-null mice had reduced reproductive efficiency, possibly due to uterine implantation problems (Roth & Fisher 1999, Dubois *et al.* 2000). Expression of MMP2 was also implicated as an important marker of endometrial remodeling since expression increased during pregnancy (Beceriklisoy *et al.* 2007).

Estrogens can activate MMPs. In rat endometrial tumor cells, estrogen exposure induced MMP2 transcript expression (Tushaus *et al.* 2003) and in mouse uterus, MMP9 activity increased with estrogen exposure (Zhang *et al.* 2007). Mechanistically, the ER has also been linked to MMP activation. Studies in human breast carcinoma MCF-7 cells revealed that membrane ERα signaling can stimulate MMP2 and MMP9 expression (Razandi *et al.* 2003). Recently, it was shown that exposure to estrogen can regulate the expression of MMP9 through interaction with transcript-bound microRNAs in order to

stimulate MMP9 protein translation (Nothnick 2008). Together, data suggest that expression of MMPs may be regulated, in part, through estrogen signaling.

L. The selective estrogen receptor modulator (SERM) zearalenone

Zearalenone (ZEA) is a mycotoxin produced by fungi in the Fusarium genus (Vesonder *et al.* 1991). ZEA contamination was detected in cereal crops including corn, barley, wheat, oats and rice, and is responsible for the mycotoxicoses of several farm animals (Diekman & Green 1992). Exposure of commercial agricultural animals to ZEA resulted in pathologies associated with reproduction including reduced egg counts in turkeys (Allen *et al.* 1983), infertility in cows (Weaver *et al.* 1986) and reduced conception rates in sows (Vanyi *et al.* 1994). Among agricultural animals affected by ZEA exposure, pigs are the most sensitive species (Diekman & Green 1992). The high sensitivity of pigs to ZEA was hypothesized to be due to hepatic and intestinal biotransformation of ZEA to its more potent metabolite alpha-zearalenol which, like ZEA, possesses estrogenic activity (Kollarczik *et al.* 1994).

ZEA, a non-steroidal SERM, can interact with ERα (Kuiper *et al.* 1998) and act as an ER agonist, mimicking the actions of steroids such as estradiol (Kiang *et al.* 1978). Binding studies show that, as compared to other phytoestrogens in the environment including genistein, enterolactones, flavones and flavonols, ZEA has the highest binding affinity for ERα (Kuiper *et al.* 1998). These studies also showed that, in addition to receptor binding, ZEA can activate ERα in a manner comparable to estradiol and DES (Kuiper *et al.* 1998, Mueller *et al.* 2004).

While exposure to ZEA is linked to reproductive disorders, its effects on neonatal uterine tissues is less well known. Pregnant sows that consume ZEA have detectable

levels of ZEA in their milk (Sandor 1983). Thus, it is likely that neonates from mothers that consume ZEA will be exposed through nursing. Whether this type of exposure to ZEA alters the developmental program of the neonatal porcine uterus is unknown.

M. Relaxin (RLX) and the RLX receptor (RXFP1)

Relaxin (RLX) is a 6 kDa peptide hormone discovered in 1926 by Frederick Hisaw and colleagues (Hisaw 1926). Structurally, RLX is a member of the insulin/RLX superfamily of structurally related peptide hormones that includes insulin, insulin-like growth factor (IGF)1 and IGF2. These hormones have a similar B-C-A peptide chain configuration where the A and B chains are linked by two inter-domain disulfide bonds and the A chain has an intra-domain disulfide bond (Sherwood 2004, Bathgate *et al.* 2006). Molecules in this family of hormones are generated as prohormones that undergo molecular processing to remove the C peptide domain required to generate the mature peptide. Pro-hormone convertases or serine proteases may be involved in this C domain cleavage (Marriott *et al.* 1992, Zhou *et al.* 1999). Nevertheless, recombinant porcine prorelaxin expressed in both Chinese hamster ovary cells and E. coli, as well as recombinant marmoset prorelaxin are bioactive (Reddy *et al.* 1992, Vu *et al.* 1993, Zarreh-Hoshyari-Khah *et al.* 2001). Thus, cleavage of the relaxin C-peptide is not required for RLX bioactivity

Corpora lutea are the major source of circulating relaxin throughout pregnancy in the mouse, rat and pig, while the placenta is an additional source of RLX during pregnancy in humans (Sherwood 2004). RLX has traditionally been recognized as a reproductive hormone that targets connective tissue of the cervix and/or interpubic ligament to facilitate remodeling around the time of parturition. Studies in estrogen-

primed mice (Steinetz *et al.* 1960), pregnant rats (Downing & Sherwood 1985) and in pigs (Winn *et al.* 1993) showed that RLX treatment elongates the interpubic ligament. Both actions on the cervix and interpubic ligament are thought to facilitate birth and reduce parturition time. Thus, early human clinical work sought to use RLX as a therapeutic agent to reduce labor duration (Evans *et al.* 1983). Since then, RLX has been implicated in many mammalian reproductive roles, including follicle growth, reducing myometrial contractility during pregnancy, uterine growth, and regulation of plasma osmolality during pregnancy (Bagnell *et al.* 1993, Weisinger *et al.* 1993, Sherwood 1994). In addition, non-reproductive tissues, including the heart, have been shown to be a target for RLX, since cardiac collagen remodeling and cellular proliferation have been shown to be modulated by RLX treatment in rats (Samuel *et al.* 2004).

In 2002, Hsu and colleagues discovered that two leucine rich guanine binding protein (LGR)-coupled receptors, LGR7 and LGR8, were receptors for RLX and insulin-like factor 3 (INSL3), respectively (Hsu *et al.* 2002). In 2006, the nomenclature for these receptors was changed from LGR7 and LGR8 to the relaxin family peptide receptor (RXFP)1 and 2, respectively (Bathgate *et al.* 2006). RLX binds with greatest affinity to cells containing RXFP1 and with low affinity to cells containing only RXFP2 receptors (Sudo *et al.* 2003). Studies on RLX signaling showed that RLX binding to RXFP1 activates the protein kinase A pathway, which increases intracellular cyclic AMP (cAMP) production (Meera *et al.* 1995). Both human (Sudo *et al.* 2003) and porcine (Frankshun *et al.* 2009) RLX induced cAMP production in human embryonic kidney cell bioassays. Transcripts for RXFP1 were detected in both immature rat (Hsu *et al.* 2000) and neonatal

porcine (Yan *et al.* 2006b) uterine tissues, suggesting a role for RLX in uterine development and function.

N. Effects of RLX on the porcine uterus

Studies in rats showed that RLX administration increased uterine wet weight and that this uterotrophic effect was similar to those induced by estrogens (Pillai *et al.* 1999), including uterine weight gain and endometrial edema. Furthermore, pre-treatment of rats with the ER antagonist ICI 182, 780 (ICI) diminished these effects. Research in pigs supported these observations by showing that treatment of neonatal gilts with RLX increased uterine VEGFA protein at PND 2 (Yan *et al.* 2008) and uterine wet weight at PND 14 (Yan *et al.* 2008). Similar to the Pillai studies (Pillai *et al.* 1999), pretreatment with ICI attenuated the expression of VEGFA at PND 2 and uterine weight gain at PND 14 in neonatal gilts. Collectively, data can be interpreted to suggest that RXFP1 signaling and subsequent trophic effects on uterine tissues are dependent on the presence and activation of uterine ERα. This crosstalk between RXFP1 and ERα signaling systems could be an important part of neonatal uterine programming.

O. Milk as a conduit for RLX transmission to the neonate

Exogenous RLX, administered from birth, affects porcine neonatal uterine developmental events as early as PND 2 (Yan *et al.* 2006a). An endogenous source of porcine RLX in the neonatal environment was identified in milk during early lactation (Yan et al., 2006) . RLX is detectable in the milk of several species, including dogs (Steinetz *et al.* 1996), rats (Steinetz et al, 2009), pigs (Yan *et al.* 2006b) and humans (Eddie *et al.* 1989). Evidence from studies in both dogs and pigs indicates that RLX is

passed to the neonate via nursing since neonatal animals that do not nurse have no detectable levels of RLX in their circulation (Goldsmith *et al.* 1994, Yan *et al.* 2006b). Detection of RLX in both milk and in the neonatal circulation during early lactation correlates with gut closure time in pigs, estimated to occur at 24-48 hours after birth (Leece 1973). It is also estimated that neonatal pigs consume 40-60ml/hr of colostrum during the first 36 hours after birth, (Coalson & Lecce 1973), equating to microgram amounts of milk-borne RLX (Yan *et al.* 2006b) before gut closure and 1000 times the necessary concentration to elicit a cellular cAMP response (Frankshun *et al.* 2009). Together, these data suggest that there is a specific 'lactocrine' pathway, defined as a mechanism through which bioactive milk-borne factors are delivered from mother to offspring as a specific consequence of nursing (Yan *et al.* 2006b, Bartol *et al.* 2008), by which maternal RLX is passed on to the neonate and affects RXFP1-positive tissues. Transmission of bioactive factors from mother to offspring during nursing represents a form of maternal signaling that can affect the neonatal uterine development program with long-term implications for adult tissue function.

P. Significance

The early days of life is a critical window of time when important developmental events occur. This time period also represents a sensitive phase during which exposure to bioactive compounds can permanently alter the course of neonatal tissue development. Given that bioactive molecules present in colostrums/milk can be passed into neonatal circulation via a lactocrine mechanism where they have potential to alter the neonatal porcine uterine program (Bartol *et al.* 2009), it is likely that molecules present in colostrum/milk, including endogenous lactocrine-acting factors such as RLX (Yan *et al.*

2006b) and exogenous factors such as ZEA (Sandor 1983), could affect uterine development. Although the effects of RLX deficiency on adult uterine function have not been investigated in the pig, porcine milk-borne RLX is bioactive, RLX levels are highest in colostrum/milk during the first two days of lactation and RLX is passed into neonatal circulation via a lactocrine mechanism (Yan *et al.* 2006b). Available data can be interpreted to suggest a functional role for colostrum/milk-borne RLX in neonatal RXFP1-positive tissues, including the porcine uterus. Alterations in the neonatal porcine uterine developmental program as a result of xenoestrogen exposure or the absence of milk-borne, lactocrine-acting factors may affect the course of uterine maturation and potentially, compromise uterine function at adulthood. Studies that identify and characterize factors present in the neonatal environment, as well as the timing and mechanism of their actions can contribute to the advancement of animal and human health and reproductive efficiency.

Q. Hypothesis and objectives

The hypothesis to be tested through research described herein is that alterations in estrogen-sensitive, ER-dependent programming of the neonatal porcine uterus by agents in the neonatal environment, including estrogens, milk-borne lactocrine-active factors exemplified by RLX, and ZEA can affect the uterine gene expression profiles associated with establishment of the uterine developmental program and trajectory that may have lasting consequences for uterine function in adult life.

Three groups of uterine developmental genes were studied, including: patterning genes - WNT7A and HOXA10 - responsible for uterine organogenesis and histoarchitecture (Bartol *et al.* 2006); growth-related genes - ERα, VEGFA and RXFP1-

that mediate uterotrophic effects in the neonatal porcine uterus (Yan *et al.* 2006a); and remodeling genes - MMP2 and MMP9 - the gelatinases, activities of which are important for connective tissue remodeling needed for growth and expansion of reproductive tissues (Lenhart *et al.* 2001). Specific objectives were to:

1. evaluate acute and long-term effects of estrogen exposure during the first two weeks of life on uterine markers and mediators of developmental patterning, growth and tissue remodeling in the porcine a) neonate (acute) and b) pregnant adult (long-term).

2. determine the effects of perinatal ZEA exposure on uterine markers and mediators of patterning, growth and tissue remodeling in the porcine neonate at PND 21.

3. study the effects of estrogens and relaxin, administered from birth, on the porcine uterus at PND 2 (co-authored work with Dr. Wenbo Yan).

4. determine effects of colostrums/milk, in the presence and absence of exogenous RLX on the porcine uterine developmental program at PND 2 and on the developmental trajectory at PND 14.

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CHAPTER II

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 alpha (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 (50µg/kg BW/day, i.m.) or corn oil (CO) 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.

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.* 2006a). However, during the first two weeks of life, uterine glands differentiate and ESR1 expression is evident in both nascent glandular epithelium (GE) 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 estrogen 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 two 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 two 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 estrogen neonatally displayed reduced uterine capacity on pregnancy day 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 pregnancy day (PxD) 12.

MATERIALS and METHODS

Materials

TRI Reagent was obtained from Sigma-Aldrich. (St. Louis, MO). RNeasy Mini Kits and RNase-Free DNase Sets were obtained from Qiagen Inc. (Valencia, CA). SuperScript III First-Strand Synthesis System for RT-PCR was from Invitrogen (Carlsbad, CA). SYBR Green PCR Master Mix was purchased from Applied Biosystems (Foster City, CA). Primers were synthesized by Sigma Genosys. Detergent-compatible protein assay kits (DC Protein Assay) were purchased from Bio-Rad Laboratories (Hercules, CA). Mouse anti-human ESR1 (Ab-15) monoclonal antibody was from NeoMarkers, Inc. (Fremont, CA). 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). Horseradish peroxidase-conjugated anti-mouse and anti-goat secondary antibodies were purchased from Zymed (Carlsbad, CA). Nitrocellulose membranes were obtained from Bio-Rad Laboratories (Hercules, CA). The Renaissance Western Blot Chemiluminescence Reagent Plus kits were acquired from Perkin Elmer Life Sciences (Waltham, MA). XOmatic films were purchased from American Imaging (South Plainfield, NJ). Zymogram gels and buffers were purchased from Invitrogen (Carlsbad, CA). EV and other chemicals were purchased from Sigma-Aldrich (St.Louis, MO) and Invitrogen (Carlsbad, CA), 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; 100 µl/kg 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 recorded. In Study 2, pregnant gilts were hysterectomized on day 12 post mating. Each uterine horn was flushed to confirm 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 30-50 mg of 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. Reverse transcription was performed with 500 ng total RNA per sample using the PTC-200 Peltier Thermal Cycler (Bio-Rad Laboratories Inc., South San Francisco, CA) and SuperScript III First-Strand Synthesis System for RT-PCR. All procedures were carried out following 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, Foster City, CA) 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, Foster City, CA) 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 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 pregnancy day 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 of lysis buffer (1% Triton X-100, 10% Glycerol, 150 mM Tris-HCl, 300 mM NaCl, 1 mM MgCl2, 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% nonfat dry milk in Tris-buffered saline containing Tween-20 (TBST;25 mM Tris [pH 7.5], 0.14 mM NaCl, 3 mM KCl,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 horseradish peroxidase-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

enhanced chemiluminescence. Protein loading was monitored using actin as a reference. Chemiluminescence signals were quantified by densitometrically from film using Scion Image for Windows (Scion Corporation, Frederick, MD).

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 (LSM) with standard errors.

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 Figures 1 and 2, respectively. Neonatal exposure to EV for two weeks from birth increased ($P<0.05$) uterine wet weight (EV: 1.40 ± 0.16g) compared to controls (C: 0.53 ± 0.07g) 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 two 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 Figures 3 and 4, respectively. EV treatment of neonatal gilts during the first two weeks of life increased ($P<0.05$) the relative abundance of a single 51 kDa uterine protein band, corresponding to ESR1 (Figure 3A), and a 46 kDa band, corresponding to dimeric VEGFA protein (Figure 3B) at PND 14. At PxD 12, transient exposure to EV for two weeks from birth decreased the relative abundance of endometrial ESR1 protein

(Figure 4A; $P<0.05$), however, there were no changes in VEGFA protein levels (Figure 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 (Figure 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 two weeks from birth in comparison to controls (data not shown). Zymographic analysis of PxD 12 adult endometrial proteins 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 (Figure 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 two 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 two 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 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 affect 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 two weeks of life (Yan *et al.* 2006a) 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 two 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 uterotrophic, long-term effects, as assessed on PxD 12, were antiuterotrophic (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.* 2006b). 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.* 2006b, Frankshun *et al.* 2009). Relaxin increased uterine growth in neonatal gilts (Yan *et al.* 2006a) 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 two weeks from birth increased uterine *RXFP1* expression at PND 14, support previous studies in both pig reproductive tract and human cell lines showing that estrogen exposure increased *RXFP1* expression (Yan *et al.* 2008, Maseelall *et al.* 2009). Importantly, data also show that the estrogen-induced increase in uterine RXFP1

expression observed on PND 14 is similar to what was 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 (Ljubica 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 with EV as neonates is associated with dysregulation of RLX-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 estrogen sensitive, 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 complement changes associated with uterine weight as a result of transient neonatal EV exposure (Tarleton *et al.* 1999, Tarleton *et al.* 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 genes associated with tissue growth, patterning and remodeling for which estrogen-

sensitive uterine expression patterns can be altered during the first two weeks of neonatal life 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. Temporo-spatial 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.* 1993). 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 important insight into mechanisms regulating reproductive efficiency and performance.

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Figure 1

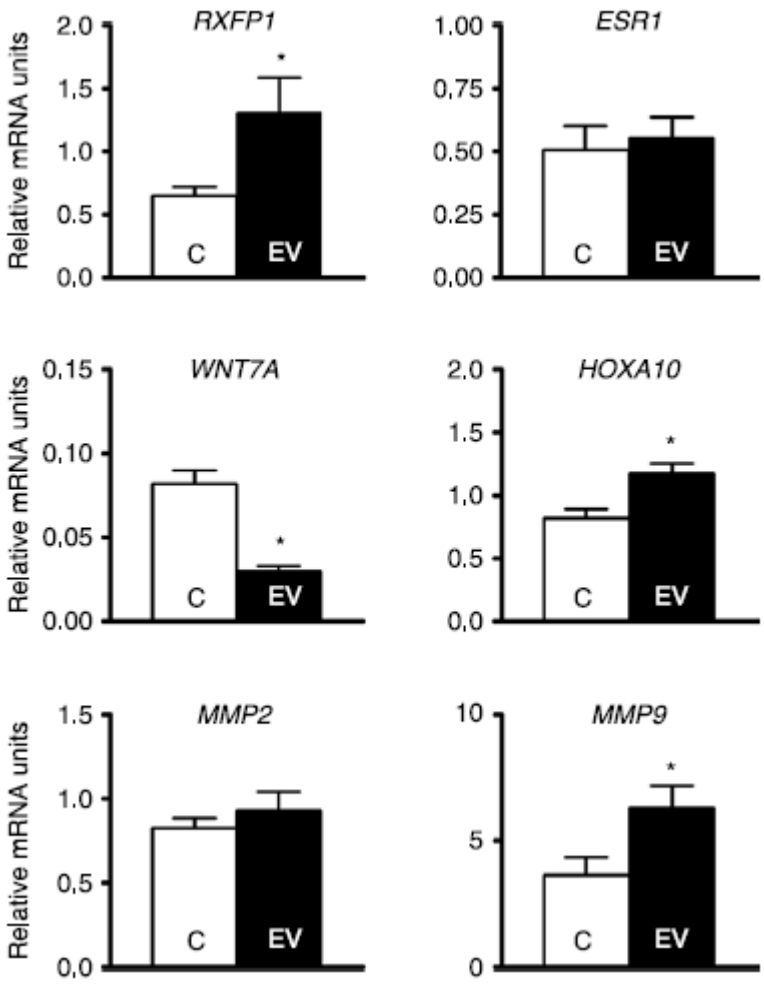

Figure 1. Effects of EV, administered from PND 0 to PND 13, on porcine uterine gene expression at PND 14. Transcripts for *RXFP*, *ESR1*, *WNT7A*, *HOXA10*, *MMP2*, *and MMP9* were quantified by qPCR and normalized to the expression of the housekeeping gene *PPIA*. Data are expressed as LSM ± SEM. N=4-6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

Figure 2

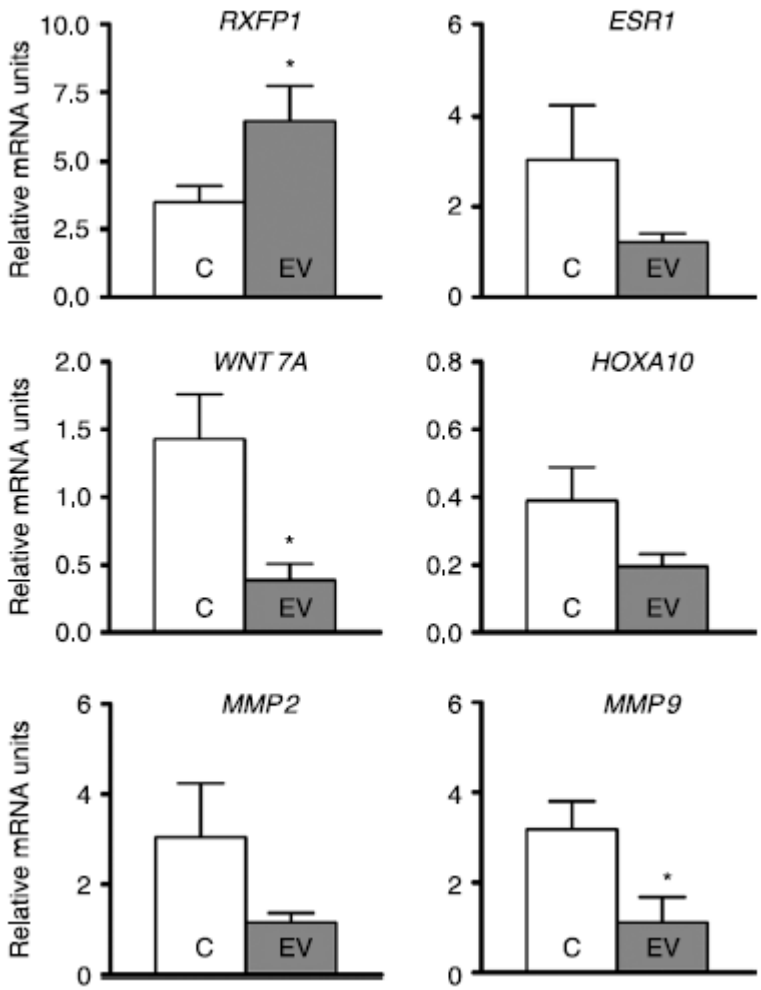

Figure 2. Effects of EV, administered from PND 0 to PND 13, on porcine endometrial levels of mRNA at PxD 12. Transcripts for *RXFP*, *ESR1*, *WNT7A*, *HOXA10, MMP2, and MMP9* were determined by qPCR and normalized to the expression of the housekeeping gene *PPIA*. Data are expressed as LSM ± SEM. N=4-6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

Figure 3

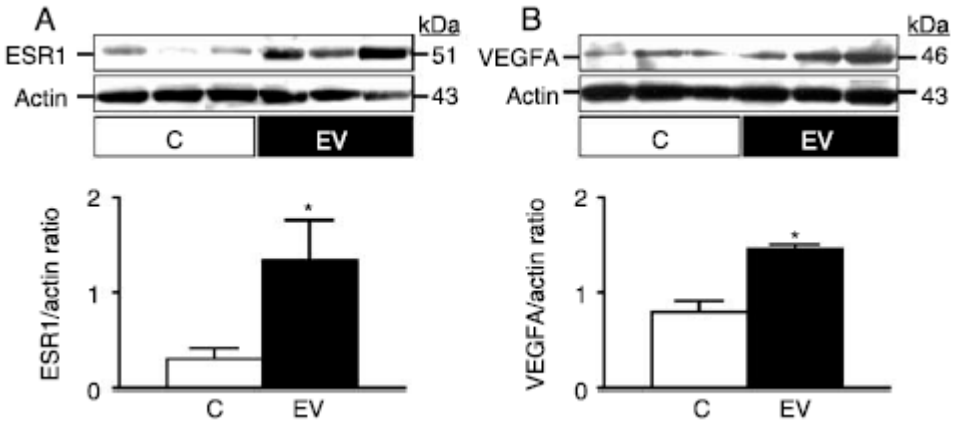

Figure 3. Effects of EV, administered from PND 0 to PND 13, on porcine uterine ESR1 (A) and VEGFA (B) protein at PND 14. Representative immunoblots for each treatment group are shown. Both 51 and 46 kDa immunoreactive bands for ESR1 and VEGFA respectively, are indicated (A and B). An immunoreactive band 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 ± SEM. N=4-6 animals per group. Asterisk indicates $P<0.05$.

Figure 4

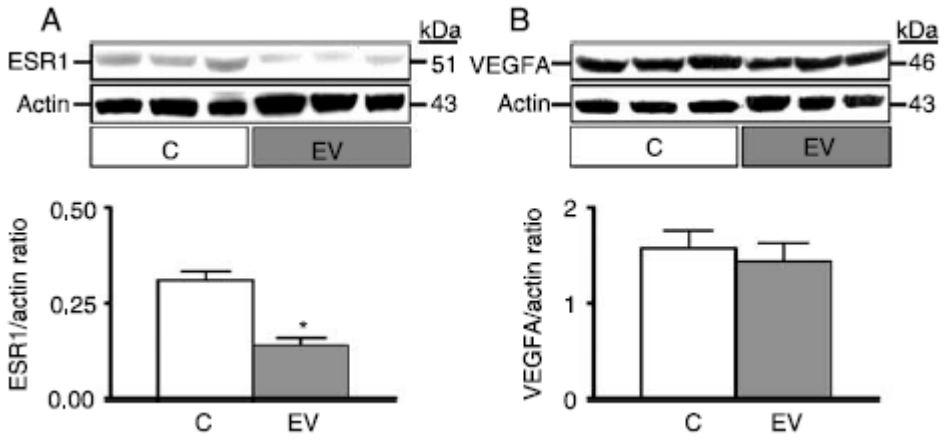

Figure 4. Effects of EV, administered from PND 0 to PND 13, on porcine uterine ESR1 (A) and VEGFA (B) protein at PxD 12. Representative immunoblots for each treatment group are shown. Both 51 and 46 kDa immunoreactive bands for ESR1 and VEGFA respectively, are indicated (A and B). An immunoreactive band 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 ± SEM. N=4-6 animals per group. Asterisk indicates $P<0.05$.

Figure 5

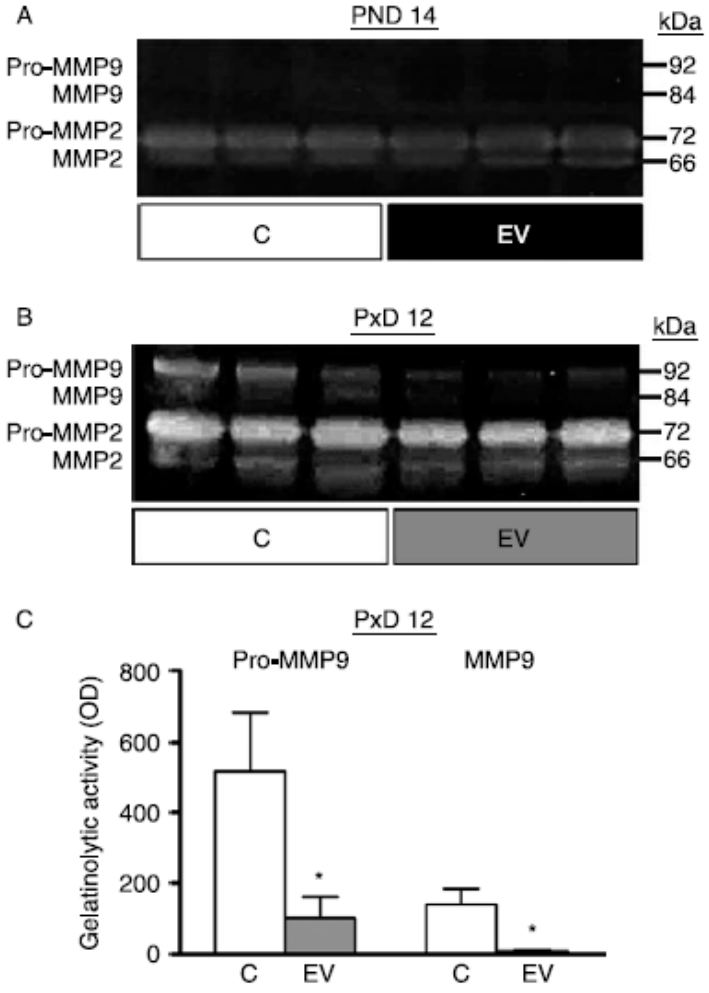

Figure 5. Effects of EV, administered from PND 0 to PND 13, on porcine uterine MMP9 and MMP2 gelatinase activity at PND 14 (A) and PxD 12 (B). Representative zymograms for each age and treatment group are shown. In the zymograms, clear zones against the dark background indicate gelatinolytic activity for pro MMP9 (92 kDa), MMP9 (82 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 ± SEM; N=4-6 animals per group). Asterisk indicates $P<0.05$.

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

Gene	Accession #	Forward Primer	Reverse Primer	Amplicon
WNT7A	**CA997684**	CACCACCAAGACC TGCTGG	TCCTTGAGCACGTAG CCCA	63 bp
HOXA10	**AF281156**	CGGCCGGAAGAAG CA	AGAAACTCCTTCTCC AGCTCCA	63 bp
ESR1	**AF035775**	AGGGAGAGGAGTT TGTGTG	TCTCCAGCAGCAGGT CATAG	306 bp
RXFP1	**CA994862**	GCATCACTTTGAGG CAGAGACA	CCTCGGCAAAGACAT TGCAT	69 bp
MMP2	**NM214192**	GAGCACCATCGAG ACCATGA	TTGTAATTGGCCACG TCGG	60 bp
MMP9	**DQ132879**	TGGATCCAAAACT ACTCGGAAGAC	CGGACAAAGGCGTC G	59 bp
PPIA	**AU058466**	TTATAAAGGTTCCT GCTTTCACAGAA	TGCCATTATGGCGTG TGAAG	77 bp

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CHAPTER III

PERINATAL ZEARALENONE EXPOSURE AFFECTS RXFP1, RXFP2 AND MORPHOREGULATORY GENE EXPRESSION IN THE NEONATAL PORCINE UTERUS

Portions of this chapter were published as a short communication in:

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ABSTRACT

The mycotoxin zearalenone (ZEA) is a selective estrogen receptor modulator (SERM) that can contaminate cereal feeds leading to reproductive disorders in domestic species, with the most severe cases occurring in the pig. To determine effects of perinatal ZEA exposure on uterine expression of genes associated with endometrial development in the neonatal gilt, pregnant sows were fed ZEA (1500 μg ZEA/kg feed/day) or vehicle from 14 days before farrowing through postnatal day (PND) 20-21, when neonatal uterine tissues were collected. At birth, gilts were cross-fostered to generate four ZEA exposure groups (n=5-6/group): unexposed controls or exposures limited to prenatal (*in utero* exposure), postnatal (exposure through nursing), or pre- and postnatal (continuous) periods. Results showed that at PND 20-21, uterine *ESR1*, *WNT7A* and *HOXA10* mRNA levels were decreased in neonates exposed continuously to ZEA ($P<0.05$). Uterine *RXFP1* transcripts were decreased in postnatally and continuously exposed groups ($P<0.05$). Data suggest that in the pig, bioactive compounds such as ZEA can be passed from the mother to the neonate during gestation and via nursing during the early weeks of life, and can affect the developmental program of the neonatal porcine uterus.

INTRODUCTION

Zearalenone (ZEA), is a nonsteroidal mycotoxin and selective estrogen receptor modulator (SERM) (Zinedine *et al.* 2007). ZEA is a product from fungi in the genus *Fusarium*, a common group of fungi that reside in temperate climates throughout the world (Vesonder *et al.* 1991). These fungi have been documented to infect a variety of crops, including soybeans, corn and wheat (Tanaka *et al.* 1986, Kuiper-Goodman *et al.* 1987). Since common agricultural animal feeds are susceptible to *Fusarium* contamination, animals that consume infected feeds are also exposed to ZEA. Studies show that exposure of ZEA to commercial agricultural animals result in pathologies associated with reproduction, such as reduced egg counts in turkeys (Allen *et al.* 1983), infertility in cows (Weaver *et al.* 1986) and reduced conception rates in sows (Vanyi *et al.* 1994). Among agricultural animals shown to be affected by ZEA, pigs have been implicated as one of the most sensitive species (Diekman & Green 1992). The high sensitivity of the pig to ZEA has been hypothesized to be due to hepatic and intestinal biotransformation of ZEA to its more potent metabolite alpha-zearalenol, both of which possess estrogenic activity (Kollarczik *et al.* 1994).

Binding studies indicate that ZEA binds with approximately 10% affinity to both estrogen receptor alpha (ESR1) and beta (ESR2) compared with estradiol (Kuiper *et al.* 1998). In addition, ZEA binds to ESR1 as a full agonist, while binding to ESR2 as a mixed agonist/antagonist (Kuiper *et al.* 1998, Mueller *et al.* 2004). Thus, exposure of ESR1-positive tissues to ZEA would likely result in ESR1 activation and downstream signaling. In the pig, functional estrogen receptor ESR1 is absent at birth and develops temporalspatially with age (Yan *et al.* 2006a), while the presence of ESR2 has been

shown to be undetectable in the pig during the first two weeks of life (Yan *et al.* 2006a). Neonatal porcine uterine ESR1 is responsive to estrogen exposure as early as postnatal day (PND) 2 (Yan *et al.* 2008) and is necessary for endometrial adenogenesis (Tarleton *et al.* 1999) during neonatal life, as well as facilitating recognition of pregnancy signaling at adulthood (Bazer & Thatcher 1977, Marengo *et al.* 1986).

Aberrant activation of ESR1 by exogenous exposure to estrogens can affect neonatal uterine programming and result in functional consequences at maturity. For example, exposure to estrogens during the first two weeks of life alters the uterine developmental program (Chen *et al.* 2010) and uterine function in adults (Tarleton *et al.* 2003), ultimately resulting in reduced uterine capacity at term (Bartol *et al.* 1993). Alterations in the neonatal uterine program were reflected by changes in the expression of uterine developmental genes associated with growth (ESR1 and the relaxin receptor RXFP1) and patterning (WNT7A and HOXA10) compared to control animals (Chen *et al.* 2010). This indicates that the porcine uterine developmental program can be disrupted by estrogenic agents and result in functional consequences at maturity.

Previous studies in the pig illustrate that consumption of ZEA by pregnant gilts results in ZEA being metabolized and secreted into colostrum/milk postpartum (Sandor 1983). These events can result in exposure of developing pigs to ZEA: prenantally *in utero* and postnatally through nursing. Whether ZEA exposure of this nature can alter the developmental program of the neonatal porcine uterus has not been investigated. Thus, the objective of this study was to determine the effects of pre- and postnatal ZEA exposure on neonatal porcine uterine developmental gene expression at post natal day (PND) 21.

MATERIALS and METHODS

Materials

ZEA was purchased from Sigma-Aldrich (Sigma-Aldrich Germany). TRI Reagent was obtained from Sigma-Aldrich. (St. Louis, MO) and RNeasy Mini Kits, RNase-Free DNase Sets and RNALater were from Qiagen Inc. (Valencia, CA). SuperScript III First-Strand Synthesis System for RT-PCR was from Invitrogen (Carlsbad, CA). SYBR Green PCR Master Mix was purchased from Applied Biosystems (Foster City, CA). Primers were synthesized by Sigma-Aldrich.

Experimental design

At puberty, gilts were synchronized for estrus and ovulation using Altrenogest, PMSG and hCG. Gilts were subsequently artificially inseminated. At day 101 of pregnancy, pregnant gilts (*Sus scrofa domesticus*) were given ZEA (1500 μg ZEA/kg feed/day) dissolved in ethanol and injected into commercially available muffins through lactation until the day of weaning at PND 21. Control gilts were given muffins without ZEA. At birth, groups of neonatal gilts were cross fostered to generate four experimental groups (n=5-6 animals/group) as illustrated in Figure 1: unexposed controls or exposures limited to prenatal, postnatal or pre and postnatal (continuous) periods. Care was taken to ensure that treatments were balanced for potential effects of litter and sows were nursing litters of similar size. Neonatal gilts were sacrificed at PND 21 and uterine tissues were collected. Animals were obtained from the Official Laboratory for Public and Veterinary Health Saxony, Leipzig, Germany and the Anhalt University of Applied Science, Bernburg, Germany. All procedures involving animals were reviewed and approved by relevant Institutional Animal Care and Use Committees.

Tissue collection, RNA isolation and cDNA generation

Neonatal uterine tissues were removed, trimmed of fat and associated tissues and ligaments and wet weights recorded. Uterine tissues are stored in RNALater solution and stored at –80°C. Total RNA was isolated from 30-50 mg of uterine tissue/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. Reverse transcription was performed with 500 ng total RNA per sample using the PTC-200 Peltier Thermal Cycler (Bio-Rad Laboratories Inc., South San Francisco, CA) and SuperScript III First-Strand Synthesis System for RT-PCR. All procedures were carried out following 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, Foster City, CA) 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, Foster City, CA) and synthesized by Sigma-Aldrich. 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 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 pregnancy day 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.

Statistics analyses

Data were subjected to analysis of variance using general linear model procedures available with SAS (SAS 2008–2009). For uterine *ESR1*, *RXFP1*, *WNT7A* and *HOXA10* transcripts, statistical models considered variation due to main effects of age at first exposure (14 days before birth vs birth) and duration of exposure (prenatal or postnatal vs continuous perinatal exposure). Preplanned contrasts included control vs prenatally exposed, control vs postnatally exposed, prenatally exposed vs postnatally exposed, prenatally exposed vs continuously exposed, postnatally exposed vs continuously exposed. Data were expressed as least square means (LSM) with standard errors (SEM).

RESULTS

Uterine expression of developmental genes in response to ZEA exposure

Results of qPCR analysis illustrating the effects of prenatal (Pre), postnatal (Post) or continuous (Both) exposure of ZEA on the expression of porcine uterine developmental genes are shown in Figure 2. Data indicate that continuous perinatal exposure to ZEA decreased ($P<0.05$) uterine *ESR1*, *WNT7A* and *HOXA10* transcript levels at PND 21 (Figure 2A, C, D) compared to unexposed, prenatally or postnatally exposed groups. Expression of *RXFP1* mRNA was decreased ($P<0.05$) in both postnatally exposed and continuously exposed groups in comparison to unexposed or prenatally exposed animals (Figure 2B).

DISCUSSION

Exposure to endocrine-disrupting compounds during embryonic and neonatal life can result in the alterations in the tissue developmental program with lasting consequences for tissue function at adulthood (Soto *et al.* 1994, Andersen *et al.* 2007). These exposures could occur prenatally *in utero* by transfer across the placenta, postnatally through nursing or both. Data obtained in the current study reinforce this idea by illustrating pregnant sows that ingest ZEA expose their embryos and nursing young to ZEA during gestation and postnatally through nursing. Kauffold and colleagues confirmed ZEA uptake, showing that there were milligram levels of ZEA in the maternal bile two days after weaning that was a thousand times higher than control animals (Kauffold 2008). Together, data suggest that only continuous perinatal ZEA exposure, during gestation and nursing, can affect changes in the expression of uterine developmental genes during the early weeks of life.

The results obtained in this study are supported by data in cows, indicating that feeding 544 mg/day ZEA to lactating heifers for 21 days resulted in nanogram levels of ZEA in the plasma and milk (Prelusky *et al.* 1990). Studies in rats also show that treatment of pregnant animals with ZEA resulted in pathological conditions in the fetuses *in utero*, such as reduced fetal body weight, delayed fetal development and inhibition of skeletal ossification (Collins *et al.* 2006). Given these data, it was not surprising that ZEA metabolism was observed in pregnant gilts fed ZEA daily and that ZEA exerted effects both *in utero* and postnatally through nursing.

The expression of uterine *ESR1*, *WNT7A* and *HOXA10* were all decreased only in animals exposed for the entire perinatal period to ZEA. This would suggest that

embryonic exposure to ZEA primed the embryonic uterus for subsequent postnatal exposures in neonatal life. This was surprising, given that expression of both porcine uterine *ESR1* mRNA and protein have previously been shown to be undetectable at birth (Tarleton *et al.* 1998, Yan *et al.* 2006a), and presumably, *in utero*. These observations could be partially explained by the fact that metabolites of estrogens have been previously shown to have the potential to interact with DNA without binding to estrogen receptors, subsequently causing DNA damage and interfering with transcription (Cavalieri *et al.* 2000, Yue *et al.* 2003, Yager & Davidson 2006). Whether metabolites of ZEA and/or alpha-zearalenol can affect DNA directly remains to be determined. Since these genes have all been implicated as critical mediators of neonatal porcine growth and morphogeneisis (Bartol *et al.* 1993, Tarleton *et al.* 1999, Bartol *et al.* 2006), alterations in their expression reflects a disrupted uterine developmental program with potential consequences at adulthood. Studies also indicate that a single intravenous administration of ZEA at 5mg/kg is usually cleared between 3-4 days (Kuiper-Goodman *et al.* 1987). Thus, the idea that ZEA is being accumulated in the developing young exposed *in utero* and postnatally through nursing, resulting in additive ZEA-induced effects is possible.

The expression of uterine *RXFP1* transcripts were decreased in both postnatal and continuously exposed groups compared to control, implying that *RXFP1* gene expression can be regulated postnatally by estrogens. Transcripts for uterine *RXFP1* are detectable at birth (Yan *et al.* 2006b), and treatment with relaxin increases uterine ESR1 as early as PND 2 (Yan *et al.* 2008) while relaxin exposure at two weeks of age increase uterine weight (Yan *et al.* 2006a). Therefore, RXFP1 could be an important mediator of uterine growth during early neonatal life. The results observed here are supported by previous

research indicating that exposure to estrogens during the first two weeks of life increases uterine *RXFP1* transcripts at PND 14 (Chen *et al.* 2010). Tissues from gilts that were exposed neonatally to estrogens also exhibited increases in endometrial RXFP1 levels (Chen *et al.* 2010) and ultimately, had reduced reproductive capacity at pregnancy (Bartol *et al.* 1993). Since altered neonatal porcine uterine *RXFP1* expression marked a negative outcome for uterine function, the expression patterns of *RXFP1* mRNA in this study could also result in a compromised trajectory of uterine development with negative outcomes for uterine capacity at adulthood.

Studies in both rodents and humans reveal that *in utero* exposure to endocrine disruptors not only induces direct effects on reproductive organ function, but may predispose those tissues to other pathological conditions such as cancer (Brown *et al.* 1998, Kaufman *et al.* 2000). However, less is known regarding the potential for endocrine disruptors to be secreted into milk and passed into the neonate through nursing. The term lactocrine was recently coined to define the transmission of milk/colostrum-derived bioactive factors into the neonate as a consequence of nursing to affect target neonatal tissues (Bartol *et al.* 2009). The results of this study extends the lactocrine concept by illustrating that ZEA in the environment could also be passed through nursing to disrupt the developmental program of sensitive tissues. Together, this research shows that perinatal exposure of developing neonatal gilts to ZEA as a consequence of ZEA ingestion by their pregnant and/or lactating mothers can affect neonatal gene expression patterns implicated in the regulation of uterine development. These effects, induced by uteroplacental and/or mammary delivery of ZEA, may alter the normal uterine

developmental trajectory with long term consequences on uterine function and reproductive success later in life.

ACKNOWLEDGEMENTS

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Figure 1

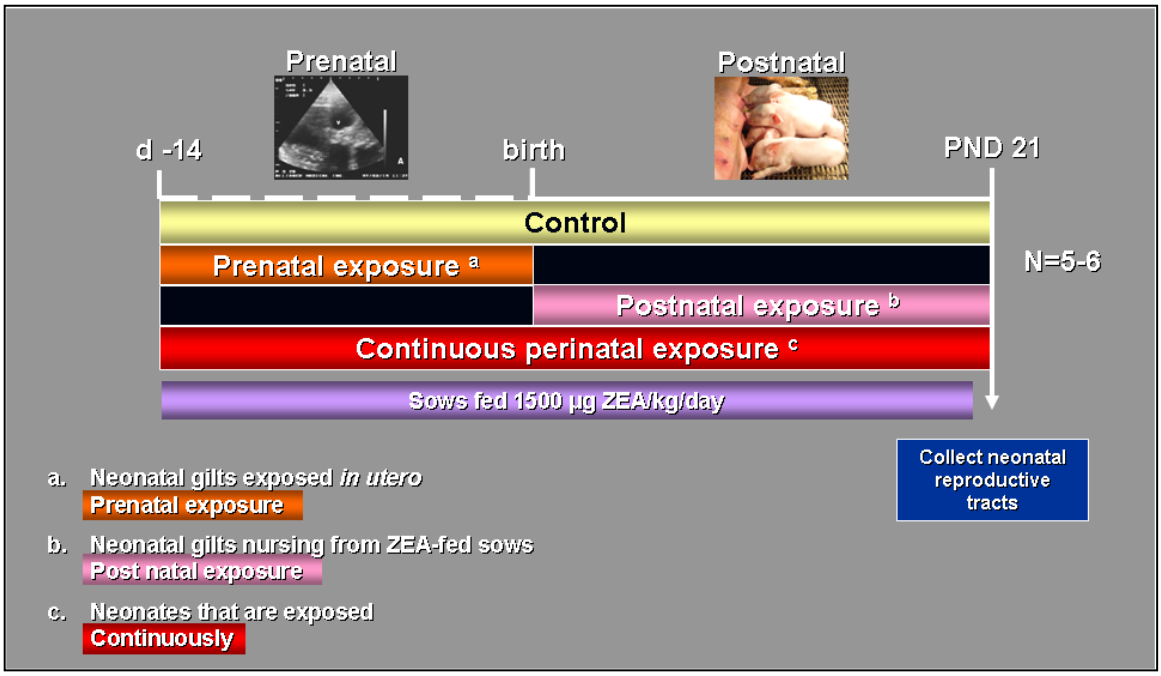

Figure 1. Experimental design to evaluate the effects of prenatal, postnatal or continuous (both) ZEA exposure on the neonatal porcine uterus. At two weeks before birth, pregnant gilts were fed ZEA (1500 µg ZEA/kg feed/day) through PND 21. At birth, neonatal gilts were cross fostered to generate 4 groups: unexposed neonates; neonates that were exposed prenatally; neonates that were exposed postnatally; neonates continuously exposed throughout the perinatal period. At PND 21, uteri were collected from neonates for analysis. N=5-6 animals per group.

Figure 2

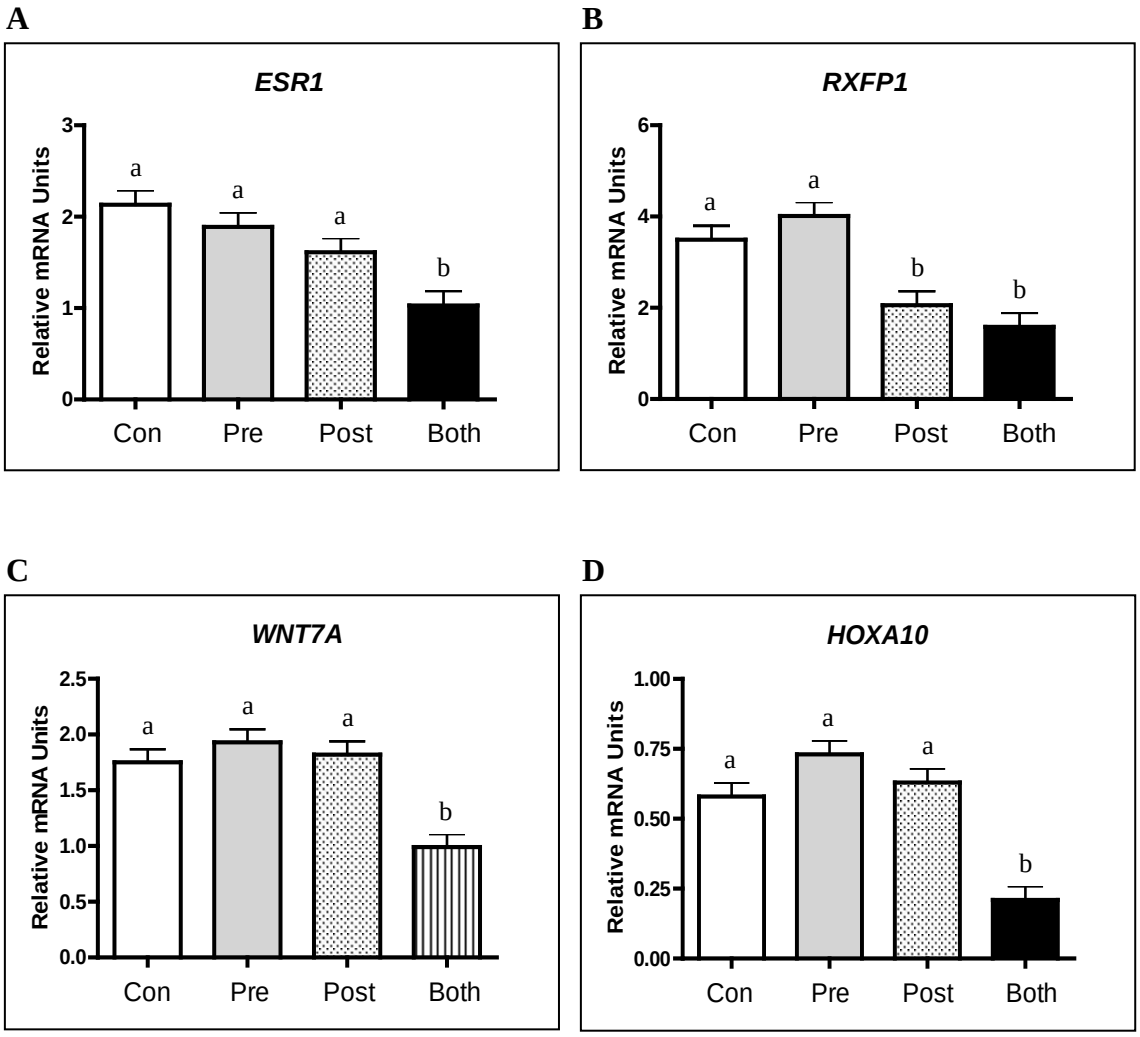

Figure 2. Effects of prenatal, postnatal or continuous (both) ZEA exposure on porcine uterine morpho-regulatory gene expression at PND 20-21. Transcripts for *ESR1*, *RXFP1*, *WNT7A* and *HOXA10* were quantified by qPCR and normalized to the expression of the housekeeping gene*PPIA*. Data are expressed as LSM ± SEM. N=5-6 animals per group. Different letters indicate P<0.05.

Table 1

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

Gene	Accession #	Forward Primer	Reverse Primer	Amplicon
WNT7A	**CA997684**	CACCACCAAGAC CTGCTGG	TCCTTGAGCACGTA GCCCA	63 bp
HOXA10	**AF281156**	CGGCCGGAAGAA GCA	AGAAACTCCTTCTC CAGCTCCA	63 bp
ESR1	**AF035775**	AGGGAGAGGAGT TTGTGTG	TCTCCAGCAGCAGG TCATAG	306 bp
RXFP1	**CA994862**	GCATCACTTTGA GGCAGAGACA	CCTCGGCAAAGACA TTGCAT	69 bp
PPIA	**AU058466**	TTATAAAGGTTC CTGCTTTCACAG AA	TGCCATTATGGCGT GTGAAG	77 bp

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CHAPTER IV

RELAXIN (RLX) AND ESTROGEN AFFECT ESTROGEN RECEPTOR α, VASCULAR ENDOTHELIAL GROWTH FACTOR, AND RLX RECEPTOR EXPRESSION IN THE NEONATAL PROCINE UTERUS AND CERVIX

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ABSTRACT

The porcine female reproductive tract undergoes estrogen receptor (ER) α-dependent development after birth (postnatal day = PND 0), the course of which can determine adult uterine function. Uterotrophic effects of relaxin (RLX) in the porcine neonate are age-specific and may involve ER activation. Here objectives were to determine effects of RLX and estrogen administered from birth on uterine and cervical growth and expression of ERα, vascular endothelial growth factor (VEGF) and the RLX receptor (RXFP1). On PND 0, gilts were treated with the antiestrogen ICI 182,780 (ICI) or vehicle alone and, two hours later, were given estradiol-17β (E) or porcine RLX for two days. Neither RLX nor E affected uterine wet weight or protein content on PND 2. However, RLX, but not E, increased cervical wet weight and protein content when compared to controls. Pretreatment with ICI did not inhibit RLX-stimulated cervical growth. Uterine and cervical ERα increased in response to RLX, but not E. Both RLX and E increased VEGF in the uterus and cervix on PND 2. Pretreatment with ICI increased VEGF in both tissues and increased RLX-induced cervical VEGF. In the uterus E, but not RLX, increased RXFP1 mRNA. In the cervix, E increased RXFP1 gene expression whereas RLX decreased it. Results indicate that the neonatal uterus and cervix are sensitive to E and RLX and that growth responses to RLX in these tissues differ by PND 2. Effects of RLX on uterine and cervical ERα and VEGF expression may be important for neonatal reproductive tract development.

INTRODUCTION

Growth of the female reproductive tract (FRT) is sensitive to both estrogen and relaxin (RLX) (Vasilenko & Mead 1987, Cullinan-Bove & Koos 1993, Hall & Anthony 1993, Pillai *et al.* 1999) and organizational events responsible for both tissue patterning and programming are estrogen receptor (ER)-dependent (Couse & Korach 1999, Tarleton *et al.* 1999). Thus, factors that affect ER expression and activation can determine the developmental trajectory of these tissues (Schonfelder *et al.* 2002, Suzuki *et al.* 2002, Nikaido *et al.* 2005). Evidence that ER antagonists block uterotrophic effects of RLX in the rat (Pillai *et al.* 1999) and neonatal pig (Yan *et al.* 2006a) and that RLX is required to support myometrial ERα expression in RLX-null mice (Siebel *et al.* 2003) indicate that RLX should be included in the list of factors with the potential to affect FRT programming.

It is well established that patterns of ER expression in FRT tissues are regulated both temporally and spatially during the course of development (Yamashita *et al.* 1989, Glatstein & Yeh 1995, Mowa & Iwanaga 2000, Okada *et al.* 2005). Moreover, aberrant activation of the ER system can have lasting consequences for FRT morphology and function (Miller *et al.* 1998, Markey *et al.* 2005). In the pig, this is supported by evidence that the neonatal uterus: (i) is ERα negative at birth (Tarleton *et al.* 1998); (ii) develops sensitivity to estrogen between birth (postnatal day = PND 0) and PND 15 that is associated with the appearance and proliferation of endometrial glands and expression of ERα in the stroma and glandular epithelium (Tarleton *et al.* 1998, Tarleton *et al.* 2001); and that (iii) transient estrogen exposure from birth affects adult endometrial

phenotype, uterine responses to conceptus signals and uterine capacity to support pregnancy (Tarleton *et al.* 2001, Tarleton *et al.* 2003).

Following identification of LGR7, now designated RXFP1 (Yan *et al.* 2006b), as the cognate RLX receptor (Hsu *et al.* 2002), its expression was confirmed in the neonatal uterus at birth, prior to the onset of ERα expression (Yan *et al.* 2006b). Taken together with evidence that uterotrophic effects of both estrogen (Spencer *et al.* 1993, Tarleton *et al.* 2001) and RLX become more pronounced with age between birth and PND 15 (Yan *et al.* 2006a), these observations indicated that functional ERα and RLX receptor systems evolve in neonatal porcine FRT tissues during the first days of neonatal life. Recent studies showed that RLX is absent from the neonatal circulation at birth, prior to nursing, but is present in colostrum or first milk at the time of parturition and is likely transmitted into the neonatal circulation during the first 48 h of postnatal life as a consequence of nursing (Yan *et al.* 2006b). Therefore, a maternally driven lactocrine mechanism was hypothesized whereby milk-borne RLX, absorbed into the neonatal circulation during the first days of postnatal life, could act directly through RXFP1 and/or indirectly through the evolving ER system to influence developmental programming events in the neonate (Yan *et al.* 2006b).

Trophic effects of RLX on reproductive tissues, including the uterus, cervix and vagina, of prepubertal and adult animals are well documented (Sherwood 2004). Given that all of the components of a classical RLX signaling system are present in neonatal gilts at birth (Yan *et al.* 2006a) and that functional crosstalk between RLX and estrogen signal transduction systems is likely to influence FRT growth (Pillai *et al.* 1999, Yan *et al.* 2006a), studies now focus on the impact of these hormones on FRT development

during early postnatal life. Here, objectives were to determine short-term effects of RLX and estradiol-17β, administered for two days from birth, on FRT growth and the expression of ERα, vascular endothelial growth factor (VEGF), a marker of estrogen (Cullinan-Bove & Koos 1993) and RLX (Unemori *et al.* 1999) action, and RXFP1 in the uterus and cervix of neonatal gilts.

MATERIALS and METHODS

Materials

Porcine RLX (CM-A fraction; 3000 U/mg) was prepared at the Department of Biomedical Sciences (University of Guelph, Ontario, Canada) by extraction and purification from the ovaries of pregnant sows (Sherwood & O'Byrne 1974). Purity was confirmed by SDS-PAGE, which revealed a single band at approximately 6.2 kDa. Biological activity of the RLX preparation was ascertained by inhibition of spontaneous uterine motility in vitro (Wiqvist & Paul 1958) and immunoreactivity was verified by RIA (Porter *et al.* 1992). Estradiol-17β was purchased from Sigma-Aldrich (St. Louis, MO, USA). ICI 182,780 was kindly provided by Dr. Alan Wakeling, Zeneca Pharmaceuticals (Macclesfield, Cheshire, UK). Mouse anti-human ERα (Ab-15) monoclonal antibody was purchased from NeoMarkers, Inc. (Fremont, CA, USA). Goat anti-human VEGF (sc-152-G) and goat anti-human β-actin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). For ERα detection an anti-mouse secondary antibody from Santa Cruz Biotechnology, Inc. (sc-2005) generated a non-specific signal at 64 kDa, similar to the size of ERα, when used alone (Marriott *et al.* 2007). Therefore, appropriate secondary antibodies conjugated to horse radish peroxidase that did not generate a nonspecific signal (Cat # 62-6620; Zymed Inc., San Francisco, CA, USA) were used for immunoblotting. Nitrocellulose membranes were obtained from Bio-Rad Laboratories (Hercules, CA, USA). The Renaissance Western Blot Chemiluminescence Reagent Plus kit was obtained from PerkinElmer Life Science (Boston, MA, USA). X-Omatic films were purchased from American Imaging (South Plainfield, NJ, USA). TRI-Reagent was obtained from Sigma-Aldrich. RNeasy Mini Kit

and RNase-Free DNase Set were obtained from Qiagen Inc. (Valencia, CA, USA). SuperScript III First-Strand Synthesis System for RT-PCR was obtained from Invitrogen (Carlsbad, CA, USA). SYBR Green PCR Master Mix was purchased from Applied Biosystems (Foster City, CA, USA). All other chemicals were purchased from Sigma-Aldrich and Invitrogen.

Animals

Yorkshire-Landrace gilts were obtained from the Swine Unit of the New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick, NJ, USA. Gilts were randomly assigned to one of six treatment groups on PND 0 as follows: (1) Control (C; vehicle; DMSO:ETOH, 4:1 and PBS; n = 6); (2) ICI 182,780 (ICI; 1 mg/kg BW, given ip in 200 μl DMSO:ETOH, 4:1 vehicle as a single injection 2 h prior to start of hormone treatment, n = 4); (3) Estradiol-17β (E; 50 μg/kg BW, given i.p. in 250 μl DMSO:ETOH, 4:1 vehicle every 24 h for 48 h, n = 6); (4) ICI/E (n = 4); (5) porcine RLX (R; 20 μg/kg BW, given i.m. in 250 μl PBS every 6 h for 48 h, n = 8); and (6) ICI/R (n = 5). During treatment, neonatal gilts were maintained with sows and allowed to suckle naturally. Timing, dosage and route of ICI administration were based on studies in rodents (Gibson *et al.* 1991, Pillai *et al.* 1999). Additionally, ICI was effective in blocking the E-induced uterine growth response observed in neonatal gilts that received the same E treatment regimen for two days prior to collection of ER-positive uterine tissues on PND 14 (Yan *et al.* 2006a). The dose and timing of RLX administration were based on studies in prepubertal gilts and rodents (Hall *et al.* 1990, Pillai *et al.* 1999). Gilts were weighed and sacrificed 3 h after the last injection on PND 2. Each uterus and cervix was trimmed free of fat and associated ligaments and weighed. Tissues were

frozen in liquid nitrogen and stored at –80 °C. All procedures involving animals were reviewed and approved as appropriate by the Rutgers University Animal Care and Facilities Committee (Protocol # 88-079). Procedures 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).

Protein Extraction and Evaluation of ERα and VEGF Expression

Tissue samples were homogenized in ice-cold lysis buffer (4 ml/g tissue) containing 1% Triton X-100, 10% glycerol, 150 mM Tris-HCl, 300 mM NaCl, 1 mM $MgCl_2$, pH 7.5. The resulting tissue lysate was passed (5 times) through a 20-gauge needle fitted to a syringe, incubated on ice for 1 h and centrifuged (12,000 g, 4 °C) for 5 min. Protein concentration of the supernatant was measured using a detergent-compatible protein assay kit (DC Protein Assay, Bio-Rad Laboratories). Tissue protein content was calculated based on the protein concentration and wet weight of the organ.

To document ERα and VEGF protein expression, uterine and cervical proteins (30 μg) were resolved on 12.0% Bis-Tris-HCl-buffered polyacrylamide electrophoresis gels under reducing conditions in the presence of SDS and transferred onto nitrocellulose membranes. After blocking in 10.0% nonfat dry milk in Tris-buffered saline containing Tween-20 [TBST; 25 mM Tris (pH 7.5), 0.14 mM sodium chloride (NaCl), 3 mM potassium chloride (KCl), 0.05% Tween-20], membranes were probed with either mouse anti-human ERα antibody (1:100) or goat anti-human VEGF antibody (1:1000) overnight at 4 °C. After washing with TBST, blots were incubated with either horseradish peroxidase-conjugated anti-mouse or anti-goat secondary antibody (1:1000) for 1 h at room temperature, and bound antibodies were detected by enhanced chemiluminescence.

Membranes were stripped with buffer (100 mM 2-β-mercaptoethanol, 2% SDS and 62.3 mM Tris-HCl) and reprobed with goat anti-human β-actin (1:1000) to determine the amount of protein loaded on the gels. Signals on films were quantified using Scion image densitometric software (Scion Corporation, Frederick, MD, USA).

RNA Isolation and cDNA generation

Total RNA was isolated from 30-50 mg of tissue samples using TRI-Reagent followed by RNeasy Mini Kit and 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. Reverse transcription was performed with 5 μg RNA per sample using the PTC-200 Peltier Thermal Cycler (Bio-Rad Laboratories, Inc., South San Francisco, CA, USA) and SuperScript III First-Strand Synthesis System for RT-PCR. All procedures were carried out following manuals/guidelines provided by the manufacturers.

Real time quantitative reverse transcription polymerase chain reaction

Real time quantitative RT-PCR (qRT-PCR) was performed using an Applied Biosystems Gene Amp 7000 Sequence Detection System and the SYBR Green method following universal thermal cycling parameters recommended by the manufacturer. Primers for quantitative qRT-PCR were designed using Primer Express Software (Applied Biosystems) and synthesized by Invitrogen Corp. Porcine RXFP1 (GenBank Accession Number: CA994862) forward - 5'-GCATCACTTTGAGGCAGAGACA-3' - and reverse - 5'-CCTCGGCAA AGACATTGCAT-3' – primers were used to generate a 69 bp amplicon. Similarly, porcine cyclophilin (GenBank Accession Number:

AU058466) forward - 5' –TTATAAAG GTTCCTGCTTTCACA GAA-3' – and reverse - 5'-TGCCATTATGG CGTGTGAAG-3' – primers were used to generate an expected 77 bp amplicon. To ensure specific amplification, multiple controls including water only, no primers and no template were included in assays. Primer quality was evaluated by amplifying serial dilutions of the cDNA template (1, 1:2, 1:5, 1:10) and dissociation curves for each set of primers were checked to ensure that there was no amplicon-independent amplification (i.e., generation of primer-dimers). PCR amplification products were analyzed by agarose gel electrophoresis to further confirm the absence of non-specific amplification. Values for cycle threshold (C_T), the point at which exponential amplification of the PCR products begins, were determined using Applied Biosystems software. Porcine cyclophilin was used as an internal control in qRT-PCR analyses to normalize cDNA input and PCR efficiency. Data were analyzed by the comparative CT method ($\Delta\Delta C_T$) for relative quantitation of gene expression in which the sample having the minimum expression level was chosen as the reference calibrator (Livak & Schmittgen 2001). Therefore, data from these analyses are presented as relative expression levels.

Statistical analyses

All quantitative data were subjected to analyses of variance using General Linear Models procedures available with SAS (SAS 2002-2003). For uterine and cervical wet weight, protein content and both ERα and VEGF gene expression data, generated by densitometry of Western blots, statistical models considered variation due to the main effects of treatment, including pretreatment with ICI. Treatment effects were identified by performing a set of preplanned contrasts that included comparisons of specific groups

as follows: control vs E; control vs RLX; E vs ICI; E vs ICI/E; and R vs ICI/R. When results of these analyses indicated a likely main effect of ICI and potential differences in the magnitude of E- and RLX-induced effects, a second set of contrasts was run with comparisons that included: control vs ICI and E vs R. Analyses of uterine and cervical RXFP1 expression data, evaluated at the transcriptional level, considered variation due to the main effects of E, RLX and their interactions. All error terms were identified based upon the expectations of the mean squares for error and data were expressed as least square means with standard errors.

RESULTS

Uterine and cervical tissue wet weights and protein content

When administered from birth, neither E nor RLX affected uterine wet weight as determined on PND 2 (Fig. 1A). Although E also had no effect on cervical wet weight, RLX treatment increased ($P<0.01$) cervical wet weight compared to controls (Fig 1B). Pretreatment of gilts with ICI did not influence the RLX-induced cervical weight gain. Results for uterine and cervical protein content mirrored those obtained for tissue wet weight data in that neither RLX nor E affected uterine protein content (Fig. 2A) whereas RLX, but not E, increased ($P<0.01$) cervical protein content on PND 2 compared to controls (Fig. 2B). Pretreatment with ICI had no effect on the RLX-induced increase in cervical protein content on PND 2.

Uterine and cervical expression of ERα and VEGF protein

Representative Western blots and results of densitometric analyses for ERα and VEGF are presented in Figures 3 and 4. Treatment with RLX, but not E, from birth increased ($P<0.04$) the relative abundance of uterine ERα protein on PND 2 when compared to controls (Fig. 3A, B). Likewise, RLX, but not E, increased ($P <0.01$) cervical ERα expression on PND 2 (Fig. 3C, D). Responses to RLX and E were unaffected by pretreatment with ICI in either the uterus or cervix (Fig. 3).

The relative abundance of dimeric VEGF protein (46 kDa) in both uterine (Fig. 4A, B) and cervical tissues (Fig. 4C, D) increased in response to RLX ($P<0.01$) and E ($P<0.01$) when compared to controls on PND 2. Interestingly, treatment with ICI alone increased VEGF expression in both the uterus ($P<0.02$) and cervix ($P<0.01$). For the uterus (Fig. 4B), ICI pretreatment had a marginal negative effect on E-induced VEGF

expression (P<0.07). In contrast, pretreatment with ICI increased (P<0.01) RLX-induced cervical VEGF expression (Fig. 4D).

Uterine and cervical expression of RXFP1 mRNA

Data illustrating effects of E and RLX administered for two days from birth on uterine and cervical RXFP1 gene expression in gilts on PND 2 are presented in Fig. 5. In the uterus (Fig. 5A), E increased (P < 0.01) RXFP1 mRNA levels when compared to tissues obtained from controls. In contrast, RLX did not affect uterine RXFP1 gene expression (Fig. 5A). In the cervix (Fig. 5B), E induced a modest increase (P<0.06) in RXFP1 gene expression, whereas RLX induced a substantial decrease (P<0.01) in this response.

DISCUSSION

While trophic effects of RLX on the reproductive tract are well documented (Sherwood 2004), the mechanism of RLX action in promoting uterine and cervical growth remains elusive. In this study we report differential effects of RLX on the uterus and cervix in the neonatal pig, in which both ERα (Tarleton *et al.* 1998) and RXFP1 expression are developmentally regulated (Yan *et al.* 2006b). Previous studies showed that the neonatal gilt is sensitive to uterine growth-promoting actions of RLX and that neonatal uterotrophic responses to RLX are both age-specific and related functionally to the relative presence and state of activation of the ERα system (Yan *et al.* 2006a). For example, RLX, administered to gilts for two days from birth, increased uterine luminal epithelial height, but not uterine weight or protein content, on PND 2. In contrast, administration of RLX for two days beginning on PND 12, after the onset of uterine ERα expression, increased uterine weight, protein content and luminal epithelial height on PND 14 (Yan *et al.* 2006a). Moreover, the latter effects were inhibited by pretreatment with ICI 182,780. These findings were consistent with data for rats showing that RLX-stimulated uterine edema is ER-dependent (Pillai *et al.* 1999).

In the present study, when treatments were initiated on PND 0, RLX increased cervical but not uterine wet weight and protein content. Additionally, cervical responses to RLX were not affected by pretreatment with ICI 182,780. These data suggest that tissue-specific trophic actions of RLX observed in the cervix of newborn gilts are unlikely to involve crosstalk with the ER signaling system at this early postnatal stage of development. Whether ER activation is involved in RLX-stimulated cervical growth later in life remains to be investigated.

Estrogen receptor-α expression was detected in both uterine and cervical tissues by PND 2 and increased in response to RLX administered from birth. To our knowledge, this is the first report that RLX increases ERα expression *in vivo.* Observations are consistent with the fact that, in the absence of RLX, myometrial ERα expression was attenuated in RLX-null mice during late pregnancy (Siebel *et al.* 2003). In contrast, studies in rats showed that RLX decreased uterine ERβ mRNA levels without affecting ERα mRNA levels (Pillai *et al.* 2002). Moreover, in the cervix and vagina of RLX null mice, chronic infusion of RLX had no effect on ERα gene expression and decreased ERβ mRNA in late gestation (Parry *et al.* 2005). Although low uterine ERβ gene expression in adult porcine tissues has been reported (Cardenas & Pope 2005), there is no evidence for porcine uterine expression of ERβ protein in the first two weeks of neonatal life (Yan *et al.* 2006a). Collectively, these observations reinforce the importance of comparative studies. Data clearly support the idea that RLX can affect patterns of ER expression in target tissues.

Although ERα protein was detectable in the uterus and cervix by PND 2, there was no evidence that treatment with ICI alone affected uterine or cervical ERα protein expression. For the uterus, this may be explained by the fact that ICI treatment was administered on PND 0, when uterine ERα expression is low to undetectable (Tarleton *et al.* 1998, Yan *et al.* 2006a), whereas tissues were obtained on PND 2. Data describing the ontogeny of porcine cervical ERα expression from birth are sorely lacking. However, if data for the uterus provide a valid reference, the absence of a detectable effect of ICI on ERα expression in neonatal FRT tissues observed here should not be surprising.

Absence of a fully functional ERα signaling system may also explain why pretreatment with ICI did not affect uterine or cervical ERα expression in response to E or RLX.

Results also indicated that VEGF expression can be used as a marker of both E and RLX action in neonatal porcine uterine and cervical tissues. Moreover, present data suggest that VEGF expression is a more sensitive marker of E action in the uterus and cervix on PND 2 than either organ wet weight or protein content. The fact that RLX stimulates both uterine and cervical VEGF expression in the neonate is consistent with similar evidence for angiogenic activity and/or VEGF expression in human endometrial stromal cells (Unemori *et al.* 1999), in the marmoset endometrium of pregnancy (Einspanier 2001) and at wound sites (Unemori *et al.* 2000).

Since ICI alone did not influence uterine or cervical wet weight, protein content, or ERα protein expression in gilts on PND 2, the observed positive effects of ICI on both uterine and cervical VEGF protein expression were somewhat unexpected. However, ICI alone was also found to increase uterine epithelial proliferation, as reflected by an increase in proliferating cell nuclear antigen (PCNA) labeling index, in gilts on PND 14 (Masters *et al.* 2007). Such effects may be explained by the fact that ICI can alter the transcription of estrogen-responsive genes via activation of Sp1 promoter elements (Kim *et al.* 2003, Fleming *et al.* 2006). Like PCNA (Shipman-Appasamy *et al.* 1991), VEGF expression is positively regulated by Sp1 activation (Milanini *et al.* 1998, Shi *et al.* 2001).

Given that objectives of the present study included evaluation of the short-term effects of E and RLX administered from birth on uterine and cervical responses, effects of these treatments on RXFP1 expression in these tissues were evaluated. Moreover,

while uterine RXFP1 expression was documented in tissues obtained at birth (Yan *et al.* 2006b), similar data for neonatal porcine cervical tissues are lacking. The fact that E administered from birth increased RXFP1 expression in both the uterus and cervix on PND 2 supports and extends previous studies indicating the importance of estrogen in sensitizing reproductive tissues to RLX (Mercado-Simmen *et al.* 1982, Downing & Hollingsworth 1992, Downing & Hollingsworth 1993). Results are consistent with the idea that estrogen priming can facilitate (Vasilenko *et al.* 1980, Vasilenko & Mead 1987) or enhance responsiveness of RLX target tissues (Adams *et al.* 1989). The observation that cervical RXFP1 expression on PND 2 was reduced following RLX administration from birth is new. These data are consistent with studies reported in rats in which infusion of unlabeled RLX reduced uterine and cervical uptake of radiolabeled RLX when compared to saline-infused controls (Downing & Hollingsworth 1993). Likewise, in RLX-null mice both cervical and vaginal RXFP1 expression increased during late pregnancy when compared to RLX-replete wild-type controls (Parry *et al.* 2005). Furthermore, continuous infusion of RLX for six days in pregnant RLX-null mice decreased cervical and vaginal RXFP1 mRNA levels on gestation day 18.5 when compared with saline-treated RLX-null controls (Parry *et al.* 2005).

Collectively, these studies point to a potential negative regulatory mechanism through which RLX down-regulates the expression of its own receptor. In studies of human endometrial cells, RLX had no effect on endometrial stromal RXFP1 expression while RLX increased RXFP1 mRNA in a dose- and time-dependent fashion in decidual cells collected at term (Mazella *et al.* 2004). However, using endometrial stromal cells under conditions known to promote decidualization *in vitro*, Ivell and colleagues reported

that RLX did not influence on RXFP1 transcript levels (Bartsch *et al.* 2004). This is consistent with data presented here showing that RLX had no effect on uterine RXFP1 expression *in vivo*. The explanation for these differences in effects of RLX on RXFP1 expression remains unclear but may be due to differences between species, endocrinological status of the tissues and/or experimental conditions.

In conclusion, data presented here show that the neonatal porcine uterus and cervix are differentially sensitive to E and RLX from birth. In addition, effects of E and RLX on cognate RLX receptor expression during this period of neonatal life are tissue specific. This study also provides further evidence for the interaction between E and RLX signaling pathways in that RLX stimulates ERα expression and E increases RXFP1 expression in the uterus and cervix of neonatal pigs. The fact that E and RLX presented to neonatal gilts from birth can increase uterine and cervical ERα expression by PND 2 has significant developmental implications. While the importance of ERα activation for cervical development remains to be investigated, it is clear that postnatal ERα expression is required for normal uterine growth and endometrial development (Bartol *et al.* 2006). Recent studies also showed that the neonatal uterus is RXFP1-positive at birth, that RLX is present in porcine milk from the first day of lactation and that RLX is detectable in the peripheral circulation of nursing pigs within 48 h of birth (Yan *et al.* 2006b). Taken together with present observations, these data suggest that critical early events associated with growth, development and programming of FRT tissues in the neonatal pig could be facilitated through the actions of milk-borne RLX delivered to the neonate from the maternal system via a lactocrine mechanism (Yan *et al.* 2006b). The extent to which

RLX or other milk-borne growth factors may be affecting the development of FRT or other RLX receptor-positive somatic tissues remains to be determined.

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Figure 1

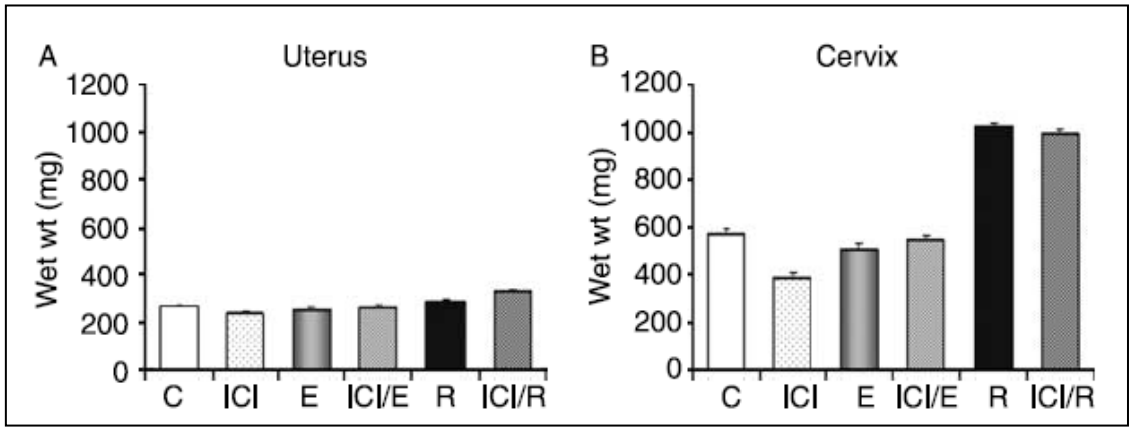

Figure 1. Treatment effects on uterine (A) and cervical (B) wet weight on PND 2. Data are expressed as LSM ± SEM. N=3-8 animals per group. Body weight was included as a covariate in statistical analyses. Results of preplanned contrasts follow for uterus: C vs E ($P<0.5$), C vs R ($P<0.4$), E vs ICI/E ($P<0.69$), R vs ICI/R ($P<0.15$) and E vs ICI ($P<0.65$); and cervix: C vs E ($P<0.41$), C vs R ($P<0.01$), E vs ICI/E ($P<0.71$), R vs ICI/R ($P<0.65$) and E vs ICI ($P<0.14$).

Figure 2

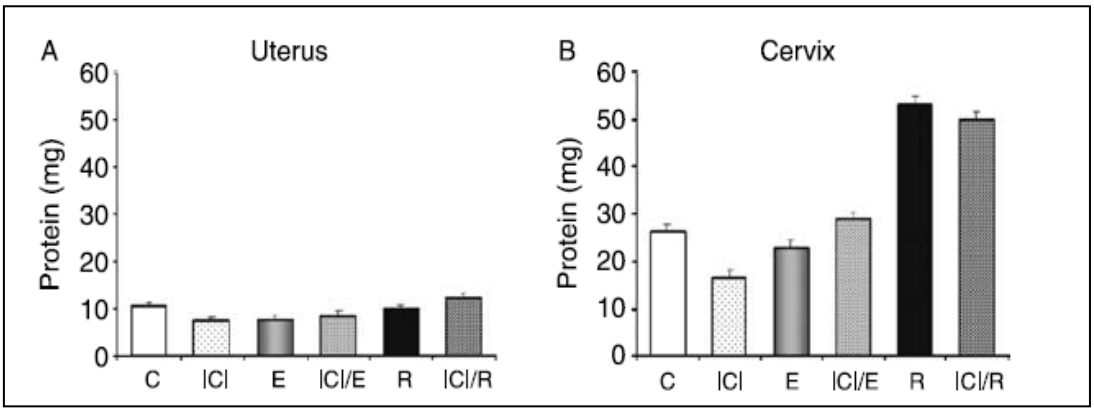

Figure 2. Treatment effects on uterine (A) and cervical (B) protein content on PND 2. Total protein content for each tissue is expressed as LSM $\pm$ SEM. N=3-8 animals per group. Results of preplanned contrasts follow for uterus: C vs E ($P<0.36$), C vs R ($P<0.89$), E vs ICI/E ($P<0.78$), R vs ICI/R ($P<0.42$) and E vs ICI ($P<0.92$); and cervix: C vs E ($P<0.54$), C vs R ($P<0.01$), E vs ICI/E ($P< 0.31$), R vs ICI/R ($P<0.56$) and E vs ICI ($P<0.35$).

Figure 3

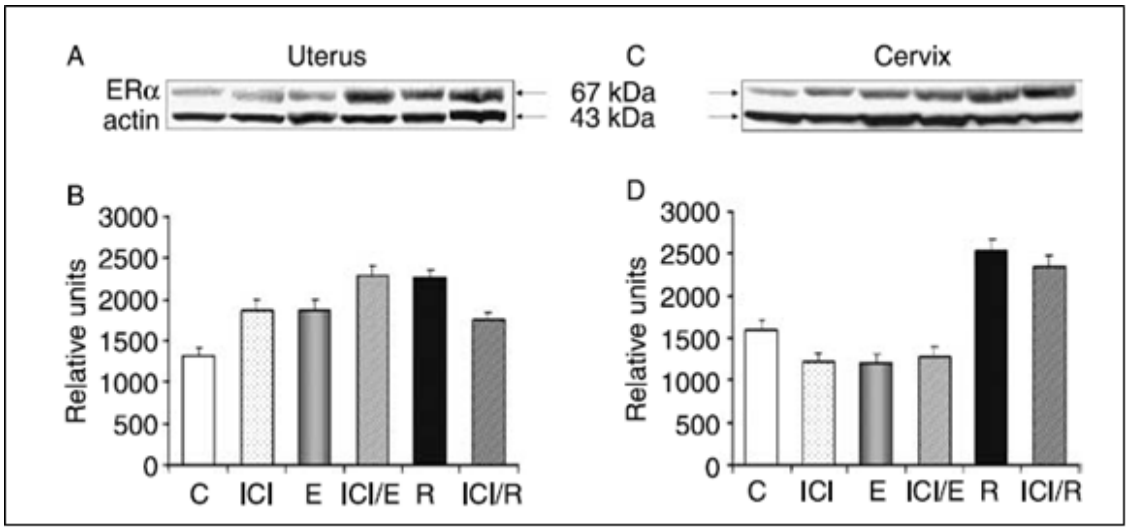

Figure 3. Treatment effects on uterine (left) and cervical (right) ERα expression on PND 2. (A, C) Representative Western blots for individual animals for each tissue and treatment category. Both 67 kDa bands indicative of immunoreactive ERα and 43 kDa bands indicative of immunoreactive beta-actin (included as a loading reference) are indicated(B, D). Summaries of densitometric data for ERα protein expressed as LSM $\pm$ SEM. N=3-8 animals per group. Data were adjusted for potential loading variation by including data for actin as a covariate in statistical analyses. Results of preplanned contrasts follow for uterus: C vs E ($P<0.28$), C vs R ($P<0.04$), E vs ICI/E ($P<0.35$), R vs ICI/R ($P<0.24$) and E vs ICI ($P<0.82$); and cervix: C vs E ($P<0.92$), C vs R ($P<0.01$), E vs ICI/E ($P <0.78$), R vs ICI/R ($P<0.75$) and E vs ICI ($P<0.55$).

Figure 4

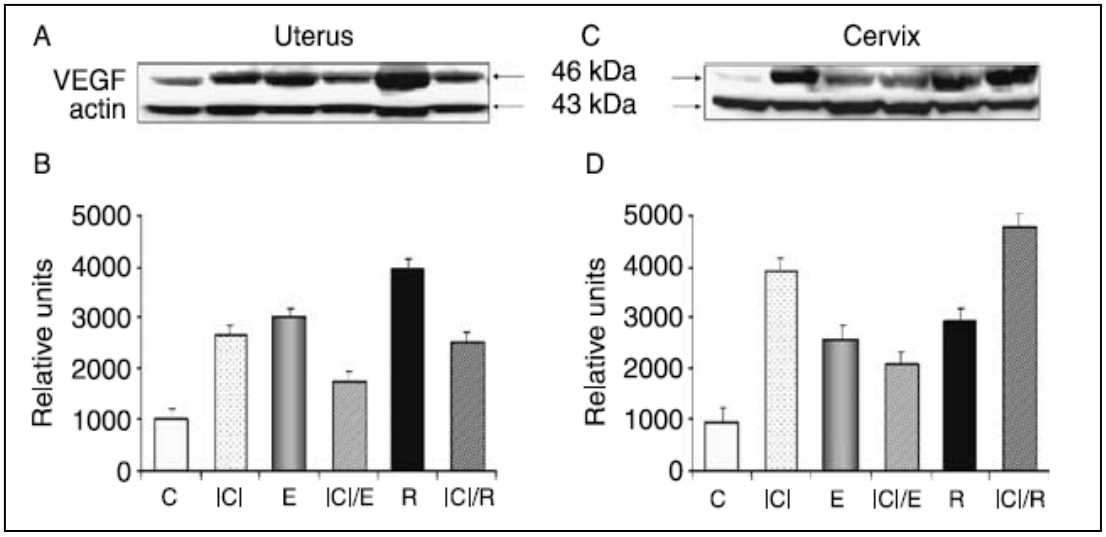

Figure 4. Treatment effects on uterine (left) and cervical (right) VEGF protein on PND 2. (A, C) Representative western blots for individual animals for each tissue and treatment category. Both 46 kDa bands indicative of immunoreactive VEGF and 43kDa bands indicative of immunoreactive beta-actin (included as a loading reference) are indicated (B, D). Summaries of densitometric data for VEGF protein expressed as LSM ± SEM. N=3-8 animals per group. Data for VEGF were adjusted for potential loading variation by including data for actin as a covariate in statistical analyses. Results of preplanned contrasts follow for uterus: C vs ICI ($P<0.02$), C vs E ($P<0.01$), C vs R ($P<0.01$), E vs ICI/E ($P<0.07$), R vs ICI/R ($P<0.05$) and E vs ICI ($P<0.73$); and cervix: C vs ICI ($P<0.01$) C vs E ($P< 0.01$), C vs R ($P< 0.01$), E vs ICI/E ($P<0.33$), R vs ICI/R ($P<0.01$) and E vs ICI ($P<0.67$).

Figure 5

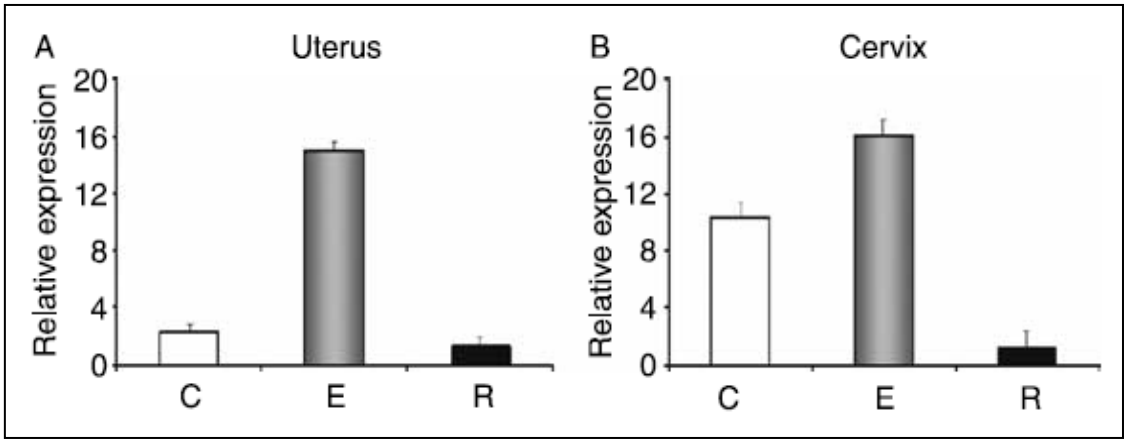

Figure 5. Treatment effects on uterine (A) and cervical (B) RXFP1 mRNA on PND 2. Data are expressed as LSM $\pm$ SEM. N=4 animals per group. Results of preplanned contrasts follow for uterus: C vs E ($P < 0.01$), C vs R ($P<0.52$), and E vs R ($P< 0.01$); and cervix: C vs E ($P<0.06$), C vs R ($P<0.01$), and E vs R ($P<0.01$).

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CHAPTER V

MILK-BORNE LACTOCRINE-ACTING FACTORS AFFECT GENE EXPRESSION PATTERNS IN THE DEVELOPING NEONATAL PORCINE UTERUS

ABSTRACT

Lactocrine communication of milk-borne bioactive factors (MbFs) from mother to offspring through nursing can affect neonatal development with lasting consequences. Relaxin (RLX), a lactocrine-active peptide found in porcine colostrum, stimulates estrogen receptor-α (ESR1) expression required for uterine development shortly after birth (postnatal day = PND 0). Whether other MbFs or cooperative lactocrine mechanisms affect the neonatal uterine developmental program is unknown. To determine effects of age, nursing and exogenous RLX on gene expression associated with uterine development, gilts (n=4-5/group) were assigned to nurse *ad libitum* or to receive milk replacer, with or without exogenous RLX (20 μg/kg BW i.m./6h for 50h), from birth to PND 2 when uteri were collected. Body and uterine weights increased ($P<0.05$) similarly from birth to PND 2 in all gilts. However, colostrum consumption was required for normal uterine ESR1, vascular endothelial growth factor (VEGFA), matrix metalloproteinase 9 (MMP9) and RLX receptor (*RXFP1*) protein and/or transcript expression on PND 2. Uterine ESR1, VEGFA and MMP9 protein levels were below ($P<0.01$) assay sensitivity in replacer-fed gilts. Supplemental RLX increased ($P<0.05$) uterine ESR1 protein and mRNA in nursed gilts, as well as VEGFA protein in nursed and mRNA in both nursed and replacer-fed gilts. Treatments did not affect uterine *MMP9* mRNA levels. When compared to replacer-fed gilts on PND 2, uterine *RXFP1* mRNA was reduced ($P<0.05$) in nursed gilts and in RLX supplemented replacer-fed gilts. Results constitute the first evidence that establishment of the neonatal porcine uterine developmental program requires maternal lactocrine support.

INTRODUCTION

Birth marks the end of intrauterine life but does not mark the end of maternal influence over events that can define the neonatal developmental program (Bartol *et al.* 2008). Evidence in several species indicates that colostrum, the first milk secreted after birth in mammals, acts as a conduit for transmission of signaling molecules from mother to offspring (Ogra *et al.* 1977, Simmen *et al.* 1990, Grosvenor *et al.* 1993, Playford *et al.* 2000, Almeida *et al.* 2008, Langer 2009). Defined as lactocrine signaling (Yan *et al.* 2006b, Bartol *et al.* 2008), neonatal consumption of bioactive factors in colostrum is recognized to affect differentiation of anterior pituitary mammotropes (Nusser & Frawley 1997), gastrointestinal tract development (Shulman 1990, Donovan & Odle 1994, Burrin *et al.* 1995) and immune system maturation (Lonnerdal 2003, Field 2005, Kosaka *et al.* 2010). The extent to which lactocrine-active factors or lactocrine signaling may contribute to establishment of the neonatal developmental program for female reproductive tract tissues is unknown.

Porcine uterine development begins *in utero* and is completed after birth (postnatal day = PND 0). Development of the neonatal porcine uterine wall is marked by age-dependent expression of estrogen receptor-α (ESR1) which is low to undetectable at birth (Yan *et al.* 2006a), evident in nascent endometrial glandular epithelium and underlying stroma by PND 2 (Yan *et al.* 2008) and continues to develop in an endometrial cell compartment-specific manner during the first two weeks of postnatal life (Tarleton *et al.* 1999). Early postnatal uterine epithelial ESR1 expression patterns are paralleled by those for proliferating cell nuclear antigen labeling, which increases markedly in nascent glandular epithelium by PND 3 (Masters *et al.* 2007). Thus, the

neonatal porcine endometrium undergoes an important morphogenetic transition between birth and PND 3, when glandular epithelium is differentiated from luminal epithelium and nascent uterine glands begin to penetrate underlying stroma (Tarleton *et al.* 1998, Masters *et al.* 2007).

Neonatal porcine uterine development is both estrogen receptor (ER) dependent and estrogen-sensitive (Tarleton *et al.* 1999, Tarleton *et al.* 2003, Bartol *et al.* 2006, Chen *et al.* 2010). Exposure to the ER antagonist ICI 182,780 (ICI) from birth can impede endometrial adenogenesis (Tarleton *et al.* 1999) while similar exposure to ER agonists can advance endometrial development, alter the neonatal uterine proteome and affect both uterine responsiveness to conceptus signaling and uterine capacity for conceptus support (Bartol *et al.* 1993, Tarleton *et al.* 1999, Tarleton *et al.* 2003, Chen *et al.* 2010). Collectively, data support the idea that factors affecting patterns of porcine uterine ESR1 expression and ER activation during early postnatal life define both the developmental program and trajectory of developing uterine tissues with lasting consequences in adults.

Relaxin (RLX), a prototypical milk-borne bioactive factor (MbF), like estrogens, increases uterine growth and connective tissue remodeling (Lenhart *et al.* 2001, Sherwood 2004, Yan *et al.* 2006a, Chen *et al.* 2010). Alterations in the porcine uterine developmental program in response to RLX or estrogens administered shortly after birth are reflected by increases in uterine weight and in uterine expression patterns of ESR1 (Yan *et al.* 2008, Chen *et al.* 2010) , as well as vascular endothelial growth factor (VEGFA) (Yan *et al.* 2008) and matrix metalloproteinase (MMP)s (Chen *et al.* 2010). These gene products are associated with critical organizational events supporting neonatal porcine uterine development (Bartol *et al.* 1993, Bartol *et al.* 2006). Whether

lactocrine signaling affects uterine expression of these markers and mediators of uterine growth and remodeling remains to be determined.

The lactocrine hypothesis was proposed to explain how bioactive factors present in colostrum could be delivered into the neonatal circulation as a consequence of nursing and influence developing neonatal tissues (Yan *et al.* 2006b, Bartol *et al.* 2008). Bioactive RLX is present in porcine colostrum (Frankshun *et al.* 2009) and RLX is detectable in the circulation of neonatal pigs allowed to nurse (Yan *et al.* 2006b). A lactocrine mechanism was envisioned in which RLX enters into the circulation of nursing animals and targets RLX receptor (RXFP1)-positive uterine cells to stimulate ESR1 expression and serve, potentially, as an ESR1 agonist, thereby supporting organizationally critical early events in porcine uterine wall development. If the lactocrine hypothesis for maternal programming of neonatal porcine uterine development is valid, neonates consuming colostrum should display a different pattern of uterine gene expression than those fed a synthetic milk-replacer devoid of RLX and other MbFs.

While evidence that ingestion of colostrum is important for neonatal health is well documented (Le Dividich & Noblet 1981, Horwood & Fergusson 1998, Blum & Hammon 2000, Stelwagen *et al.* 2009), the extent to which lactocrine signaling affects development of neonatal somatic tissues is unknown. More specifically whether lactocrine signaling is required to: (i) establish the neonatal uterine developmental program; (ii) determine uterine developmental trajectory; and (iii) set the stage for uterine developmental success as reflected by functional uterine capacity for support of pregnancy has not been investigated. Here, the goal was to address the first of these issues. Thus, objectives were to determine effects of age and consumption of colostrum,

in the presence and absence of exogenous RLX for two days from birth, on the expression of molecular markers and mediators of neonatal porcine uterine development at PND 2. Results provide the first direct test of the lactocrine hypothesis for maternal regulation of neonatal uterine development.

MATERIALS and METHODS

Materials

Advance Liqui-Wean, a nutritionally complete commercial piglet milk replacer, was purchased from MSC Specialty Nutrition (Carpentersville, IL). Porcine RLX was kindly provided by Dr. OD Sherwood (University of Illinois, Urbana, IL). Beuthanasia-D Special was purchased from Intervet/Schering Plough Animal Health (Whitehouse Station, NJ). TRI Reagent was obtained from Sigma-Aldrich (St. Louis, MO) and RNeasy Mini Kits, RNase-Free DNase Sets, and RNALater were from Qiagen Inc. (Valencia, CA). SuperScript III First-Strand Synthesis System for RT-PCR was from Invitrogen (Carlsbad, CA). SYBR Green PCR Master Mix was purchased from Applied Biosystems (Foster City, CA). Primers were synthesized by Sigma-Aldrich. Detergent-compatible protein assay kits (DC Protein Assay) were purchased from Bio-Rad Laboratories (Hercules, CA). Mouse anti-human ESR1 (Ab-15) monoclonal antibody was from NeoMarkers, Inc. (Fremont, CA). Rabbit anti-human VEGFA (sc-152-G) and goat anti-human actin (sc-1615) polyclonal antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse anti-human MMP9 (IM09L) and MMP2 (IM33) antibodies were purchased from Calbiochem (Gibbstown, NJ). Horseradish peroxidase-conjugated anti-mouse and anti-goat secondary antibodies were purchased from Zymed (Carlsbad, CA). 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). XOmatic films were purchased from American Imaging (South Plainfield, NJ).

Experimental design and tissue collection

At birth, crossbred gilts (*Sus scrofa domesticus*) (n=5-10/group) were assigned randomly to either nurse *ad libitum* or to receive milk replacer by gavage (50 ml/2h for 50h), with or without exogenous RLX (20µg/kg BW, given i.m. in PBS every 6h for 50h). The dose and timing of RLX administration were based on previous studies in neonatal gilts (Yan *et al.* 2008). Uteri were collected 3h after the last treatment on PND 2. In addition, uteri were obtained from gilts prior to their consumption of milk at birth on PND 0. Neonatal gilts were euthanized for tissue collection using Beuthanasia-D Special while under surgical anesthesia. Care was taken to ensure that treatments were balanced for potential effects of litter and that sows were nursing litters of similar size.

Neonatal uterine tissues were removed, trimmed of fat and associated tissues and ligaments and wet weights recorded. Tissues were then immersed in RNALater and stored at –80°C. Animals were obtained from the Auburn University Swine Research and Education Center, or from the Swine Unit of the New Jersey Agricultural Experiment Station, Rutgers University. 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).

RNA isolation, cDNA generation, and real-time RT-PCR

Total RNA was isolated from 30-50 mg of 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. Reverse transcription was performed with 500 ng total RNA per sample using the PTC-200 Peltier Thermal Cycler (Bio-Rad Laboratories) and SuperScript III First-Strand Synthesis System for RT-PCR. All procedures were carried out following manufacturer's instructions.

Real-time quantitative RT-PCR (qPCR) was performed using an Applied Biosystems Gene Amp 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) 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, Foster City, CA) and synthesized by Sigma-Aldrich. 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 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. 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 quantification 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 2 uteri (Larionov *et al.* 2005). Efficiencies for the reaction were calculated using the slope of the standard curves, and ranged between 90 to 110% for each primer set. Target gene expression was normalized to the expression of porcine *18*S. Data from qPCR analyses are presented as relative mRNA units. Data generated by

qPCR for *18S* mRNA from each sample were analyzed to confirm the absence of treatment effects on this reference gene.

Protein extraction and evaluation of ESR1, VEGFA, MMP2 and MMP9 expression

Uterine tissues (20-50 mg) were homogenized in 200 µl of lysis buffer (1% Triton X-100, 10% Glycerol, 150 mM Tris-HCl, 300 mM NaCl, 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 Laboratories). To document target protein expression, uterine proteins (30µg) were resolved on 12.0% total monomer, Bis-Tris-HCl-buffered polyacrylamide gels under reducing conditions (non-reducing conditions for MMP9 to detect both the latent and active forms, as suggested by the manufacturer) and transferred onto nitrocellulose membranes. After blocking in 10.0% nonfat dry milk in Tris-buffered saline containing Tween-20 (TBST;25 mM Tris, pH 7.5, 0.14 mM NaCl, 3 mM KCl,0.05% Tween-20), membranes were probed with either mouse anti-human ESR1 antibody (1:200), mouse anti-human MMP2 or MMP9 antibodies (1:2000), or rabbit anti-human VEGFA antibody (1:1000) overnight at 4°C. After washing with TBST, blots were incubated with either horseradish peroxidase-conjugated anti-mouse secondary antibody (1:1000) or anti-rabbit secondary antibody (1:1000) for 1h at room temperature. Bound antibodies were detected by enhanced chemiluminescence. Protein loading was monitored using actin as a reference. Chemiluminescence signals were quantified by densitometry from film using Scion Image for Windows (Scion Corporation, Frederick, MD). In circumstances where immunoblot signals were below

background signals under identical exposure conditions, data were recorded as being non-detectable (nd).

Statistics analyses

Data were subjected to analyses of variance using general linear model procedures available with SAS (SAS 2009–2010). For weights, and uterine ESR1, VEGFA, MMP2 and MMP9 protein and mRNA levels, statistical models considered variation due to main effects of age, nursing and RLX treatments. Preplanned contrasts were performed to determine effects of neonatal age (PND 0 vs PND 2) and treatments for tissues obtained on PND 2, including: (a) nursing vs replacer; (b) nursing vs nursing + RLX; and (c) replacer vs replacer + RLX. All error terms were identified based upon expectations of the mean squares for error. Data were expressed as least square means with standard errors.

RESULTS

Neonatal body and uterine weights in newborn and PND 2 animals

Data for body weights and uterine wet weights are presented in Figure 1. Newborn gilt body weight (Figure 1A) averaged 1.4 kg and increased ($P<0.05$) by PND 2 (2.1 kg ± 0.01 kg) to a similar extent in both nursing and replacer-fed animals (Figure 1A). At PND 2, there were no differences in body weight between any of the treatment groups. Uterine wet weights for gilts at birth (Figure 1 B) averaged 0.22g and increased ($P<0.05$) by PND 2 (0.3 g ± 0.01 g) to a similar extent for both nursing and replacer-fed animals. Administration of RLX from birth in nursing gilts further increased ($P<0.05$) uterine weight compared to all other PND 2 groups (Figure 1 B).

Neonatal uterine ESR1 and VEGFA protein levels in nursed and replacer-fed gilts

Neonatal gilts that nursed during the first two days of life expressed a single 51 kDa uterine protein band, corresponding to ESR1 (Figure 2A), and a 46 kDa band, corresponding to dimeric VEGFA protein (Figure 2B) at PND 2. Surprisingly, neither uterine ESR1 or VEGF protein were detectable ($P<0.01$) in gilts that ingested milk replacer in lieu of nursing for two days from birth (Figure 2A and 2B). Similar results (data not shown) were obtained using a different ESR1-specific primary antibody, (DAKO mouse anti-human ESR1; Carpinteria, CA) (Nishihara *et al.* 2000).

Effects of age, nursing, and RLX on neonatal uterine ESR1 and VEGFA protein

At birth, prior to nursing, uterine ESR1 and VEGFA protein bands were undetectable. However, by PND 2, uterine ESR1 and VEGFA, consistently and clearly detectable in animals that nursed from birth, were undetectable in uteri of replacer-fed animals (Figure 3A). RLX administration increased ($P<0.05$) uterine expression of both

ESR1 and VEGFA protein in nursing animals (Figure 3B and 3C) whereas similar treatment with RLX failed to induce expression of either of these uterine proteins in gilts fed milk replacer from birth.

Effects of age, nursing and RLX on neonatal uterine MMP9 and MMP2 protein

Immunoblot analyses were used to identify the type and measure the relative abundance of MMP2 and MMP9 proteins in neonatal uterine tissues (Figure 4). At PND 0, prior to nursing, uterine MMP9 was undetectable. However, by PND 2, protein bands at 92 and 84 kDa, the reported sizes of proMMP9 and active MMP9, respectively (O'Connell *et al.* 1994) were induced in uteri of gilts nursed from birth (Figure 4A) but remained undetectable in replacer-fed gilts. Exogenous RLX administration did not affect expression of uterine MMP9 protein at PND 2 in nursed or replacer-fed gilts (Figure 4B). A 72 kDa protein band, corresponding to proMMP2 and to a lesser extent, a 66 kDa band corresponding to active MMP2 (Crabbe *et al.* 1993) was detected in uteri from all groups (Figure 4A). Neither age, nursing or RLX treatment affected the relative expression of uterine proMMP2 or MMP2 (Figure 4A and 4C).

Effects of age, nursing and RLX on neonatal uterine gene expression

As illustrated in Figure 5A, uterine expression of *ESR1* mRNA was detected at PND 0 and, to a greater extent in nursing but not in replacer-fed gilts at PND 2. RLX treatment of nursing animals further increased uterine *ESR1* transcripts compared to nursing alone, whereas exposure to RLX had no effect on uterine *ESR1* mRNA in replacer-fed animals. Uterine *VEGFA* mRNA expression was low in PND 0, nursing and replacer-fed groups, but increased ($P<0.05$) in response to exogenous RLX from birth in both nursed and replacer-fed gilts (Figure 5B). Neither age, nursing or RLX treatment

affected the relative expression of uterine *MMP*9 (Figure 5C) or *MMP2* gene expression (data not shown). Detectable at birth, *RXFP1* mRNA expression remained at PND 0 levels in replacer-fed gilts on PND 2, but was reduced ($P<0.05$) at PND 2 in gilts that nursed from birth and in response to exogenous RLX in both groups (Figure 5D).

DISCUSSION

Results of the present study provide the first test of the lactocrine hypothesis for maternal regulation of postnatal uterine development. Data show that maternal lactocrine signaling is necessary to support normal patterns of gene expression associated with establishment of the neonatal porcine uterine developmental program within the first two days of postnatal life. Evidence presented here in support of the lactocrine hypothesis constitutes a critical first step in efforts aimed at defining the extent to which lactocrine signaling is required to set uterine developmental trajectory and determine functional uterine capacity. Evidence indicating that uterine ESR1, VEGFA, MMP9 and RXFP1 can be modulated predictably by RLX in nursing, but not in replacer-fed animals suggests that cooperating lactocrine-active factors may be required to support the actions of specific MbFs, including RLX.

Similarities in both body weights and uterine weights observed for nursed and replacer-fed gilts on PND 2 (Figure 1) indicate that replacer feeding did not compromise growth of neonatal gilts. Thus, treatment-related uterine responses reported here were not biased by differences in either body or organ weights. Consistent with previous reports (Yan *et al.* 2006a, Yan *et al.* 2008), exogenous RLX increased uterine weight on PND 2 in nursing gilts. The fact that uterotrophic effects of exogenous RLX were more pronounced in nursing than in replacer-fed gilts (Figure 1) supports a role for cooperative, lactocrine-acting factors in such responses.

Neonatal porcine uterine development is both ESR1-dependent and estrogen-sensitive (Bartol *et al.* 1993, Tarleton *et al.* 1999, Tarleton *et al.* 2003, Chen *et al.* 2010). Therefore, it was important to determine the extent to which uterine ESR1 expression

might be regulated via a lactocrine mechanism. Present data show clearly that the increase in uterine ESR1 expression expected by PND 2 (Yan *et al.* 2008) occurred only in those gilts allowed to consume colostrum from birth. Results support the idea that lactocrine regulation of neonatal uterine ESR1 expression involves complementary actions at transcriptional and translational levels. Interestingly, both prototypical MbFs estradiol and RLX (Osterlundh *et al.* 1998, Frankshun *et al.* 2009) can increase *ESR1* mRNA stability and decrease transcript degradation independently (Hod & Hanson 1988, Ing & Ott 1999). Such actions could contribute to conditions associated with reduced uterine *ESR1* mRNA levels observed in replacer-fed as compared to nursing gilts on PND 2. Consistent with data reported here for uterine wet weight (Figure 1), exogenous RLX did not affect uterine *ESR1* mRNA levels in replacer-fed gilts. Data can be interpreted to suggest a post-transcriptional mechanism involving lactocrine-acting factors cooperating in support of normal uterine ESR1 expression dynamics. For example, like RLX, both estradiol and IGF1, detectable in porcine colostrum (Donovan & Odle 1994, Osterlundh *et al.* 1998), stimulated increases in uterine ESR1 protein levels (Aronica & Katzenellenbogen 1993, Katzenellenbogen 1996, Tsai *et al.* 2004). Such lactocrine-acting factors may function in concert to support normal uterine ESR1 expression in neonatal porcine uterine tissues.

A marker of both RLX (Unemori *et al.* 1999) and estrogen (Cullinan-Bove & Koos 1993) action, VEGFA supports angiogenesis and related events associated with the growth of many tissues (Neufeld *et al.* 1999, Cebe-Suarez *et al.* 2006), including rodent and porcine endometrium (Shao Longjiang *et al.* 2001, Welter *et al.* 2003). Data for neonatal uterine VEGFA expression reported here, particularly at the protein level, were

complementary to those observed for ESR1. The dramatic reduction in VEGFA protein expression observed for replacer-fed as compared to nursing gilts on PND 2 (Figures 1 and 2), considered in light of the absence of a similar effect for *VEGFA* mRNA levels (Fig. 5B), suggests that lactocrine regulation of uterine VEGFA expression occurs primarily at the translational level. Consistently, RLX administration increased uterine *VEGFA* mRNA levels to a similar extent in both nursed and replacer-fed gilts on PND 2, but increased uterine VEGFA protein levels only in nursed gilts. The latter observation agrees with data reported previously for nursing gilts (Yan *et al.* 2008). Present data indicate that a cooperative lactocrine mechanism is required to support translational events necessary to achieve a normal uterine expression level for this peptide growth factor. Relationships reported here may also be explained, in part, by data showing that secretion of VEGFA protein by human endometrial stromal cells was dependent on ESR1 expression (Huang *et al.* 1998). Here uterine ESR1 expression was suppressed and, therefore, effectively delayed in replacer-fed animals.

Gelatinases, MMP2 and MMP9, digest Type IV and V collagens in basement membranes to facilitate tissue growth and remodeling (Zeng *et al.* 1999, O'Farrell & Pourmotabbed 2000). Both MMP2 and MMP9 were detected in the murine uterus (Hu *et al.* 2004) and human endometrial cells (Martelli *et al.* 1993, Iwahashi *et al.* 1996). In the prepubertal porcine uterus RLX increased secretion of both MMP2 and MMP9 (Lenhart *et al.* 2001). Given that RLX is a component of porcine colostrum/milk (Yan *et al.* 2006b, Frankshun *et al.* 2009) it was hypothesized that uterine expression of these enzymes could be sensitive to lactocrine regulation.

Consistent with this hypothesis, neonatal uterine expression of MMP9, but not MMP2, was clearly lactocrine-dependent at the protein level. Like ESR1 and VEGFA, normal expression of MMP9 protein required lactocrine support, as uterine MMP9 protein levels were below assay sensitivity in replacer-fed gilts. However, in contrast to data for *ESR1* and *VEGFA*, mRNA levels for *MMP9* were unaffected by age, the absence of colostrum/replacer-feeding and administration of exogenous RLX. Thus, as suggested for VEGFA, lactocrine regulation of neonatal uterine MMP9 expression may involve translational control. The absence of an effect of exogenous RLX on uterine expression of either MMP2 or MMP9 at PND 2 suggests that RLX sensitivity associated with regulated expression of these proteins develops later in life. Patterns of MMP2 expression observed here support the idea that some neonatal uterine gene expression events are not sensitive to lactocrine regulation between birth and PND 2.

Data for VEGFA and MMP9 illustrate some discordance between uterine transcriptional and translational events. While mechanisms were unclear, dissimilarities between mRNA measurements and both protein expression and activity patterns were described by others for both VEGFA and MMP9 (Kuo *et al.* 1999, Zhang *et al.* 2007). Discordance between transcriptional and translational events does occur and evidence for gene-specific translational control is well documented (Pavitt 2005, Sonenberg & Hinnebusch 2009). Translational control of gene expression can be particularly important during development and differentiation of eukaryotic cells and tissues (Sonenberg & Hinnebusch 2009). As is the case for other transcripts, translation of both *VEGFA* (De Benedetti & Graff 2004, Zhou *et al.* 2006) and *MMP9* (Graff *et al.* 1995, De Benedetti & Graff 2004) involves eukaryotic initiation factor eIF-4E, a critical

component of cap-dependent translation (Sonenberg & Hinnebusch 2009). Expression of both of these lactocrine-sensitive factors may be regulated translationally to a significant degree.

Recently, RXFP1 was identified as the central element of a feed-forward, lactocrine-driven mechanism regulating establishment of the neonatal porcine uterine developmental program (Bartol *et al.* 2009). Present data corroborate this scheme, in which milk-borne RLX, acting via RXFP1 expressed in uterine tissues at birth (Yan *et al.* 2006b), supports or induces uterine expression of ESR1 and the parallel expression of VEGFA and other relaximedins and estromedins (Bartol *et al.* 2009). This, in turn, may insure continued uterine expression of both RXFP1 and ESR1, as well as propagation of related signaling events essential to the success of endometrial development. Lactocrine regulation of uterine RXFP1 expression was also clear. However, in contrast to data presented for *ESR1* mRNA (Figure 5), uterine *RXFP1* transcript levels were reduced in nursed as compared to replacer-fed gilts on PND 2. Interestingly, RLX administered to replacer-fed gilts reduced uterine *RXFP1* expression to levels similar to those observed for nursed gilts, but not different from those observed for nursed gilts supplemented with exogenous RLX. These relationships suggest a RLX-specific effect not further modulated by cooperating lactocrine-active factors. Data are also consistent with the idea that *RXFP1* expression can be regulated by its cognate ligand (Parry *et al.* 2005, Yan *et al.* 2008).

Evidence reviewed here supports the lactocrine hypothesis for maternal programming of porcine neonatal uterine development and reinforces the role of RLX as a lactocrine-acting MbF (Bagnell *et al.* 2009, Bartol *et al.* 2009). Of course, many other

factors found in colostrum/milk are likely to be acting via a lactocrine mechanism to affect development of the uterus and other reproductive and somatic tissues. It is well known that steroid and peptide hormones, cytokines and growth factors are present in colostrum/milk (Grosvenor *et al.* 1993, Osterlundh *et al.* 1998, Playford *et al.* 2000, Salmon *et al.* 2009). Recently microRNAs, which can regulate gene expression by silencing translation (Alvarez-Garcia & Miska 2005), were identified in human and bovine milk (Hata *et al.* 2010, Kosaka *et al.* 2010). Thus, microRNAs must also be added to the list of MbFs with the potential to affect neonatal development. Likewise, bioactive peptides encrypted in colostral/milk proteins (Hartmann *et al.* 2007) may also contribute to lactocrine signaling events. Evidence indicating that neonatal consumption of milk-replacer can affect patterns of gastrointestinal tract maturation and function (Simmen *et al.* 1990, Zabielski *et al.* 2008, Chapkin *et al.* 2010) should also be considered, as such differences could affect the efficiency of lactocrine signaling processes which are yet to be defined.

Results presented here indicate that initiation of the porcine uterine developmental program during the first two days of life is supported, in part, by lactocrine-acting MbFs, delivered to the neonate as a consequence of nursing and the ingestion of colostrum. Such lactocrine effects may be necessary to establish a trajectory of uterine development that will define the mature uterine phenotype. The stage is now set for studies designed to evaluate the extent to which maternally-driven, lactocrine-mediated signaling affects development and function of reproductive and non-reproductive tissues in both males and females. Much remains to be learned about the nature of lactocrine-active factors and their role in regulating the development of mammalian neonates.

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Figure 1

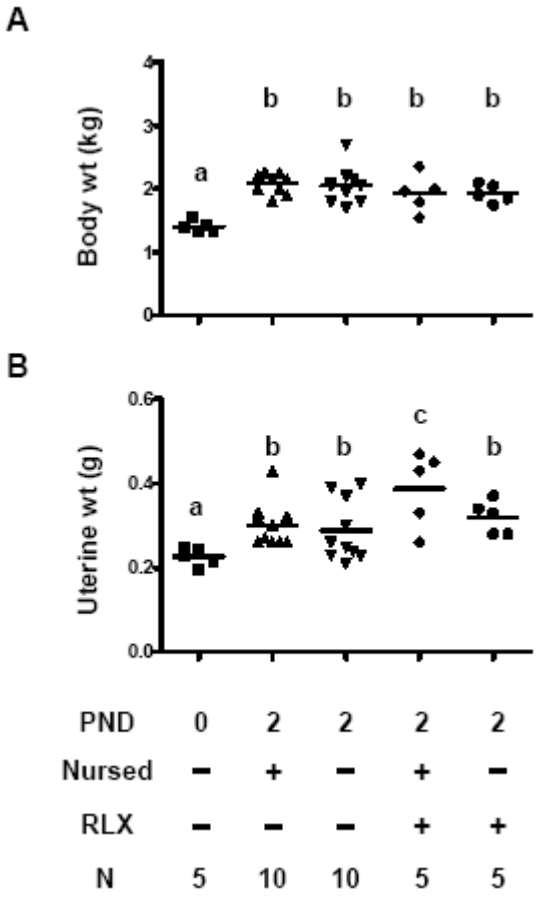

Figure 1. Effects of age, nursing and RLX on neonatal porcine body (A) and uterine (B) weight. Individual weights are presented, horizontal bars indicate LSM. N=animals per group. Different letters indicate differences at $P<0.05$.

Figure 2

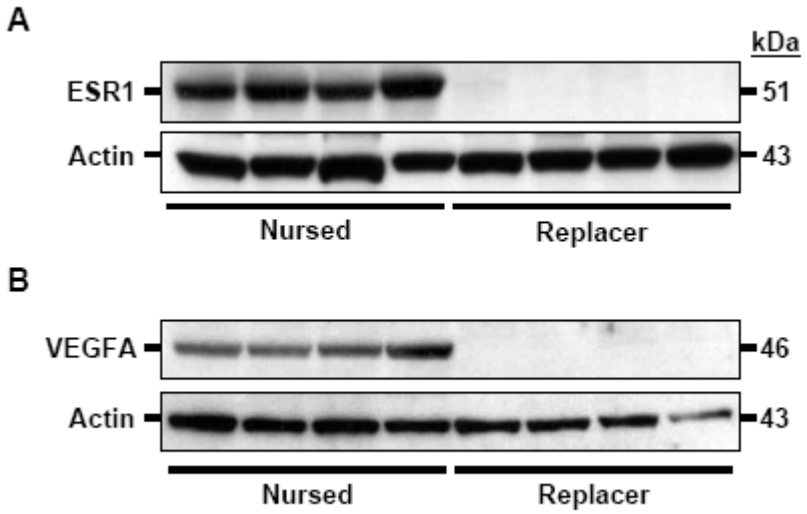

Figure 2. Effects of nursing vs milk replacer ingestion on porcine uterine ESR1 (A) and VEGFA (B) proteins at PND 2. Representative immunoblots are shown. Both 51 and 46 kDa immunoreactive bands for ESR1 and VEGFA respectively, are indicated. An immunoreactive band for actin was detected at 43 kDa and included as a loading reference. N=5-10 animals per group.

Figure 3

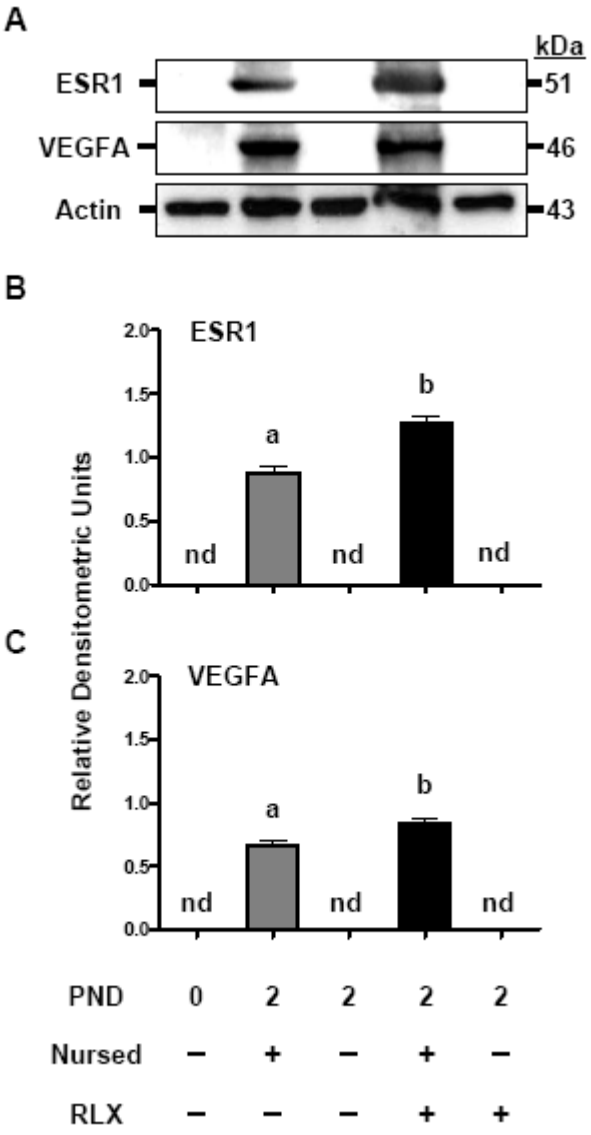

Figure 3. Effects of age, nursing and RLX on neonatal porcine uterine ESR1 and VEGFA protein levels. Representative immunoblots (A) are shown. Densitometric data for the relative expression of ESR1 (B) and VEGFA (C) are presented as LSM ± SEM. N=4-5 animals per group. Signals below the detection range are marked non-detectable (nd). Different letters indicate differences at $P<0.05$.

Figure 4

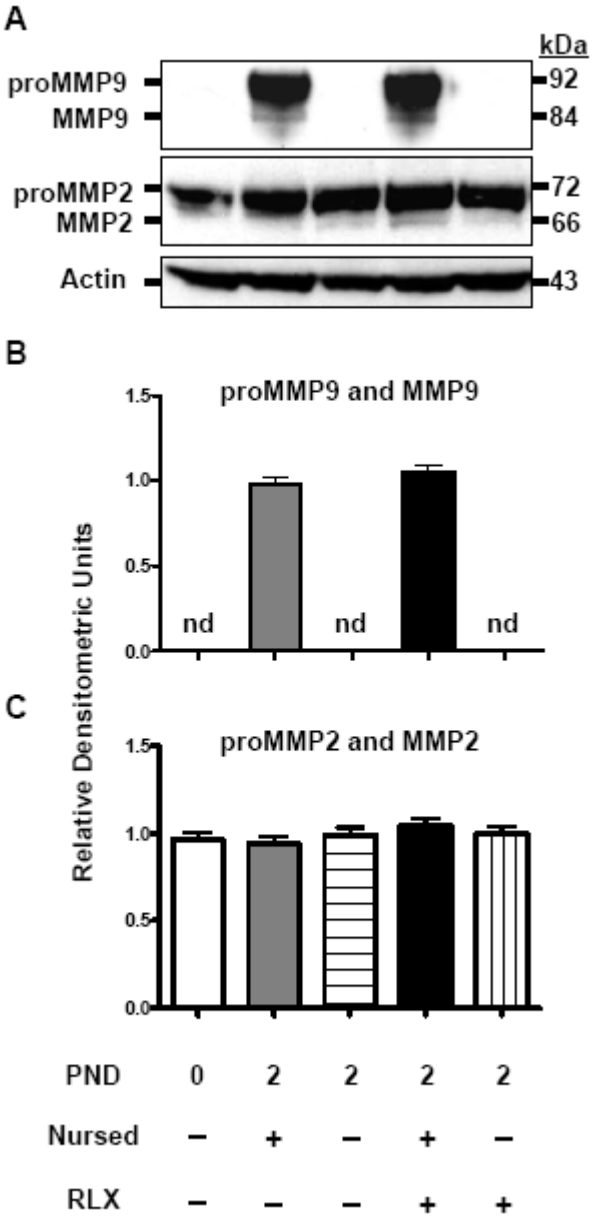

Figure 4. Effects of age, nursing and RLX on neonatal porcine uterine MMP9 and MMP2 protein. Representative immunoblots (A) are shown. Immunoreactive bands for proMMP9 (92 kDa) and MMP9 (86 kDa), as well as proMMP2 (72 kDa) and MMP2 (66 kDa) are indicated. An immunoreactive band for actin was detected at 43 kDa and included as a loading reference. Densitometric data for the relative expression of MMP9 (B) and MMP2 (C), including respective proforms, are presented as LSM ± SEM. N=4-5 animals per group. Signals below the detection range are marked as non-detectable (nd).

Figure 5

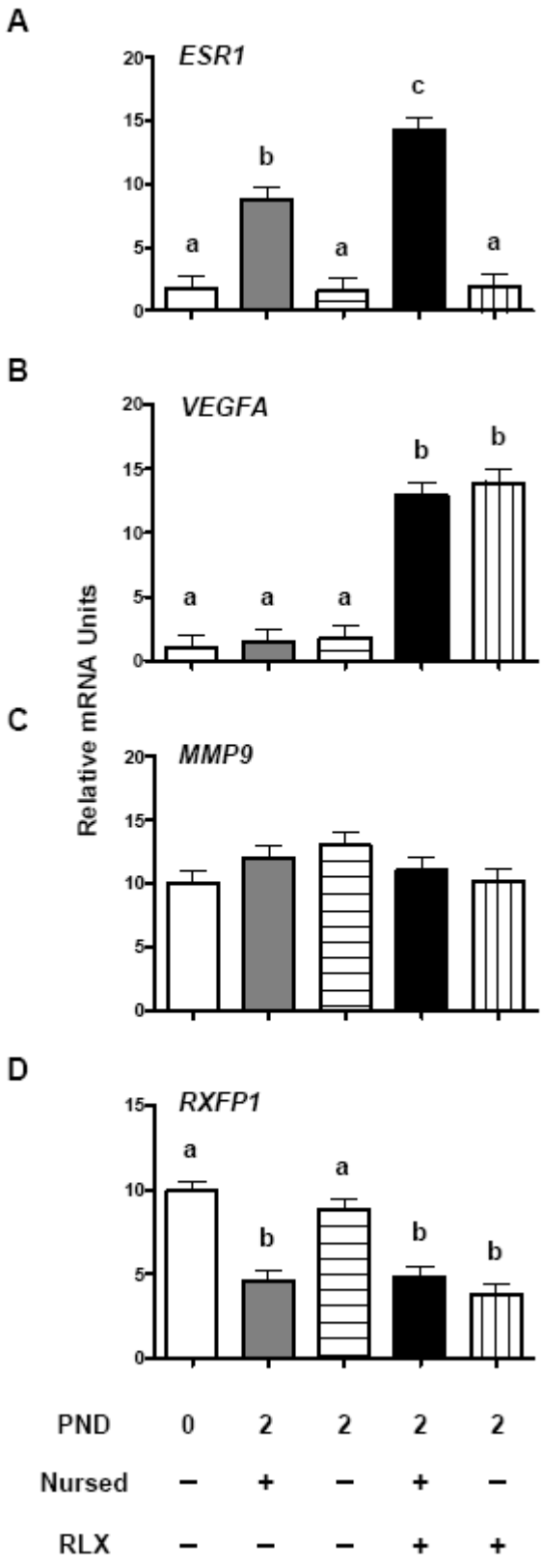

Figure 5. Effects of age, nursing and RLX on neonatal porcine uterine expression of *ESR1* (A), *VEGFA* (B), *MMP9* (C) *RXFP1* (D) transcripts were determined by qPCR. Data were normalized to *18S* gene expression and are presented as LSM ± SEM. N=4-5 animals per group. Letters a and b indicate $P<0.05$; c indicates $P=0.09$.

Table 1. Porcine primer accession numbers and primer sequences for targeted uterine genes.

Gene	Accession #	Forward Primer	Reverse Primer
ESR1	**AF035775**	AGGGAGAGGAGTTTG TGTG	TCTCCAGCAGCAGGTC ATAG
VEGFA	**AF318502**	AAGATCCGCAGACGTG TAAA	CACATCTGCAAGTACG TTCG
MMP9	**DQ132879**	TGGATCCAAAACTACTCGGA AGAC	CGGACAAAGGC GTCG
MMP2	**NM214192**	GAGCACCATCGAGACC ATGA	TTGTAATTGGCCACG TCGG
RXFP1	**CA994862**	GCATCACTTTGAGGCAGA GACA	CCTCGGCAAAGACATT GCAT
18S	**AF102857**	CCGCGGTTCTATTTTGTTGG TTTT	CGGGCCGGGTGAGG TTTC

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CHAPTER VI

LACTOCRINE SIGNALING SUPPORTS ESTABLISHMENT OF DEVELOPMENTAL TRAJECTORY IN THE NEONATAL PORCINE UTERUS

ABSTRACT

Colostrum (first milk) is rich in milk-borne bioactive factors (MbFs) that include estrogens, relaxin and IGFs. Nursing during the first two days of life is necessary for expression of genes supportive of and required for normal uterine development, including estrogen receptor alpha (ESR1), at postnatal day (PND) 2. Thus, lactocrine signaling, through which MbFs delivered from mother to offspring as a consequence of nursing affect development of neonatal tissues, could be important for establishment of an optimal uterine developmental trajectory, with consequences for adult uterine function. Here, objectives were to determine if duration of nursing and age at first nursing influence porcine uterine developmental trajectory as assessed on PND 14. At birth, neonatal gilts (n=4-5/group) were: (a) allowed to nurse *ad libitum*; (b) fed milk replacer ad libitum; (c) nursed for 2 days from birth and switched to milk replacer; or (d) fed milk replacer for 2 days from birth and switched to nursing. Uterine tissues were collected on PND 14. Compared to nursed gilts, expression of uterine ESR1, vascular endothelial growth factor (VEGFA) and matrix metalloproteinase 9 (MMP9) protein levels decreased ($P<0.05$) in gilts that ingested only replacer during the first two days or two weeks of life. Similar patterns ($P < 0.05$) were observed for *VEGFA* and *MMP9* transcripts. Importantly, nursing after the first two days of life by gilts fed replacer from birth did not affect protein or mRNA levels for ESR1, VEGFA or MMP9. Zymographic analysis indicated that nursing during the first two days or two weeks of life increased ($P<0.05$) uterine MMP9 activity compared to gilts that consumed only replacer from birth through PND 2. Nursing after the first two days of life did not affect MMP9 activity compared to fed only replacer. Results suggest that a lactocrine window exists whereby nursing

during the first two days of life results in the consumption of colostrum-specific MbFs that are necessary for the establishment of the porcine uterine developmental trajectory.

INTRODUCTION

Lactocrine signaling was recently defined as the mechanism through which milk-borne bioactive factors (MbFs), communicated from mother to offspring as a specific consequence of nursing, enter the neonatal circulation and affect events associated with growth and development of somatic tissues (Yan *et al.* 2006b, Bartol *et al.* 2009). Based upon data for porcine relaxin (RLX), a prototypical MbF in the pig, the lactocrine hypothesis was proposed to describe how lactocrine-acting factors might affect the developmental program of the porcine uterus (Yan *et al.* 2006b, Bartol *et al.* 2009). Direct support for this hypothesis was provided by data indicating that nursing during the first two days of life is required to support normal expression of gene products recognized to be required for, or likely to be involved in neonatal porcine uterine development between birth (postnatal day = PND 0) and PND 2, including estrogen receptor alpha (ESR1), vascular endothelial growth factor (VEGFA) and matrix metalloproteinase (MMP) 9 (Chen et al submitted). The lactocrine hypothesis for maternal programming of neonatal development also gains support from studies of the tammar wallaby which showed that patterns of neonatal intestinal development can be determined by milk composition (Joss *et al.* 2009). Similarly, studies involving humans showed that molecular profiles of intestinal cells in newborn babies allowed to nurse are different from those fed milk replacer (Chapkin *et al.* 2010). Since porcine colostrum contains a variety of MbFs including estrogen (Osterlundh *et al.* 1998), IGFs (Donovan & Odle 1994) and relaxin (Yan *et al.* 2006b), it is, perhaps, not surprising that lactocrine signaling was found to be required for establishment of a normal uterine developmental program in the pig between birth and PND 2 (Figure 1) (Chen et al. submitted).

The first two days of postnatal life in the neonatal gilt represent an important developmental period during which both morphogenetic and cytodifferentiative events occur in the uterine wall. During this period, differentiation of glandular epithelium (GE) from luminal epithelium (LE) (Tarleton *et al.* 1999), is accompanied by onset of ESR1 expression in nascent GE, detectable by PND 2 (Yan *et al.* 2008). Uterine expression of vascular endothelial growth factor (VEGFA) and matrix metalloproteinase-9 (MMP9), mediators of angiogenesis (Neufeld *et al.* 1999, Cebe-Suarez *et al.* 2006) and tissue remodeling (Zeng *et al.* 1999), is also undetectable at birth but evident by PND 2 (Chen et al submitted). Additionally, appearance of GE is marked by a dramatic increase in glandular epithelial cell proliferation, evident clearly by PND 3 as reflected by immunostaining of neonatal uterine tissues for proliferating cell nuclear antigen (Masters *et al.* 2007). Thus the period from birth to PND 3 defines an unique transitional window for porcine endometrial development.

Development of the neonatal porcine uterine wall is both estrogen-sensitive and ESR1-dependent (Bartol et al, 1993, 2006; Tarleton *et al.* 1999). Alterations in uterine expression of ESR1, VEGFA and MMP9, evident on PND 14 after estrogen exposure from birth (Chen *et al.* 2010) are indicative of a divergent uterine developmental trajectory (Figure 1) and potentially predictive of uterine dysfunction later in life (Bartol *et al.* 1993, Tarleton *et al.* 2003, Chen *et al.* 2010). Thus, factors affecting expression of these genes also affect the neonatal uterine developmental program and have the potential to define the uterine developmental trajectory with lasting consequences for reproductive performance (Figure 1).

It is likely that lactocrine signaling required to establish the uterine developmental program involves the cooperative actions of multiple MbFs by PND 2. (Chen et al., submitted). Whether duration of lactocrine signaling from birth, or lactocrine signaling initiated after the first two days of neontal life can affect uterine developmental trajectory as reflected by divergent patterns of gene expression on PND 14 has yet to be investigated. Here, objectives were to determine if duration of nursing and age at first nursing affect porcine uterine developmental trajectory as assessed on PND 14 (Figure 1).

MATERIALS and METHODS

Materials

Isoflurane was obtained from Butler Schein Animal Health (Dublin, OH). TRI Reagent was obtained from Sigma–Aldrich (St. Louis, MO). RNeasy Mini kits, RNase-Free DNase sets and RNALater were obtained from Qiagen Inc (Germantown, MD). SuperScript III First-Strand Synthesis System for qPCR was obtained from Invitrogen (Carlsbad, CA). SYBR Green PCR Master Mix was purchased from Applied Biosystems (Foster City, CA). Primers were synthesized by Sigma-Aldrich (St. Louis, MO). Detergent-compatible protein assay kits (DC Protein Assay) were purchased from Bio-Rad Laboratories (Hercules, CA). Mouse anti-human ESR1 (Ab-15) monoclonal antibody was obtained from NeoMarkers, Inc. (Fremont, CA, USA). Mouse anti-human MMP9 (IM09L) and mouse anti-human MMP2 (IM33) monoclonal antibodies were acquired from Calbiochem (Gibbstown, NJ). Rabbit anti-human VEGFA (sc-152-G) and goat anti-human Actin (SC-1615) polyclonal antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). HRP-conjugated anti-mouse and anti-goat secondary antibodies were purchased from Zymed (Carlsbad, CA). Nitrocellulose membranes were obtained from Bio-Rad Laboratories (Hercules, CA). Renaissance Western Blot Chemiluminescence Reagent Plus kit was acquired from Perkin Elmer Life Sciences (Waltham, MA). XOmatic films were purchased from American Imaging (South Plainfield, NJ, USA). Zymogram gels and buffers were purchased from Invitrogen (Carlsbad, CA). Advance Liqui-Wean, a nutritionally complete commercial piglet milk replacer, was purchased from MSC Specialty Nutrition (Carpentersville, IL).

Experimental design and tissue collection

At birth, crossbred-gilts (*Sus scrofa domesticus*) were assigned randomly into one of four treatment groups (n = 4-5/group) and: (a) allowed to nurse *ad libitum*; (b) pan-fed milk-replacer *ad libitum*; (c) allowed to nurse for 2 days from birth then switched to milk replacer; or (d) pan-fed milk replacer for 2 days from birth then switched to nursing until PND 14, when tissues were collected. Care was taken to ensure that treatments were balanced for potential effects of litter and that sows were nursing litters of similar size. Animals were euthanized on PND 14 by exsanguination after anesthetization by isoflurane.

Neonatal uterine tissues were removed, trimmed of fat and associated tissues and ligaments and wet weights recorded. Tissues were then immersed in RNALater and stored at –80°C. Animals were obtained from the Auburn University Swine Research and Education Center, and from the Swine Unit of the New Jersey Agricultural Experiment Station, Rutgers University. 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).

Protein isolation and immunoblotting

Uterine proteins were quantified using procedures described previously (Chen *et al.* 2010)(Chen et al submitted). Uterine tissues (20-50 mg) were homogenized in 200 µl of lysis buffer (1% Triton X-100, 10% Glycerol, 150 mM Tris-HCl, 300 mM NaCl, 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 Laboratories). To document target protein expression, uterine proteins (30μg) were resolved on 12.0% total monomer, Bis-Tris-HCl-buffered polyacrylamide gels under reducing conditions (non-reducing conditions for MMP9 to detect both the latent and active forms, as suggested by the manufacturer) and transferred onto nitrocellulose membranes. After blocking in 10.0% nonfat dry milk in Tris-buffered saline containing Tween-20 (TBST; 25 mM Tris [pH 7.5], 0.14 mM NaCl, 3 mM KCl,0.05% Tween-20), membranes were probed with either mouse anti-human ESR1 antibody (1:200), rabbit anti-human VEGFA antibody (1:1000), mouse anti-human MMP2 or MMP9 antibodies (1:4000) overnight at 4°C. After washing with TBST, blots were incubated with either horseradish peroxidase-conjugated anti-mouse secondary antibody (1:2000 or 1:6000 for MMP9) or anti-rabbit secondary antibody (1:2000) for 1h at room temperature. Bound antibodies were detected by enhanced chemiluminescence. Protein loading was monitored using actin as a reference. Chemiluminescence signals were quantified by densitometry from scans of the film using Scion Image for Windows (Scion Corporation, Frederick, MD).

Zymography

Gelatinase activity was determined for MMP2 and MMP9. Zymography was performed as described previously (Lenhart *et al.* 2001, Ho *et al.* 2007). SDS sample buffer was mixed with 20 μg of protein sample and run on 1% gelatin polyacrylamide zymogram gels under non-denaturing conditions by electrophoresis. Gels were then incubated in renaturing conditions overnight and stained with Comassie Blue (0.05% Comassie Blue R-250, 30% methanol, 10% acetic acid) followed by destaining with Gel Destaining Solution (30% ethanol, 10% acetic acid, 60% water). After the staining

process, areas of gelatinase activity showed up as clear lysis bands on a blue background. Porcine uterine protein extract from day 12 of pregnancy was used as a positive control. Areas of protein degradation were quantified by densitometry from scans of the gel using Scion Image for Windows.

RNA isolation, cDNA synthesis and real time RT-PCR

RNA isolation and real-time quantitative RT-PCR (qPCR) were performed as described previously (Chen *et al.* 2010)(Chen et al submitted). Uterine tissues samples (50mg each) from PND 14 were homogenized in TRI reagent. The RNeasy Mini kit was used to isolate RNA and traces of DNA were removed using the RNase-free DNase kit. RNA purity and concentration were determined by spectrophotometery, with an ideal A_{260}/A_{280} ratio of a pure RNA sample between 1.9-2.1 and an A_{260} absorbance of 1 corresponding to 40 μg RNA/mL. RNA integrity was checked by agarose gel electrophoresis with ethidium bromide staining to aid in visualization of distinct 18S and 28S rRNA bands. Samples of RNA were then diluted to a concentration of 100 ng/μL and stored at -20°C until use. Reverse transcription was carried out using 500 ng of total RNA per sample using the SuperScript III First-Strand Synthesis System for qPCR and the PTC-200 Thermocycler (Bio-Rad Laboratories Inc.). All procedures were carried out according to the manufacturers' instructions.

Quantitative real-time PCR (qPCR) was performed using an Applied Biosystems Gene Amp 7500 Sequence Detection System with the SYBR Green method. Forward and reverse primers for qPCR were designed using Primer Express software (Applied Biosystems) and synthesized by Sigma-Aldrich. All primer sequences were directed to the porcine genome (Table 1). Dissociation curves for each set of primers were checked

to ensure no amplicon-independent amplification. Data were analyzed using the relative standard curve method for quantification of gene expression as described by Applied Biosystems. Standard curves were generated for each gene using two fold dilutions of cDNA from PND 14 uterus (Larionov *et al.* 2005). Target gene expression was normalized to the expression of porcine *18S* and data from qPCR analyses are presented in relative mRNA units.

Statistical analyses

All quantitative data were subjected to analyses of variance using general linear model procedures available with SAS (SAS 2009–2010). For weights, and uterine expression of ESR1, VEGFA, RXFP1, MMP9 and MMP2 protein, activity and mRNA levels, statistical models considered variation due to main effects of duration of nursing and age at first nursing. Preplanned contrasts were performed to determine effects of two days or two weeks nursing duration, as well as age at first nursing (either PND 0 or PND 2) and included: (a) nursing vs replacer; (b) nursing vs nursing to replacer; (c) nursing vs replacer to nursing; (d) replacer vs replacer to nursing. All error terms were identified based upon expectations of the mean squares for error. Data were expressed as least square means with standard errors.

RESULTS

Neonatal porcine uterine weights at PND 14

Data for PND 14 uterine weights, shown here as mg/kg BW ratios, are presented in Figure 2. At PND 14, the ratios were similar between animals that nursed, with no effects of duration of nursing or age at first nursing. However, animals that were only fed replacer for the first two weeks of life had reduced ($P<0.05$) uterine weight ratios compared to the other groups (Figure 2).

Neonatal porcine uterine ESR1and VEGFA protein expression at PND 14

Neonatal gilts that nursed for two weeks expressed a single 51 kDa uterine protein band, corresponding to ESR1 and a 46 kDa band, corresponding to dimeric VEGFA protein (Figure 3A) at PND 14. Both uterine ESR1 and VEGFA protein were reduced ($P<0.05$) in gilts that ingested milk replacer for the two week duration (Figure 3B and C). Nursing animals that were switched to replacer after the first two days of life (N to R) had similar levels of ESR1 and VEGFA protein compared to gilts that nursed for two weeks, while switching replacer-fed gilts to nursing after the first 50h of life (R to N) could not restore ESR1 or VEGFA protein expression (Figure 3B and C).

Neonatal porcine uterine ESR1 and VEGFA gene expression at PND 14

At PND 14, uterine *ESR1* mRNA expression was detectable in all treatment groups and unaffected by duration of nursing or age at first nursing (Figure 4A). Uterine *VEGFA* mRNA expression was detectable in gilts that nursed for two weeks and reduced ($P<0.05$) in all other treatment groups, indicating an effect of duration of nursing and age at first nursing (Figure 4B).

Neonatal porcine uterine MMP9 and MMP2 protein expression at PND 14

Immunoblot analyses were used to identify the type and measure the relative abundance of MMP2 and MMP9 proteins in neonatal uterine tissues. In PND 14 gilts protein bands at 92 and 84 kDa, the reported sizes of proMMP9 and MMP9, respectively (O'Connell *et al.* 1994) were present in uteri of gilts that nursed for the two week duration (Figure 5A). Gilts that pan-fed replacer for the entire two week period showed reduced ($P<0.05$) uterine MMP9 (Figure 5B). Nursing gilts that were switched to replacer after the first 50h of life had similar levels of MMP9 protein when compared to gilts that nursed for two weeks (Figure 5B). Switching replacer-fed gilts to nursing after the first two days after birth could not restore MMP9 protein expression (Figure 5B). A 72 kDa protein band, corresponding to proMMP2, and a 66 kDa band, corresponding to active MMP2, (Crabbe *et al.* 1993) were detected in uteri from all treatment groups (Figure 5A). Neither age at first nursing or duration of nursing affected the relative expression of uterine MMP2 (Figure 5C).

Neonatal porcine uterine MMP9 and MMP2 protein activity at PND 14

Gelatin zymography was used to identify the type and measure the activity of gelatinases in neonatal uterine tissues at PND 14. A representative zymogram illustrating the gelatinolytic activity of neonatal uterine protein extracts is shown (Figure 6A). Zymographic analysis of PND 14 uterine proteins from gilts that nursed for two weeks revealed the presence of lysis bands at 92 and 84 kDa (Figure 6A), the reported sizes of proMMP9 and MMP9 respectively (O'Connell *et al.* 1994). Gelatinolytic activity was reduced ($P<0.05$) in gilts that were fed replacer for two weeks (Figure 6B). Nursing gilts that were switched to replacer after the first 50h of life had similar pro and active MMP9 activity when compared to gilts that nursed for two weeks (Figure 6B). Switching

replacer-fed gilts to nursing after the first two days of life could not restore pro or active MMP9 protein activity (Figure 5B). Lysis bands at 72 and 66 kDa, corresponding to proMMP2 and MMP2 respectively (Crabbe *et al.* 1993), showed uterine gelatinolytic activity in all treatment groups (Figure 6C), and was unaffected by duration of nursing or age at first nursing.

Neonatal porcine uterine MMP9 and MMP2 gene expression at PND 14

At PND 14, both uterine *MMP9* and *MMP2* mRNA expression were detectable in gilts that nursed for two weeks (Figure 7A and B). Gilts pan-fed replacer for two weeks showed reduced ($P<0.05$) expression of uterine *MMP9* and *MMP2* mRNA expression (Figure 7A and B). Nursing animals that were switched to replacer after the first two days of life had similar levels of *MMP9* and *MMP2* mRNA transcripts compared to gilts that nursed for two weeks (Figure 7A and B). Switching replacer-fed gilts to nursing after the first 50h of life could not restore *MMP9* and *MMP2* mRNA expression (Figure 7A and B).

DISCUSSION

Studies show that nursing during the first two days of life is necessary to support normal patterns of uterine developmental gene expression in the neonatal gilt at PND 2 (Chen et al submitted). Results obtained here extend those observations by illustrating that the first two days of life represents a critical window of lactocrine signaling that establishes the developmental program and sets the trajectory for tissue development at PND 14. This is supported by data indicating that lactocrine support during the first two days of life is necessary for a normal uterine developmental trajectory at PND 14, reflected by changes in the expression of uterine ESR1, VEGFA and MMP9. Given that alterations in the uterine developmental trajectory at PND 14 are indicative of abnormal uterine function at adulthood (Chen *et al.* 2010), it is likely that lactocrine signaling plays a vital role in the maturation of the porcine uterus and is necessary for the evolution of the normal uterine phenotype.

Neonatal gilts fed replacer during the first two days of life exhibited no differences in uterine weight in comparison to nursing animals at PND 2, despite radical differences in the expression of uterine developmental markers (Chen et al submitted). This indicates that lactocrine signaling does not affect uterine growth during the first two days of life . However, data obtained here show uterine weight was reduced in gilts fed replacer exclusively during the first two weeks of life compared to all other treatment groups. These results suggest that there are factors present in mature milk that can assist in the growth of the neonatal porcine uterus. For example epidermal growth factor is detectable in mature porcine milk (Jaeger *et al.* 1987) and can stimulate uterine mitogenesis in mice (Nelson *et al.* 1991). Thus, the potential exists for growth factors

present in mature milk to promote trophic effects in the uterus and partially explain why nursing after PND 2 supported uterine growth.

Expression of porcine uterine ESR1, shown to be absent at birth and develops temporalspatially during the first two weeks of life (Tarleton *et al.* 1999, Yan *et al.* 2006a), is dependent on nursing during the first two days post partum. Neonatal gilts fed a synthetic milk replacer had undetectable levels of uterine ESR1 protein compared to their nursing littermates at PND 2 (Chen et al submitted), while supplemental RLX could not restore ESR1 expression in replacer-fed animals. Given these data, it was logical to determine whether ESR1 would be affected by duration of nursing, or restored in replacer-fed animals that nursed after the first two days of life, at PND 14. Results obtained here indicate that animals that were prevented from nursing during the first two days of life continue to express reduced ESR1 protein compared to nursing animals at PND 14. Replacer-fed animals that nursed after the first two days of life failed to express ESR1 levels comparable to those animals that did nurse during the first two days post partum. Similar patterns were observed for VEGFA protein, MMP9 protein and MMP9 activity. Results suggest that there are unique MbFs and/or higher concentrations of MbFs present in colostrum needed to support the expression of uterine developmental genes. This is supported by the fact that in pigs, colostrum has higher levels of hormones and growth factors compared to mature milk, produced after PND 2. These molecules include estrogens (Osterlundh *et al.* 1998, Foisnet *et al.* 2010), relaxin (Yan *et al.* 2006b) and IGF1 (Donovan *et al.* 1994). Both estrogen and relaxin can stimulate expression of porcine uterine ESR1 and VEGFA (Yan *et al.* 2008, Chen *et al.* 2010), as well as porcine uterine MMP9 (Lenhart *et al.* 2001, Chen *et al.* 2010). Exposure of IGF1 can also

stimulate expression of ESR1 (Leclercq *et al.* 2006), VEGFA (Menu *et al.* 2004) and MMP9 (Mira *et al.* 1999). The high levels of these MbFs in colostrum correlates to a critical time-frame before gut closure, which occurs in pigs at 24-36h of life (Leece 1973). These studies support the idea that a lactocrine programming window exists whereby colostrum-derived MbFs are passed into neonatal circulation as a consequence of nursing and absorbed by the neonates before gut closure in order to affect target neonatal tissues (Figure 1).

Since ESR1 is critical for porcine endometrial development and morphogenesis (Tarleton *et al.* 1999) and previous work documents that aberrant ESR1 activation by transient neonatal estrogen exposure can result in negative outcomes for uterine function (Tarleton *et al.* 2003), lactocrine support for neonatal uterine ESR1 expression could be an important developmental component that determines the mature uterine phenotype. This is further supported by the fact that ESR1 can regulate, in part, the expression of VEGFA (Huang *et al.* 1998, Hyder *et al.* 2000) and MMP9 (Zhang *et al.* 2007, Chen *et al.* 2010). The expression of VEGFA, a mediator of tissue angiogenesis and marker of estrogen action (Hyder *et al.* 1996), is important for endometrial development (Shao Longjiang *et al.* 2001, Welter *et al.* 2003) and whose altered expression levels in the neonatal uterus at PND 14 of gilts exposed to estradiol valerate for two weeks from birth is indicative of aberrant hormone exposure (Chen *et al.* 2010). Similarly, expression patterns of MMP9, a regulator of connective tissue remodeling (Visse & Nagase 2003), are also associated with normal uterine development. MMP9 null mice experienced reduced reproductive efficiency due to uterine implantation problems during pregnancy (Roth & Fisher 1999, Dubois *et al.* 2000). Given that VEGFA and MMP9 are both

expressed in the neonatal porcine uterus and regulated in part, by ESR1, it is likely that ESR1 is an important upstream mediator of developmental events that support neonatal uterine morphogenesis, angiogenesis and tissue remodeling.

The expression for *VEGFA* and *MMP9* transcripts were similar to the expression of VEGFA and MMP9 protein levels, thus suggesting a correlation between transcript and protein expression. This trend was not observed for *ESR1*, since the expression of mRNA was similar at PND 14 in all the treatment groups, yet there were differences in ESR1 protein expression. One possible explanation is that in the current system, ESR1 is regulated at the post-transcriptional level. For example, ESR1 mRNA has been shown to be stabilized by exposure to estradiol (Ing & Ott 1999). This could result in increases in translated ESR1 protein. The since replacer-fed animals are not exposed to colostrum-derived estradiol (Osterlundh *et al.* 1998, Foisnet *et al.* 2010), it is possible that translation of ESR1 protein was reduced.

There likely exist many factors that are unique to colostrum that may also affect developmental gene expression, or work cooperatively with colostrum-derived MbFs, such as estrogens, relaxin and IGF1 to establish the uterine developmental program. For example, microRNAs, which regulate gene expression by silencing translation (Alvarez-Garcia & Miska 2005), have been identified in human and bovine milk (Hata *et al.* 2010, Kosaka *et al.* 2010) . Likewise, bioactive peptides encrypted in colostral/milk proteins have been shown to have cytomodulatory effects, influencing cellular proliferation and differentiation (Kampa *et al.* 1997, Hartmann *et al.* 2007). These molecules must be included in the list of MbFs that could affect neonatal tissue development. Together, data obtained from the current study indicate that lactocrine signaling through the ingestion of

colostrum-specific MbFs during the first two days of life is necessary to support the uterine developmental trajectory at PND 14. Much more remains to be deciphered to attain a complete understanding on the role of lactocrine signaling in neonatal development. However, this study extends previous findings and sets the foundation for future work to investigate how lactocrine modulation of the neonatal porcine uteru affect mature uterine function, as well as how MbFs can influence the development of non-reproductive neonatal tissues.

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Figure 1

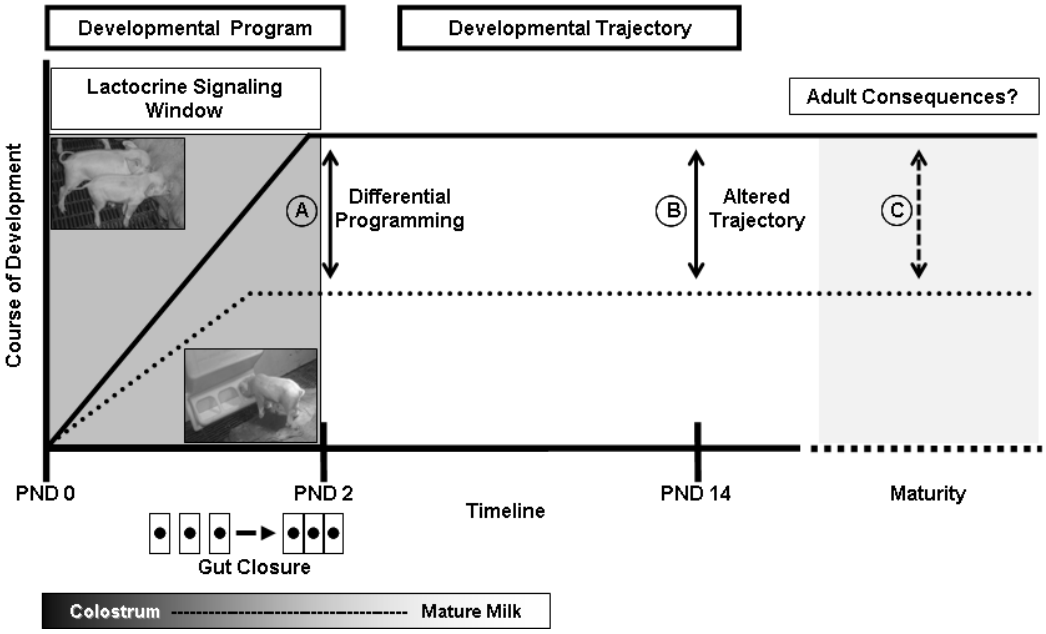

Figure 1. Schematic representation of the lactocrine hypothesis in the pig. The first two days of life represents a critical window for lactocrine programming. Nursing during this period, before gut closure occurs at 36-48h post partum, results in the ingestion of colostrum, which is rich in growth factors, hormones and other bioactive molecules. Nursing after this window results in ingestion of mature milk, produced in the pig after the first two days of life, which has been shown to have a different molecular composition. Animals that don't nurse during the window of lactocrine programming are colostrum-deprived and has an altered uterine developmental program, reflected by changes in markers of uterine growth and remodeling (A). These alterations distorts the uterine developmental trajectory at PND 14, shown in the current study (B). The objectives of future work will be to evaluate the functional outcomes of the mature uterus as a result of these early, lactocrine-mediated changes in program and trajectory (C).

Figure 2

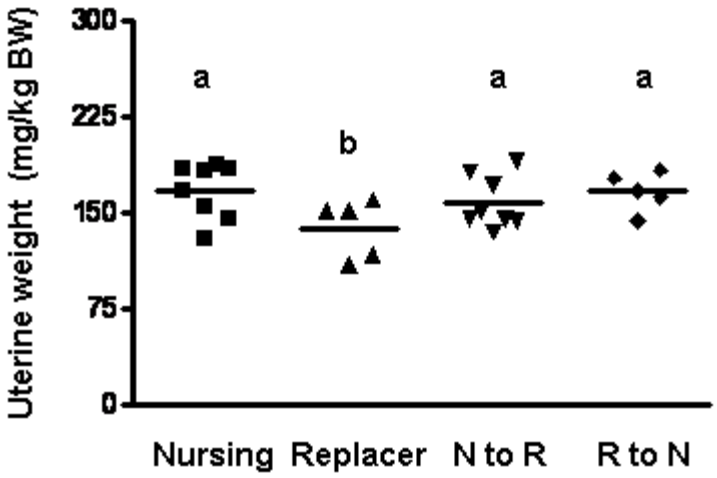

Figure 2. Effects of duration of nursing and age at first nursing on neonatal porcine uterine weight at PND 14. Data are presented as uterine to body weight ratios (mg/kg). Individual values are presented, horizontal bars indicate LSM. N=5-8 animals per group. N to R represents animals that nursed during the first two days of life, and switched to replacer at PND 2. R to N represents animals fed replacer during the first two days of life, and switched to nursing at PND 2. Different letters indicate differences at $P<0.05$.

Figure 3

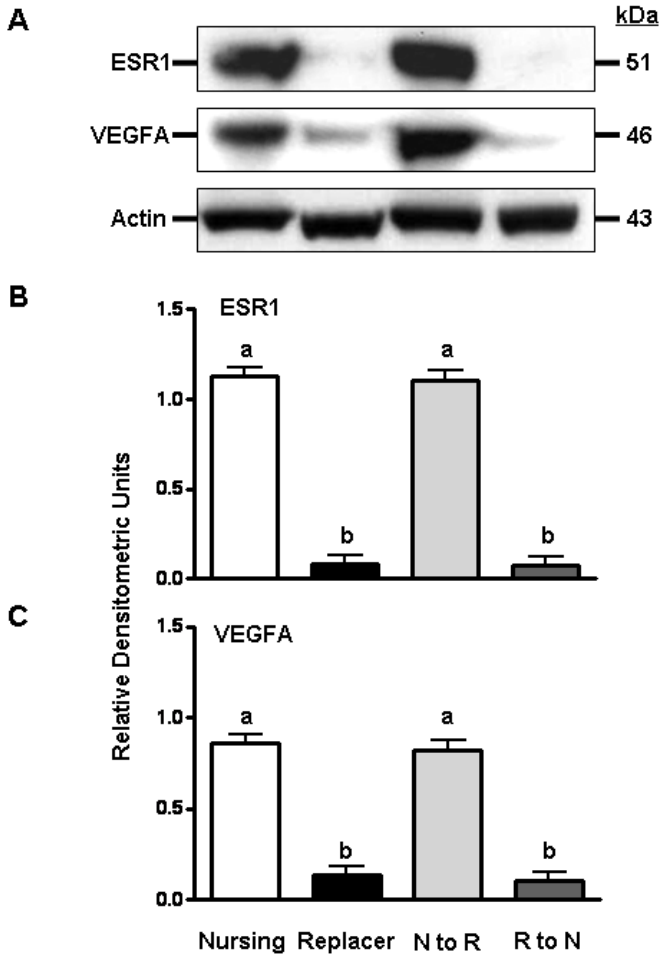

Figure 3. Effects of duration of nursing and age at first nursing on neonatal porcine uterine ESR1 and VEGFA protein expression at PND 14. Representative immunoblots are shown (A). Both 51 and 46 kDa immunoreactive bands for ESR1 and VEGFA respectively, are indicated. An immunoreactive band for actin was detected at 43 kDa and is included as a loading reference. Densitometric data for the relative expression of ESR1 (B) and VEGFA (C) are presented as LSM ± SEM. N=4-5 animals per group. Different letters indicate differences at $P<0.05$.

Figure 4

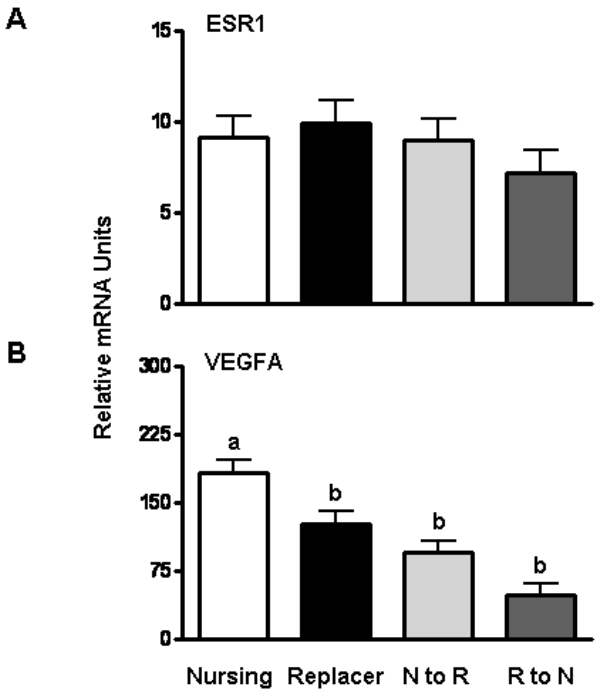

Figure 4. Effects of duration of nursing and age at first nursing on neonatal porcine uterine expression of *ESR1* (A) and *VEGFA* (B) mRNA at PND 14. Transcripts were determined by qPCR. Data were normalized to the expression of *18S*, the reference gene and are presented as LSM ± SEM. N=4-5 animals per group. Different letters indicate differences at $P<0.05$.

Figure 5

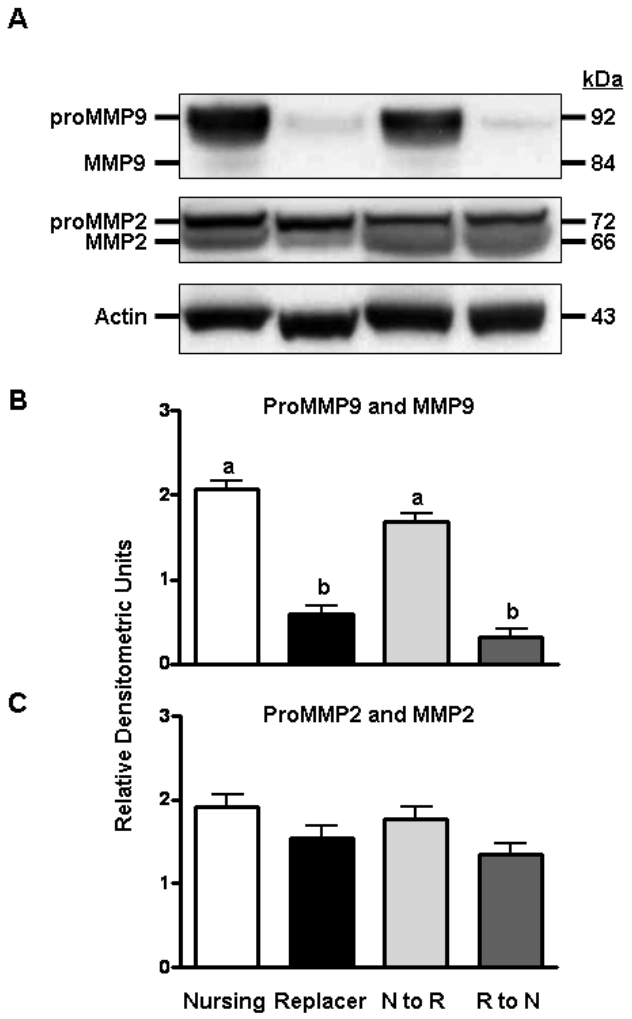

Figure 5. Effects of duration of nursing and age at first nursing on neonatal porcine uterine MMP9 and MMP2 protein expression at PND 14. Representative immunoblots are shown (A). Immunoreactive bands for proMMP9 (92 kDa) and MMP9 (86 kDa), as well as proMMP2 (72 kDa) and MMP2 (66 kDa) are indicated. An immunoreactive band for actin was detected at 43 kDa and is included as a loading reference. Densitometric data for the relative expression of MMP9 (B) and MMP2 (C), including respective pro forms, are presented as LSM ± SEM. N=4-5 animals per group. Different letters indicate differences at $P<0.05$.

Figure 6

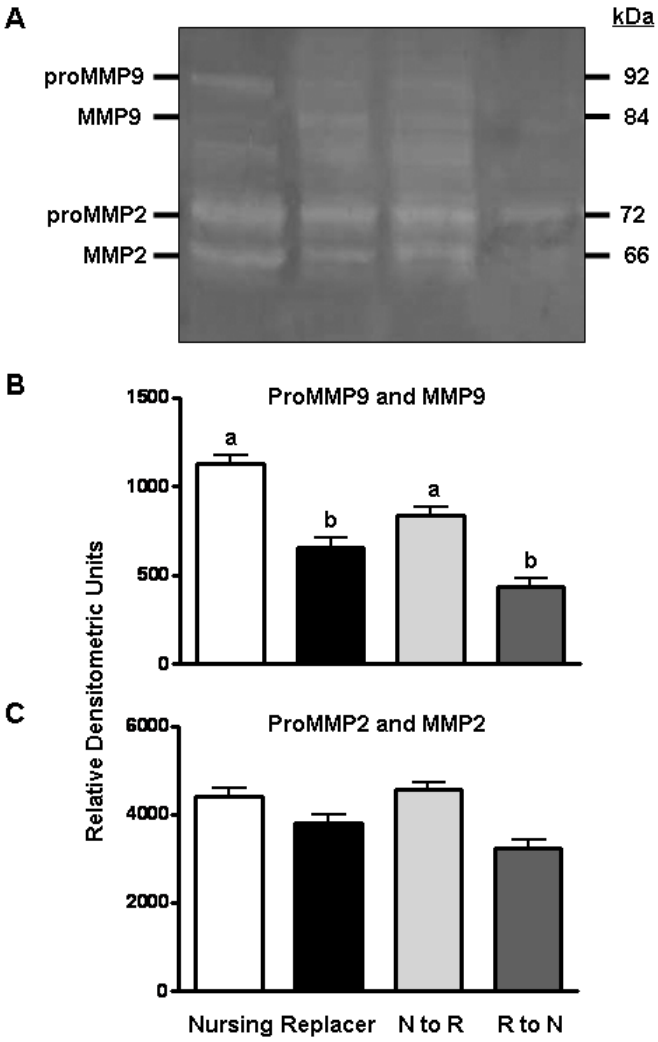

Figure 6. Effects of duration of nursing and age at first nursing on neonatal porcine uterine MMP9 and MMP2 protein activity at PND 14. A representative zymogram is shown (A). Lysis bands for proMMP9 (92 kDa) and MMP9 (86 kDa), as well as proMMP2 (72 kDa) and MMP2 (66 kDa) are indicated. Densitometric data for the gelatinolytic activity of MMP9 (B) and MMP2 (C), including respective pro forms, are presented as LSM ± SEM. N=4-5 animals per group. Different letters indicate differenes at $P<0.05$.

Figure 7

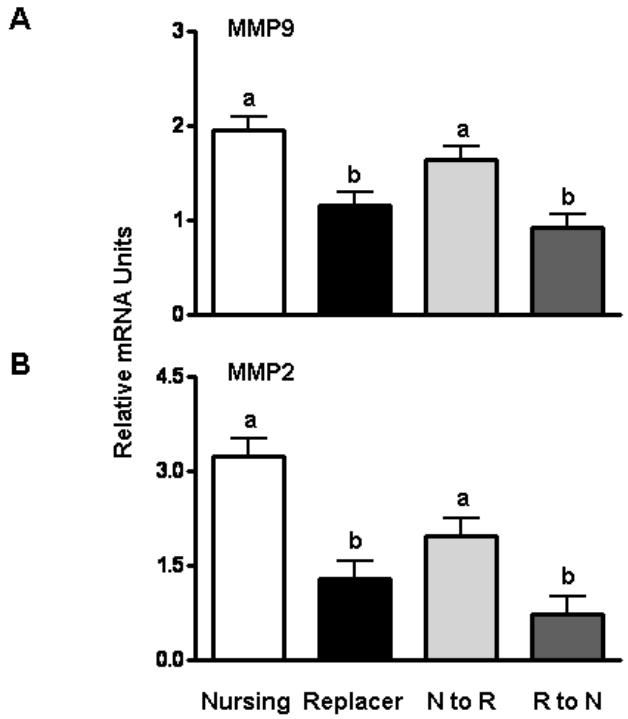

Figure 7. Effects of duration of nursing and age at first nursing on neonatal porcine uterine expression of *MMP9* (A) and *MMP2* (B) mRNA at PND 14. Transcripts were determined by qPCR. Data were normalized to the expression of *18S,* the reference gene and are presented as LSM ± SEM. N=4-5 animals per group. Different letters indicate differences at $P<0.05$.

Table 1. Porcine primer accession numbers and primer sequences for targeted uterine genes.

Gene	Accession #	Forward Primer	Reverse Primer
ESR1	**AF035775**	AGGGAGAGGAGTTTG TGTG	TCTCCAGCAGCAGGTC ATAG
VEGFA	**AF318502**	AAGATCCGCAGACGTG TAAA	CACATCTGCAAGTACG TTCG
MMP9	**DQ132879**	TGGATCCAAAACTACTCGGA AGAC	CGGACAAAGGC GTCG
MMP2	**NM214192**	GAGCACCATCGAGACC ATGA	TTGTAATTGGCCACG TCGG
18S	**AF102857**	CCGCGGTTCTATTTTGTTGG TTTT	CGGGCCGGGTGAGG TTTC

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CHAPTER VII

DISSERTATION CONCLUSIONS

DISSERTATION CONCLUSIONS

The existence of endocrine-disrupting compounds in the neonatal environment and their potential to negatively impact neonatal development is well documented (Soto *et al.* 1994, Humfrey 1998). However, the effects of these agents on the expression profiles of genes associated with uterine growth, patterning and remodeling during the early days of life is less well known. These effects can define the developmental program and set the developmental trajectory for immature tissues and ultimately, affect the mature tissue phenotype. The goal of this research was to identify factors in the environment of neonatal gilts that can alter the expression of genes regulating uterine growth [estrogen receptor alpha (ESR1), vascular endothelial growth factor (VEGFA) and the relaxin receptor (RXFP1)], patterning (WNT7A and HOXA10) and remodeling [matrix metalloproteinase (MMP) 9 and 2].

In studies by Tarleton et al. (2003), EV was used as a tool to disrupt estrogen-sensitive developmental events in the neonate in order to identify long term outcomes of this developmental disruption on the uterus in cyclic and early pregnant gilts (Tarleton *et al.* 2003). The objectives of the studies described in Chapter II were to extend these observations by determining: 1) short-term effects of EV, administered daily from birth through PND 13, on molecular mediators of uterine growth, patterning, and remodeling at PND 14; and2) long-term effects of neonatal estrogen exposure on these markers of endometrial development in adults at PxD 12. The results of this research demonstrated that expression of estrogen-sensitive genes regulating neonatal porcine uterine growth, patterning, and remodeling were altered by neonatal EV exposure acutely at PND 14 and

also resulted in long-term changes in uterine gene expression in adulthood. These studies support and extend previous research showing that neonatal estrogen-induced disruption of uterine function (Tarleton *et al.* 2003) and capacity (Bartol *et al.* 1993) are marked by alterations in uterine expression patterns of developmental genes at both neonatal and adult time periods.

In Chapter III, the hypothesis that exposure of developing gilts pre- and postnatally to the estrogenic mycotoxin ZEA (Kiang *et al.* 1978, Kuiper *et al.* 1998) results in changes in the expression of uterine growth and patterning genes was tested. Results from these studies supported the hypothesis by showing that neonatal gilts exposed to ZEA *in utero* and postnatally through nursing via mothers that consumed ZEA during pregnancy and lactation exhibited an altered pattern of uterine *ESR1*, *RXFP1*, *WNT7A* and *HOXA10* mRNA expression at PND 21. Thus, perinatal exposure of developing female pigs to ZEA as a consequence of ingestion by pregnant and/or lactating females affects neonatal gene expression patterns implicated in regulation of uterine development. These effects, induced by uteroplacental and/or lactocrine delivery of estrogenic endocrine disrupting agents, such as ZEA, may alter the normal uterine developmental program, with the potential for long-term consequences on uterine function and reproductive success later in life.

The results from Chapter III support the lactocrine hypothesis, a proposed mechanism whereby MbFs are delivered to into the circulaton of nursing offspring to influence growth and development of neonatal tissues (Yan et al., 2006). Since RLX is a prototypical milk-borne growth factor in the pig, the goal of studies described in Chapter IV was to determine the effects of RLX, administered for two days from birth, on uterine

growth and expression of ESR1, VEGFA and RXFP1. Results indicated that RLX treatment during the first two days of life increased uterine VEGFA protein expression. This increase was attenuated by pretreatment of gilts with the antiestrogen ICI. These results suggest that RLX can affect mediators of uterine growth during the first two days of life and furthermore, these actions may be mediated in part through uterine ESR1, detectable by PND 2. Taken together with previous observations, these data indicate that critical early events associated with uterine development in the neonatal pig could be facilitated through the actions of milk-borne RLX delivered to the neonate from the maternal system via a lactocrine mechanism.

Building on the results of Chapter IV, if the lactocrine hypothesis for maternal programming of neonatal porcine uterine development is valid, neonates consuming colostrum should display a different pattern of uterine gene expression than those fed a synthetic milk-replacer devoid of RLX and other MbFs. While evidence that ingestion of colostrum is important for neonatal health is well documented (Le Dividich & Noblet 1981, Horwood & Fergusson 1998, Blum & Hammon 2000, Stelwagen *et al.* 2009), the extent to which lactocrine signaling affects development of neonatal somatic tissues is unknown. More specifically whether lactocrine signaling is required to: (i) establish the neonatal uterine developmental program; (ii) determine uterine developmental trajectory; and (iii) set the stage for uterine developmental success as reflected by functional uterine capacity for support of pregnancy has not been investigated. Thus, focus of Chapters V and VI was to investigate the first two issues. Studies described in Chapter V support the lactocrine hypothesis by illustrating that nursing during the first two days of life is required to support the normal uterine developmental program at PND 2, reflected by the

expression of genes associated with uterine development, including ESR1, VEGFA, MMP9 and RXFP1. Supplemental RLX administration to animals fed milk replacer did not restore the uterine phenotype to that of nursing animals at PND 2. Studies described in Chapter VI showed that nursing during the first two days of life is critical for the normal expression patterns of uterine developmental markers and the establishment of the uterine developmental trajectory at PND 14. Together, these data suggest that a lactocrine programming window is open during the first two days of life, during which MbFs uniquely present in colostrum are ingested and absorbed before gut closure (Leece 1973), to support the uterine developmental program and trajectory.

The goal of future studies should be focused on examining whether lactocrine-mediated alterations in the developmental program and trajectory of the porcine uterus, induced by exposure to milk-borne ZEA or the absence of colostrum-specific lactocrine signaling, results in functional consequences on uterine function and reproductive efficiency in adults. In addition, the role of lactocrine signaling on the development of non-reproductive tissues should also be examined, especially since there is evidence indicating that neonatal consumption of milk-replacer can affect patterns of gastrointestinal tract maturation and function (Simmen *et al.* 1990, Zabielski *et al.* 2008, Chapkin *et al.* 2010). Studies from our laboratory indicate that the expression of developmental markers in neonatal porcine cardiac tissues is also sensitive to lactocrine signaling, since neonatal gilts fed milk replacer from birth exhibited an altered cardiac ESR1 profile compared to nursing animals (Sanchez *et al.* 2010). While the studies described in this dissertation focused on genes important for uterine growth, development and remodeling, investigations into global changes in uterine transcript and/or protein

expression in response to lactocrine signaling could provide information about pathways affected by ingestion of MbFs and lead to a better understanding of their mechanism of action on developing tissues.

There exists a myriad of bioactive molecules in the environment that could potentially alter the developmental program and trajectory of neonatal tissues. However, several important sources of exogenous signaling were identified in this research, including xenoestrogens and estrogenic mycotoxins. Exposure to these compounds predictably altered the expression of developmental genes that regulate growth, patterning and remodeling in the estrogen-sensitive neonatal porcine uterus. Importantly, a novel discovery was made showing that nursing from birth and thus lactocrine signaling is necessary for the expression of uterine developmental markers in neonatal gilts associated with growth and remodeling. Together, data presented here show that aberrant exposure to bioactive agents or the absence of maternally driven lactocrine signaling can alter the developmental program and trajectory of neonatal uterine tissues. Reproductive capacity is a perpetual concern in the agricultural production industry, as well as in clinical settings . Studies that can advance the knowledge of uterine development could greatly contribute to both fields of study.

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