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MILK-BORNE BIOACTIVE FACTORS:

EFFECTS ON NEONATAL PORCINE REPRODUCTIVE TISSUES

by

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ABSTRACT OF THE DISSERTATION

Milk-Borne Bioactive Factors:

Effects on Neonatal Porcine Reproductive Tissues

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Milk-borne bioactive factors (MbFs) are delivered to nursing offspring via a lactocrine mechanism to affect development of somatic tissues, including the uterus. In the pig, lactocrine-sensitive events associated with postnatal uterine development between birth (postnatal day = PND 0) and PND 2 define the developmental program and can determine developmental trajectory and function. However, lactocrine-sensitive elements of the neonatal porcine uterine transcriptome have not been defined during this period. The extent to which MbFs, including insulin-like growth factor (IGF)-I, affect development of the porcine uterus postnatally is unknown. Furthermore, whether nursing supports testicular development in pigs by PND 2 remains to be determined. Research goals were to (1) define the lactocrine-sensitive uterine transcriptome in nursed gilts compared to animals fed replacer from birth until PND 2; (2) determine whether feeding colostrum, with or without IGF-I supplementation, supports endometrial cell proliferation at 12 h postnatal; and (3) assess whether nursing affects Sertoli and Leydig cell

development as well as expression of markers important for testicular development within two days after birth. Results showed that in uteri of nursed as compared to replacer-fed gilts, 896 genes were differentially expressed and multiple elements of biological processes and pathways affected by PND 2. Additionally, data indicated that when compared to replacer-fed gilts, a single dose of colostrum at birth increased endometrial cell proliferation at 12 h postnatal. Furthermore, oral IGF-I increased uterine cell proliferation when co-administered with replacer, but not with colostrum. Sertoli cell number and proliferation were increased in boars nursed for two days compared to newborn or replacer-fed boars at PND 2. However, testicular *RXFP1* expression was increased in replacer-fed animals as compared to boars collected at birth or after two days of nursing. Collectively, data presented here reinforce and extend previous findings that lactocrine signaling supports neonatal porcine reproductive development. Results can be used to refine and focus the lactocrine hypothesis to identify key MbFs as well as cellular and molecular mechanisms that regulate postnatal male and female reproductive development in the pig.

DEDICATION

"Family is the most important thing in the world."

~Princess Diana

I dedicate this work to my family.

Thank you for your unconditional love and support.

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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 better understand the impact of nursing on the development of both female and male reproductive tissues in pigs. Colostrum, or first milk, contains many biologically active factors that have the ability to enter into neonatal circulation to affect tissue development. Although the role of colostrum in imparting passive immunity to the neonate is well understood, the impact of nursing on development of somatic tissues, including the reproductive tract, is less well defined. Thus, the purpose of this literature review is to explain how neonatal tissues can be influenced by nursing as proposed in the lactocrine hypothesis. Studies indicating that biologically active factors, present in colostrum, can influence neonatal development and adult function will be reviewed. Further, postnatal development of uterine and testicular tissues will be explored. Finally, the significance of this work as it relates to aspects of neonatal porcine reproductive development and long-term reproductive performance will be discussed.

B. Developmental programming

The notion that the relationship between genotype and phenotype is not linear but rather a complex interplay between genes and environmental cues was first put forth by Stockard (Stockard 1921) and defined as the epigenome by Waddington (Waddington 1942). Epigenetics, in a general sense, may be defined as the study of stable changes in gene expression or phenotype that do not result from changes to the DNA sequence (Goldberg *et al.* 2007). The ability of the genome to respond to environmental cues that alter the expressed phenotype is called “plasticity” and occurs during development

(Hochberg *et al.* 2011). The idea of plasticity goes hand-in-hand with "programming", which is defined as the capacity of developing tissues to adapt to conditions present in early life (Langley-Evans 2006). Taken together, developmental programming describes the process whereby environmental factors present during a critical window of development can elicit a response that produces long-term alterations in tissue structure and/or function (Langley-Evans 2006). Evidence supporting developmental programming comes from epidemiological studies that established a relationship between early cues and the risk of developing diseases in adulthood. Barker and Osmond (1986) suggested that environment in early life can determine risk of disease in adulthood. They recognized that fetal undernutrition during pregnancy, indicated by low birth weight, was correlated with later development of ischaemic heart disease (Barker 1993, 1995). Further evidence relating undernutrition during pregnancy and disease comes from retrospective studies of the Dutch Hunger Winter during the Second World War, where populations were subjected to periods of severe undernutrition (Langley-Evans 2006). It is clear that prenatal undernutrition programmed greater risk of adult coronary heart disease (Roseboom *et al.* 2000) and obesity (Ravelli *et al.* 1999). Together, these studies indicate that fetal nutrition during early life can affect physiology in adult life.

The notion of developmental origins of adult disease is an evolving concept now recognized to include neonatal as well as fetal stages of development. For instance, neonatal nutrition, such as fat content in milk, has an impact on adult tissue function in rats (Khan *et al.* 2005). Kahn et al., (2005) observed that adult offspring were hypertensive and hyperinsulinemic after nursing from dams fed a high-fat diet compared to control rats. Therefore, nutrition during the postnatal programming stage effected a

change in development resulting in an alteration in adult tissue function and metabolism. Observations suggest that events occurring during the neonatal period, such as altering nutrition, can affect postnatal developmental programming with lasting effects on adult tissue function.

C. Colostrum as a conduit for delivery of biologically active factors

Lactation is the defining characteristic of all mammals and involves milk production by the mammary glands for delivery to nursing offspring (Peaker 2002). It is through nursing that maternal influence on offspring development is extended into the postnatal period. Colostrum (first milk) is the initial secretions from the mammary glands following onset of parturition. Compared to mature milk, colostrum contains higher concentrations of crude protein and lower concentrations of fat and lactose in pigs (Klobasa *et al.* 1987) and other species (Langer 2009), and is rich in milk-borne bioactive factors (MbFs) (Blum and Baumrucker 2008). As in other mammals (Langer 2009), colostrum composition in pigs changes over time during early lactation (Klobasa *et al.* 1987, Bartol *et al.* 2012). For instance, the total protein content of colostrum declines by 50% within the first 12 h of lactation (Klobasa *et al.* 1987). The transition from colostrum to mature milk occurs by 24-48 h after birth when concentrations of immunoglobulin (Ig) G drops and IgA becomes more prevalent (Klobasa *et al.* 1987).

Studies show that colostrum is important for imparting passive immunological protection to newborn mammals (Brambell 1966). For example, milk from vaccinated sows fed to ten day old pigs provided passive protection against *Esherichia coli* infections by increasing the survival time compared to animals fed milk from

unvaccinated sows (Wilson and Svendsen 1971). Similarly, newborn calves challenged orally with bovine rotavirus that were fed colostrum from vaccinated cows were protected against infection and did not develop disease symptoms compared to calves fed colostrum from uninoculated cows (Saif *et al.* 1983). Furthermore, Saif and colleagues (1983) determined that the colostral IgG content was higher in inoculated as compared to uninoculated cows. Results show that immunization of the mother against diseases boosts colostrum concentrations of antibodies that, once ingested, serve to protect the neonates from disease.

Transfer of passive immunity, specifically Igs, can occur *in utero* and/or via colostrum intake. Whether passage of Igs from the maternal to the fetal circulation occurs *in utero* depends upon placental type. Histologically, placental classifications are based on the number of cell layers that separate fetal and maternal blood supplies (Enders and Blankenship 1999). The least invasive type of placentation, epitheliochorial, consists of six layers including the fetal endothelial cells, connective tissue and chorionic epithelial cells apposed to maternal uterine epithelial cells, connective tissue, and endothelial cells. In the endotheliochorial placenta involves erosion of the endometrial epithelium, leaving the maternal capillary endothelium in direct contact with chorionic epithelium. In hemochorial placentation, chorionic epithelium is in direct contact with the maternal blood (Enders and Blankenship 1999). Only in hemochorial and endotheliochorial placentae can large molecules, including immunoglobulins, cross the placenta and enter the fetal blood stream (Enders and Blankenship 1999). Malek and colleagues (1996) demonstrated that IgG readily crosses the human placenta into the fetal circulation during the second and third trimesters of pregnancy by measuring concentrations in maternal

serum and fetal cord blood. Results showed that there was a positive correlation between the ratio of fetal to maternal IgG levels and gestational age, suggesting that the amount of IgG transported across the placenta continues to increase as pregnancy continues (Malek *et al.* 1996). Unlike human infants, the nature of the epitheliochorial placenta prevents pigs, horses, and ruminants from receiving Igs *in utero*. Instead, these species acquire passive immunity by ingestion of colostrum after birth (Rooke and Bland 2002). For instance, Markowska-Daniel and colleagues (2010) measured serum Ig concentrations in pigs immediately following birth, prior to nursing, and on PND 7, 14, and 56. Results showed that Ig levels were below assay sensitivity at birth, but were detectable in the serum following nursing at PND 7, 14, and 56 (Markowska-Daniel *et al.* 2010). Together these studies demonstrate the importance of lactation for acquisition of Ig transmission in the newborn pig and indicate that such large molecules are absorbed during this time.

Neonatal gut permeability is an important factor affecting absorption of intact macromolecules from colostrum. Intestinal cells in neonatal animals have the ability to take up macromolecules, such as Ig, by endocytosis and transport them intact into the circulation (Sangild 2003). Increased neonatal intestinal permeability in young offspring compared to older animals is observed in a variety of mammalian species including humans (Weaver *et al.* 1984), rodents, and ungulates (Pacha 2000, Sangild 2003). Loss of macromolecular gut permeability, which occurs over time in neonatal mammals, is termed “gut closure”. Timing of gut closure is species-specific (Pacha 2000). By feeding lactulose and mannitol and measuring their levels in infant urine, Weaver and colleagues (1984) were able to estimate the timing of gut closure in humans. Results indicated that a daily decrease in lactulose and mannitol excretion occurred gradually following birth and

PND 4, indicating that the molecules were not being taken up from the gut and transported into circulation for excretion in the urine (Weaver *et al.* 1984). This was interpreted to indicate reduced permeability of the intestine. In ungulates, including the pig, the ability of the gut to absorb macromolecules ceases within the first few days after birth as well (Sangild 2003). In nursing pigs, the ability of the gut to absorb polyvinylpyrrolidone, a nonprotein macromolecule, ceases at 24-36 hours after birth (Lecce and Morgan 1962). Therefore, a window of opportunity for the uptake of large molecules in nursing neonatal pigs is only open within the first few days after birth.

D. Nursing can impact development of the neonate

Although the importance of colostrum in imparting passive immunity to the neonate is widely understood and accepted, the role of other MbFs in influencing neonatal development is less well known. Colostrum contains a variety of MbFs including growth factors, antimicrobial peptides, and hormones (Grosvenor 1992, Donovan and Odle 1994, Playford *et al.* 2000) that can affect development of neonatal tissues. Evidence to support this comes from elegant studies conducted in marsupials. Unlike eutherian mammals, marsupials have comparatively short gestation periods, give birth to altricial young, and have long lactation periods (Trott *et al.* 2003). To accommodate the nutritional needs of growing neonates (called pouch young), the composition of their milk changes progressively during lactation and is characterized in three distinct phases (Green *et al.* 1980). These changes in milk composition correlate with the ages of pouch young and can dictate their development. Evidence of this comes from studies conducted where pouch young consume milk out-of-phase with their age. In one study, pouch young were

fostered onto mothers at more advanced phases of lactation and, ultimately, displayed advanced development including substantial covering of fur and improved motor coordination compared to animals ingesting milk that was in phase with their developmental ages (Trott *et al.* 2003). Kwek and colleagues (2009) showed that young at PND 120 that were fostered to mothers at a later stage of lactation [lactation day (LD) 170] displayed more advanced fore-stomach maturation (Kwek *et al.* 2009).

There is also evidence of MbFs dictating development in eutherian animals. Nusser and Frawley (1997) showed that mammotrope development in rats coincides with exposure to early milk. Newborn rat pups were fostered onto dams that had given birth a week prior and were therefore deprived of milk factors from early lactation (Nusser and Frawley 1997). Fostered pups displayed a delay in development of mammotropes that, in adulthood, resulted in altered prolactin secretory activity (Nusser and Frawley 1997). To determine which milk-borne agent(s) influenced mammotrope development, newborn rat pituitary cells were cultured with early milk protein fractions of different molecular size (Porter and Frawley 1991). Results suggested that cell differentiation was stimulated by peptides of 2-6 kDa in size (Porter and Frawley 1991). In 2013, Liu and colleagues showed that offspring of heterozygous or homozygous tumor necrosis factor-α (TNFα) knockout mice exhibited enhanced memory in adulthood compared with offspring of wild-type parents (Liu et al., 2013). When wild-type offspring were cross fostered onto TNFα-deficient mothers, their memory as adults was improved. Results were interpreted to indicate that elements of the postnatal environment were regulating this effect (Liu et al., 2013). Analysis of milk revealed that several chemokines were down-regulated in secretions obtained from TNFα knockout mice compared to controls. Supplementing

pups born to TNFα-deficient mothers with those chemokines reduced memory performance in adulthood. These observations were interpreted to indicate that murine hippocampal development is lactocrine-sensitive (Liu et al., 2013). Taken together, these studies illustrate that maternal lactocrine programming of somatic cells and tissues can affect neonatal development with consequences on adult phenotype.

E. The lactocrine hypothesis

The lactocrine hypothesis was proposed to describe a mechanism whereby MbFs, communicated from mother to offspring as a specific consequence of nursing, affect the developmental program of somatic tissues during early neonatal life with the potential to affect the developmental trajectory of such tissues and, consequently, the functional capacity of those tissues later in life (Bartol *et al.* 2008). This hypothesis, upon which investigations described in this dissertation are based, was developed through studies designed to determine how the peptide hormone relaxin (RLX) was involved in porcine neonatal uterine development.

Relaxin, a 6 kDa protein hormone, acts through its cognate receptor, the RLX family peptide receptor-1 (RXFP1) (Hsu et al., 2002). Expression of RXFP1 was detected in porcine uterine tissues at birth (Yan *et al.* 2006b). Administration of exogenous RLX to neonatal pigs increased uterine and cervical wet weights, as well as expression of estrogen receptor-α (ESR1) and vascular endothelial growth factor (VEGF) A, recognized to support uterine and cervical developmental (Yan *et al.* 2008). Immunoreactive RLX was found in porcine colostrum, with highest concentrations identified during the first 24h of lactation (Yan *et al.* 2006b). In gilts allowed to nurse

normally from birth, serum RLX levels were elevated on PND 0 and 1, but were undetectable by PND 2. Immunoreactive RLX was undetectable in the serum of newborn pigs prior to nursing, and in the serum of piglets fed porcine milk replacer in lieu of nursing (Yan *et al.* 2006b). The inability of nursing piglets to take up RLX on and after PND 2 is consistent with the observation that gut closure occurs within three days of birth (Lecce and Morgan 1962). Milk-borne RLX bioactivity was highest in LD 0 secretions and decreased to undetectable levels by LD 4 (Frankshun *et al.* 2010). Thus, high levels of bioactive RLX are present in colostrum/milk during the first few days of lactation.

Overall, data for the neonatal pig indicate that: (1) bioactive RLX is present in porcine colostrum at highest levels within two days of birth; (2) milk-borne RLX can be absorbed into circulation of nursing offspring when consumed within two days of birth; (3) RXFP1 is present in the porcine endometrium at birth; and (4) both exogenous and milk-borne, lactocrine-active RLX support endometrial development as reflected by events including increased endometrial cell proliferation and cell compartment-specific ESR1 expression associated with endometrial maturation (Yan et al., 2006, Yan et al., 2008, Miller et al., 2013). Collectively, these observations provided evidence to support the lactocrine hypothesis for maternal programming of FRT development (Bartol *et al.* 2008).

F. Evidence supporting the lactocrine hypothesis in pigs

Evidence supporting the lactocrine hypothesis for maternal programming of uterine development in pigs comes from a study designed to determine effects of imposition of a lactocrine-null state for two days from birth, by feeding porcine milk replacer in lieu of

nursing, on neonatal porcine uterine development at PND 2 and PND 14. Nursing through PND 2 supported endometrial cytodifferentiative and morphogenetic events associated with the genesis of nascent uterine glands, including transcription of genes important for uterine development (Miller *et al.* 2013). Further, imposition of the lactocrine-null state altered patterns of uterine gene expression at transcriptional and translational levels on PND 2 (Miller *et al.* 2013). These effects persisted to PND 14, even after gilts were returned to nursing on PND 2. Moreover, in the absence of lactocrine signaling for two days from birth, endometrial growth and uterine gland development were markedly reduced by PND 14 (Miller *et al.* 2013). Collectively, these data indicate that disruption of normal lactocrine signaling by imposition of the lactocrine null state for two days from birth alters the neonatal porcine uterine developmental program and may also alter the developmental trajectory of uterine tissues with lasting consequences.

Vallet and colleagues developed an immunocrit assay to measure the concentration of Ig in the serum of nursing piglets within one day of birth (Vallet *et al.* 2012). Since milk-borne Ig traverses the intestinal barrier and enters the neonatal circulation rapidly, the quantity of Ig in the blood can be used as an indicator of the amount of colostrum ingested (Vallet *et al.* 2012). Using immunocrit data, a retrospective test of the lactocrine hypothesis was conducted to determine if there are long-term consequences of colostrum ingestion on reproductive performance in adulthood. Immunocrit values were obtained from 381 gilts on the first day of life. The number of pigs born alive for the same gilts across four parities was recorded (total ~1525 litters). Results showed that pigs with low immunocrit values as neonates, corresponding to lower colostrum intake or quality on

PND 0, had fewer pigs born alive in adulthood (Bartol *et al.* 2013). Thus, as predicted by the lactocrine hypothesis, colostrum consumption early in life can alter the uterine development program that has lasting consequences on adult reproductive capacity.

G. Insulin-like growth factor (IGF)-I: Presence in colostrum and neonatal uptake

The insulin family of hormones and growth factors, including insulin, IGF-I, IGF-II, and relaxin and are mediators of tissue growth and development (Jones and Clemmons 1995). Like RLX, IGF-I has been identified in colostrum from many species including humans (Baxter *et al.* 1984), rats (Donovan *et al.* 1991), cows (Francis *et al.* 1986), goats (Faulkner 1999), and pigs (Simmen *et al.* 1988). It was also demonstrated that IGF-I concentrations are highest in colostrum and decrease over the course of lactation with the transition to mature milk production (Baxter *et al.* 1984, Francis *et al.* 1986, Simmen *et al.* 1988, Donovan *et al.* 1991). In the pig, IGF-I concentrations are highest in colostrum on lactation day 0, although levels reported vary (Simmen *et al.* 1990, Donovan and Odle 1994). It was suggested that the variation in colostral IGF-I concentrations may be attributed to breed differences and/or timing of colostrum collection (<12 h of lactation vs >12 h of lactation). An early study showed that insulin, like RLX, is able to pass through the wall of the gastro-intestinal tract of neonatal rats (Mosinger *et al.* 1959). Mosigner and Placer (1959) showed that oral administration of insulin in 2- and 8-day old neonatal rats induced hypoglycemia. Authors suggest that insulin was able to avoid digestion and pass, intact, through the intestine to regulate glucose concentrations in blood.

Studies have also been conducted on another member of the insulin family of hormones and growth factors, IGF-I. Burrin and colleagues (1996) fed recombinant human IGF-I (rhIGF-I; 10 ug/ml on PND 1; 20 ug/ml PND 2-4 per feeding) in formula to pigs from birth to PND 4. Plasma rhIGF-I levels were measured on PND 1, prior to feeding, and then daily through PND 4. There were no differences in plasma IGF-I levels in pigs fed formula supplemented with IGF-I compared to animals fed formula alone (Burrin *et al.* 1996). However, in rats, Philipps and colleagues (2000) fed suckling neonates rh^{125}I-IGF-I and measured IGF-I levels in portal blood at 5, 10, 20, and 30 minute intervals. Results indicated that IGF-I is absorbed into the portal circulation at maximal concentrations by 20 and 30 minutes post ingestion (Philipps *et al.* 2000). Studies also showed that orally supplemented IGF-I is biologically active in the neonate and can support tissue development. Houle and colleagues (1997) fed pigs formula alone or supplemented with rhIGF-I from birth through PND 4 and collected intestinal tissue on PND 7 and PND 14. Results indicated that orally administered IGF-I did not affect whole body growth, but did increase intestinal enzyme activity and ileal villus growth (Houle *et al.* 1997). Another group fed formula alone or supplemented with rhIGF-I for the first four days after birth and collected intestinal tissues at PND 4 (Burrin *et al.* 1996). Data from this study suggested that oral administration of IGF-I increased small intestinal weight, protein and DNA content, and villus height in neonatal pigs (Burrin *et al.* 1996). Extra-intestinal effects of oral IGF-I supplementation were recorded at even earlier neonatal ages for pigs. For instance, pigs given IGF-I orally for 24 h displayed an increase in pancreatic weight and DNA content at PND 1 (Xu *et al.* 1994). Data on IGF-I

uptake and effects on neonatal tissue development are mixed, and whether oral supplementation of IGF-I can support neonatal uterine development is unknown.

H. Neonatal uterine development in pigs

Development of the porcine uterus, begins prenatally and continues into postnatal life (Bartol *et al.* 1993). The initial changes occurring in the neonatal uterus between birth and PND 60 are not dependent on ovarian signals since ovariectomy on PND 0 did not affect uterine growth and or uterine wall development (Tarleton *et al.* 1998). Ovary-independent maturation of the porcine endometrium is characterized by significant remodeling of uterine histoarchitecture. At birth, the histology of the porcine uterus is rudimentary consisting of simple columnar luminal epithelial (LE) supported by the stromal mesenchyme (Spencer *et al.* 1993). Absent at birth, uterine glands begin to form (a process termed adenogenesis) with differentiation of glandular epithelium (GE) from the LE (Bartol *et al.* 1993). Nascent uterine glands continue to mature, elongate, and penetrate underlying stroma, eventually extending to the border of the myometrium (Spencer *et al.* 1993). By PND 56, endometrial folds are apparent in the uterine wall and uterine glands have coiled and branched throughout the stroma (Spencer *et al.* 1993). The porcine uterus is functionally mature by PND 120 since it has the ability to support pregnancy (Dziuk and Gehlbach 1966, Bartol *et al.* 1993).

Adenogenesis in the neonatal porcine uterus is both estrogen receptor-α (ESR1) - dependent and estrogen sensitive (Tarleton *et al.* 1998, Tarleton *et al.* 1999, 2001). Absent at birth, ESR1 expression is evident in nascent glandular epithelium as early as 24 h postnatal (Bartol *et al.* 2012) and consistently evident at PND 15 (Tarleton *et al.* 1998,

Cooke *et al.* 2013). Administered from birth, estradiol valerate (EV) advanced and the type-II antiestrogen ICI 182,780 inhibited endometrial development and adenogenesis in neonatal pigs (Tarleton *et al.* 1999). Administration of EV from birth also altered expression of genes important for uterine development at PND 14 as reflected by decreased *WNT7A* expression and increased expression of *HOXA10, RXFP1*, and *MMP9* (Chen *et al.* 2010). Overall, data can be interpreted to indicate that ESR1 and ESR1-medited signaling is required for normal neonatal porcine uterine development. In this respect, ESR1 is not only a marker, but also a mediator of uterine development in the pig.

Given the importance of the ESR1 system in determining neonatal uterine developmental fate, it is reasonable to propose that factors acting through or conditions affecting ESR1 mediated signaling have the potential to affect uterine developmental programming events. Yan and colleagues (2006) reported that treatment with RLX from birth altered neonatal uterine development in pigs most overtly only after ESR1 expression had been initiated. While, for example, treatment of gilts with RLX daily from birth increased LE height on PND 2, effects were most more pronounced at PND 12, after onset of ESR1 expression, such that both uterine weight and protein content were increased (Yan *et al.* 2006a). Administration of exogenous RLX or estradiol-17β (E) from birth to PND 2 not only increased uterine weight and protein content, but also increased uterine VEGFA expression (Yan *et al.* 2008, Chen *et al.* 2010), a protein important for angiogenesis and endometrial growth (Welter *et al.* 2003), as well as expression of MMP9 (Yan *et al.* 2008, Chen *et al.* 2010), a proteolytic enzyme that targets the extracellular matrix and is important for uterine tissue growth and remodeling (Lenhart *et al.* 2001). Furthermore, treatment of neonatal gilts with either RLX or E

increased endometrial cell proliferation, as indicated by proliferating cell nuclear antigen (PCNA) immunostaining (Masters *et al.* 2007). Interestingly, pretreatment with ICI 182,780, an estrogen receptor antagonist, attenuated the effects of administering E or RLX alone on uterine weight, uterine luminal epithelial height, and cell proliferation (Yan et al., 2006 Repro; Masters et al., 2007). Consistently, Pillai and colleagues (1999) observed that pretreatment of ovariectomized rats with ICI attenuated RLX-induced uterine edema. Authors interpreted this finding to indicate that RLX could be acting, in part, through crosstalk with the ER system (Pillai et al., 1999). Altogether, these observations suggest that effects of RLX on uterine development in the pig: (1) are enhanced in the presence of a functional ESR1 system; and (2) indicate crosstalk between the RXFP1 and ESR1 signaling systems.

I. Global gene expression profiling of the developing uterus

Our knowledge of mechanisms governing neonatal uterine development is limited. Recent and emerging technologies enable comprehensive examination of the array of transcripts expressed by cells and tissues, called the transcriptome (Wang *et al.* 2009). Microarray technology employs a technique where an unknown sample is hybridized to an array of immobilized nucleic acids for which the sequences are known, allowing for the identification of thousands of genes simultaneously (Gershon 2002, Wang *et al.* 2009). Although the microarray method supports reasonably high-throughput analyses, it does have several limitations including high background levels due to cross-hybridization (Wang *et al.* 2009). RNA-Sequencing (RNAseq) is a high throughput technique that involves conversion of the entire array of mRNA transcripts obtained from cells or

tissues into a complementary DNA (cDNA) library which can then be sequenced. The resulting sequences, which can be quantitated, are then aligned to a reference genome (Wang *et al.* 2009). Advantages to RNAseq over microarray are substantial. There are no physical limitations delimiting the array of transcripts that can be detected. Alternate transcript splicing can be identified and described. There are effectively no complications associated with background signal and no upper limits on quantification and number of unique sequences that can be identified. Additionally, results are highly reproducible (Wang *et al.* 2009, Wilhelm and Landry 2009). Consequently, RNAseq is currently the method of choice for transcriptome profiling (Wang et al., 2009).

Regardless of the method employed, novel information about the developing uterine transcriptome has been revealed. Results from studies using microarray technology suggest that neonatal mouse uterine development involves coordinated changes in expression of genes coding for transcription factors, growth factors, enzymes, and appropriate receptors (Hu *et al.* 2004, Filant and Spencer 2013). Microarray analysis of LE and GE isolated from normal and aglandular, progesterone-induced uterine gland knockout mice at PND 10 revealed greater expression of genes in the LE compared to GE, with GE-expressed genes related to morphogenesis, development, migration, and retinoic acid signaling (Filant and Spencer 2013). RNAseq was used to define the porcine endometrial transcriptome on pregnancy day (PxD) 14 (Samborski *et al.* 2013). Analysis revealed that as compared to cyclic controls, overrepresented terms of differentially expressed genes that were lower in pregnancy included extracellular region, ion transport, and cell adhesion. Those that were higher including defense response, activation of immune response, and response to hormone stimulus (Samborski et al., 2014). RNAseq

technology has yet to be used in efforts to define the neonatal porcine devleopmetnal transcriptome.

J. Neonatal testicular development in pigs

In male pigs the testes also undergo development during the neonatal period. The testes contain two prominent somatic cell types. Sertoli cells, located within the seminiferous tubules, function in support of spermatogenesis by nurturing germ cell development. Early Sertoli cell development differs in pigs as compared to rodents. In rats and mice, Sertoli cell proliferation is maximal during the late fetal and early neonatal period (Steinberger and Steinberger 1971, Orth 1982, Kluin *et al.* 1984) whereas the maximal rate of Sertoli cell proliferation in boars occurs after birth, between PND 1 and PND 14 (McCoard *et al.* 2001). In boars, greater testicular weight at PND 14 is associated with an increase in Sertoli cell number (McCoard *et al.* 2001). Sertoli cell number determines adult testes size and subsequent capacity of the testes to produce sperm (McCoard *et al.* 2001). These observations suggest that Sertoli cell number, established during the early neonatal period in the pig, can determine mature testicular size and adult fertility. Further, Sertoli cell proliferation during this early neonatal period in pigs is sensitive to estrogens. Reducing endogenous estrogens increased proliferation of porcine Sertoli cells during the first two months of life (At-Taras *et al.* 2006).

The second prominent somatic cell type within the testis, Leydig cells are found in the interstitial compartment and function in production of steroid hormones. Like Sertoli cells, these cells also undergo discrete phases of development. In most species there are two distinct populations of Leydig cells, fetal and adult. The fetal population appears

first, immediately following early differentiation of the fetal testis (Lejeune *et al.* 1998, Ivell *et al.* 2013). In rodents, fetal Leydig cells involute postnatally and a new population of Leydig cells emerges within the testes (Ivell *et al.* 2013). This new population further differentiates into adult Leydig cells, with the process completed by 7-8 wk of age (Ivell *et al.* 2013). In pigs, as in humans (Bay *et al.* 2007), there is an intermediate population of Leydig cells present during the perinatal period that persists until 2.5 weeks after birth (Van Straaten and Wensing 1978). During this phase of Leydig cell development the neonatal porcine testes produce significant amounts of steroid hormones (Schwarzenberger *et al.* 1993) including androgens (Colenbrander *et al.* 1978) and estrogens (Ford 1983). Consistently, Leydig cells express steroidogenic enzymes required to catalyze production of steroid hormones (Choi *et al.* 2009). Neonatal porcine Leydig cell development and steroidogenic activity are negatively regulated by estrogens, since reducing endogenous estrogen levels leads to increased Leydig cell testosterone production during the first months of life in pigs (At-Taras *et al.* 2008).

Both Sertoli (At-Taras *et al.* 2008) and Leydig (Griswold 1998) cells are necessary for normal testicular function, including spermatogenesis. A previous report demonstrated that reducing endogenous estrogens during the neonatal period in pigs alters testicular function later in life (At-Taras *et al.* 2006). This, therefore, suggests that a critical period for proper neonatal testicular development exists postnatally and can affect subsequent mature reproductive efficiency. Whether nursing, occurring during this time, can alter testicular development is unknown.

K. Mediators of Testicular Development

A number of factors, including ESR1, RXFP1, and VEGFA, are recognized to be involved in mediation of testis development (Korpelainen *et al.* 1998, Hess and Carnes 2004, Krajnc-Franken *et al.* 2004). In the pig, ESR1 was undetectable immunohistochemically on PND 1-2 (Nielsen *et al.* 2001). However, by 3 months of age, ESR1 was localized to Sertoli, Leydig, peritubular myoid, and germ cells in the porcine testis (Ramesh *et al.* 2007). The adult ESR1 knockout mouse is infertile because of increased germ cell apoptosis and atrophy within seminiferous tubules (Gould *et al.* 2007) and dilution of cauda epididymal sperm (Hess 2003). These observations support the idea that ESR1-mediated events affect development and function of the male reproductive tissues, including the testis.

The receptor for relaxin, RXFP1, is a transmembrane protein belonging to the leucine-rich G protein receptor family (Hsu et al., 2002) and is present in male reproductive tissues, including the testis, throughout development (Kato *et al.* 2010). Both RXFP1 mRNA and protein were localized to Sertoli, Leydig, and germ cells in boar testis (Kato *et al.* 2010). Compared to wild-type controls, RXFP1-null mice exhibited increased germ cell apoptosis in the testis, as well as spermatogenic arrest, resulting in reduced fertility suggesting that RXFP1 is a regulator of male reproductive efficiency (Samuel *et al.* 2003, Krajnc-Franken *et al.* 2004).

A major regulator of angiogenesis, VEGFA was observed to be important in male reproductive tissues during postnatal life. In neonatal bovine testes, VEGFA protein was localized to mitotically active spermatogonia, as well as to Sertoli and Leydig cells (Caires *et al.* 2009). Culturing explanted bovine testicular tissue with VEGF increased

germ cell survival and number compared to controls (Caires *et al.* 2009). Additionally, grafting of explanted testicular tissues after culture with VEGFA increased spermatid production (Caires *et al.* 2009). Results of bovine studies suggest that neonatal VEGFA supports spermatogonial survival leading, ultimately, to greater sperm production (Caires *et al.* 2009). However, mice overexpressing VEGFA are infertile due to spermatogenic arrest (Korpelainen *et al.* 1998). This suggests that appropriate levels of VEGFA are necessary, during development and, perhaps, later in life to insure optimal capacity for spermatogenesis.

L. Significance and Objectives

The importance of colostrum in providing passive immunological protection to the neonate is well documented. Although it is also recognized that colostrum contains many other biologically active molecules including hormones, growth factors, and antimicrobial peptides (Grosvenor 1992, Donovan and Odle 1994, Playford *et al.* 2000), the functional roles of such molecules in support of neonatal development are not well understood. Nevertheless, it is clear that nursing supports development of somatic tissues beyond the gut and, in the pig, that neonatal uterine tissues are sensitive to lactocrine signaling that can have lasting consequences for reproductive performance. The long-term goal of this research is to better understand the importance of nursing in maternal programming of neonatal development.

Working hypotheses underlying research presented herein is that: (1) nursing from birth affects the neonatal uterine transcriptome; (2) MbFs, known to be present in colostrom, exemplified by IGF-I, can be orally active in the neonatal gilt as reflected by

effects on neonatal uterine development; and that (3) nursing supports reproductive development in males as well as females. S**pecific objectives** were to:

1. Define the lactocrine-sensitive neonatal uterine transcriptome on PND 2 by comparing RNAseq results generated from uterine tissues obtained from gilts that were either nursed or milk replacer-fed from birth.
2. Determine the impact of feeding colostrum, with or without IGF-I supplementation, on uterine cell proliferation at 12 h postnatal.
3. Determine effects of nursing from birth on porcine testicular development at PND 2.

M. References

At-Taras, EE, T Berger, MJ McCarthy, AJ Conley, BJ Nitta-Oda, and JF Roser 2006 Reducing estrogen synthesis in developing boars increases testis size and total sperm production. *J Androl* **27** 552-559.

At-Taras, EE, IC Kim, T Berger, A Conley, and JF Roser 2008 Reducing endogenous estrogen during development alters hormone production by porcine Leydig cells and seminiferous tubules. *Domest Anim Endocrinol* **34** 100-108.

Barker, DJ 1993 Fetal origins of coronary heart disease. *Br Heart J* **69** 195-196.

Barker, DJ 1995 Fetal origins of coronary heart disease. *Bmj* **311** 171-174.

Bartol, FF, AA Wiley, and CA Bagnell 2008 Epigenetic programming of porcine endometrial function and the lactocrine hypothesis. *Reprod Domest Anim* **43 Suppl 2** 273-279.

Bartol, FF, AA Wiley, DJ Miller, AJ Silva, KE Roberts, ML Davolt, JC Chen, AL Frankshun, ME Camp, KM Rahman, JL Vallet, and CA Bagnell 2012 Lactocrine signaling and developmental programming. *J Anim Sci*.

Bartol, FF, AA Wiley, DJ Miller, AJ Silva, KE Roberts, ML Davolt, JC Chen, AL Frankshun, ME Camp, KM Rahman, JL Vallet, and CA Bagnell 2013 Lactation Biology Symposium: lactocrine signaling and developmental programming. *J Anim Sci* **91** 696-705.

Bartol, FF, AA Wiley, TE Spencer, JL Vallet, and RK Christenson 1993 Early uterine development in pigs. *J Reprod Fertil Suppl* **48** 99-116.

Baxter, RC, Z Zaltsman, and JR Turtle 1984 Immunoreactive somatomedin-C/insulin-like growth factor I and its binding protein in human milk. *J Clin Endocrinol Metab* **58** 955-959.

Bay, K, HE Virtanen, S Hartung, R Ivell, KM Main, NE Skakkebaek, AM Andersson, and J Toppari 2007 Insulin-like factor 3 levels in cord blood and serum from children: effects of age, postnatal hypothalamic-pituitary-gonadal axis activation, and cryptorchidism. *J Clin Endocrinol Metab* **92** 4020-4027.

Blum, JW, and CR Baumrucker 2008 Insulin-like growth factors (IGFs), IGF binding proteins, and other endocrine factors in milk: role in the newborn. *Adv Exp Med Biol* **606** 397-422.

Brambell, FW 1966 The transmission of immunity from mother to young and the catabolism of immunoglobulins. *Lancet* **2** 1087-1093.

Burrin, DG, TJ Wester, TA Davis, S Amick, and JP Heath 1996 Orally administered IGF-I increases intestinal mucosal growth in formula-fed neonatal pigs. *Am J Physiol* **270** R1085-1091.

Caires, KC, J de Avila, and DJ McLean 2009 Vascular endothelial growth factor regulates germ cell survival during establishment of spermatogenesis in the bovine testis. *Reproduction* **138** 667-677.

Chen, JC, AA Wiley, TY Ho, AL Frankshun, KM Hord, FF Bartol, and CA Bagnell 2010 Transient estrogen exposure from birth affects uterine expression of developmental markers in neonatal gilts with lasting consequences in pregnant adults. *Reproduction* **139** 623-630.

Choi, I, JY Kim, EJ Lee, YY Kim, CS Chung, J Chang, NJ Choi, HJ Chung, and KH Lee 2009 Ontogeny of expression and localization of steroidogenic enzymes in the neonatal and prepubertal pig testes. *J Androl* **30** 57-74.

Colenbrander, B, FH de Jong, and CJ Wensing 1978 Changes in serum testosterone concentrations in the male pig during development. *J Reprod Fertil* **53** 377-380.

Cooke, PS, TE Spencer, FF Bartol, and K Hayashi 2013 Uterine glands: development, function and experimental model systems. *Mol Hum Reprod* **19** 547-558.

Donovan, SM, RL Hintz, DM Wilson, and RG Rosenfeld 1991 Insulin-like growth factors I and II and their binding proteins in rat milk. *Pediatr Res* **29** 50-55.

Donovan, SM, and J Odle 1994 Growth factors in milk as mediators of infant development. *Annu Rev Nutr* **14** 147-167.

Dziuk, PJ, and GD Gehlbach 1966 Induction of Ovulation and Fertilization in the Immature Gilt. *J Anim Sci* **25** 410-412.

Enders, AC, and TN Blankenship 1999 Comparative placental structure. *Adv Drug Deliv Rev* **38** 3-15.

Faulkner, A 1999 Changes in plasma and milk concentrations of glucose and IGF-1 in response to exogenous growth hormone in lactating goats. *J Dairy Res* **66** 207-214.

Filant, J, and TE Spencer 2013 Cell-specific transcriptional profiling reveals candidate mechanisms regulating development and function of uterine epithelia in mice. *Biol Reprod* **89** 86.

Ford, JJ 1983 Serum estrogen concentrations during postnatal development in male pigs. *Proc Soc Exp Biol Med* **174** 160-164.

Francis, GL, LC Read, FJ Ballard, CJ Bagley, FM Upton, PM Gravestock, and JC Wallace 1986 Purification and partial sequence analysis of insulin-like growth factor-1 from bovine colostrum. *Biochem J* **233** 207-213.

Frankshun, AL, TY Ho, DC Reimer, J Chen, S Lasano, BG Steinetz, FF Bartol, and CA Bagnell 2010 Characterization and biological activity of relaxin in porcine milk. *Reproduction* **141** 373-380.

Gershon, D 2002 Microarray technology: an array of opportunities. *Nature* **416** 885-891.

Goldberg, A, C Allis, and E Bernstein 2007 Epigenetics: A Landscape Takes Shape. *Cell* **128** 635-638.

Gould, ML, PR Hurst, and HD Nicholson 2007 The effects of oestrogen receptors alpha and beta on testicular cell number and steroidogenesis in mice. *Reproduction* **134** 271-279.

Green, B, K Newgrain, and J Merchant 1980 Changes in milk composition during lactation in the tammar wallaby (Macropus eugenii). *Aust J Biol Sci* **33** 35-42.

Griswold, MD 1998 The central role of Sertoli cells in spermatogenesis. *Semin Cell Dev Biol* **9** 411-416.

Grosvenor, CE, Picciano, M.R., and Baumrucker, C.R. 1992 Hormones and Growth Factors in Milk. *Endocrine Reviews* **14** 710-728.

Hess, RA 2003 Estrogen in the adult male reproductive tract: a review. *Reprod Biol Endocrinol* **1** 52.

Hess, RA, and K Carnes 2004 The role of estrogen in testis and the male reproductive tract: a review and species comparison. *Anim. Reprod.* **1** 5-30.

Hochberg, Z, R Feil, M Constancia, M Fraga, C Junien, JC Carel, P Boileau, Y Le Bouc, CL Deal, K Lillycrop, R Scharfmann, A Sheppard, M Skinner, M Szyf, RA Waterland, DJ Waxman, E Whitelaw, K Ong, and K Albertsson-Wikland 2011 Child health, developmental plasticity, and epigenetic programming. *Endocr Rev* **32** 159-224.

Houle, VM, EA Schroeder, J Odle, and SM Donovan 1997 Small intestinal disaccharidase activity and ileal villus height are increased in piglets consuming formula containing recombinant human insulin-like growth factor-I, Pediatr Res, pp. 78-86.

Hu, J, CA Gray, and TE Spencer 2004 Gene expression profiling of neonatal mouse uterine development. *Biol Reprod* **70** 1870-1876.

Ivell, R, JD Wade, and R Anand-Ivell 2013 INSL3 as a biomarker of Leydig cell functionality. *Biol Reprod* **88** 147.

Jones, JI, and DR Clemmons 1995 Insulin-like growth factors and their binding proteins: biological actions. *Endocr Rev* **16** 3-34.

Kato, S, Siqin, I Minagawa, T Aoshima, D Sagata, H Konishi, K Yogo, T Kawarasaki, H Sasada, H Tomogane, and T Kohsaka 2010 Evidence for expression of relaxin hormone-receptor system in the boar testis. *J Endocrinol* **207** 135-149.

Khan, IY, V Dekou, G Douglas, R Jensen, MA Hanson, L Poston, and PD Taylor 2005 A high-fat diet during rat pregnancy or suckling induces cardiovascular dysfunction in adult offspring. *Am J Physiol Regul Integr Comp Physiol* **288** R127-133.

Klobasa, F, E Werhahn, and JE Butler 1987 Composition of sow milk during lactation. *J Anim Sci* **64** 1458-1466.

Kluin, PM, MF Kramer, and DG de Rooij 1984 Proliferation of spermatogonia and Sertoli cells in maturing mice. *Anat Embryol (Berl)* **169** 73-78.

Korpelainen, EI, MJ Karkkainen, A Tenhunen, M Lakso, H Rauvala, M Vierula, M Parvinen, and K Alitalo 1998 Overexpression of VEGF in testis and epididymis causes infertility in transgenic mice: evidence for nonendothelial targets for VEGF. *J Cell Biol* **143** 1705-1712.

Krajnc-Franken, MA, AJ van Disseldorp, JE Koenders, S Mosselman, M van Duin, and JA Gossen 2004 Impaired nipple development and parturition in LGR7 knockout mice. *Mol Cell Biol* **24** 687-696.

Kwek, JH, RD Iongh, MR Digby, MB Renfree, KR Nicholas, and M Familari 2009 Cross-fostering of the tammar wallaby (Macropus eugenii) pouch young accelerates fore-stomach maturation. *Mech Dev* **126** 449-463.

Langer, P 2009 Differences in the composition of colostrum and milk in eutherians reflect differences in immunoglobulin transfer. *Journal of Mammalogy* **90** 332-339.

Langley-Evans, SC 2006 Developmental programming of health and disease. *Proc Nutr Soc* **65** 97-105.

Lecce, JG, and DO Morgan 1962 Effect of dietary regimen on cessation of intestinal absorption of large molecules (closure) in the neonatal pig and lamb. *J Nutr* **78** 263-268.

Lejeune, H, R Habert, and JM Saez 1998 Origin, proliferation and differentiation of Leydig cells. *J Mol Endocrinol* **20** 1-25.

Lenhart, JA, PL Ryan, KM Ohleth, SS Palmer, and CA Bagnell 2001 Relaxin increases secretion of matrix metalloproteinase-2 and matrix metalloproteinase-9 during uterine and cervical growth and remodeling in the pig. *Endocrinology* **142** 3941-3949.

Malek, A, R Sager, P Kuhn, KH Nicolaides, and H Schneider 1996 Evolution of maternofetal transport of immunoglobulins during human pregnancy. *Am J Reprod Immunol* **36** 248-255.

Markowska-Daniel, I, M Pomorska-Mol, and Z Pejsak 2010 Dynamic changes of immunoglobulin concentrations in pig colostrum and serum around parturition. *Pol J Vet Sci* **13** 21-27.

Masters, RA, BD Crean, W Yan, AG Moss, PL Ryan, AA Wiley, CA Bagnell, and FF Bartol 2007 Neonatal porcine endometrial development and epithelial proliferation affected by age and exposure to estrogen and relaxin. *Domest Anim Endocrinol* **33** 335-346.

McCoard, SA, DD Lunstra, TH Wise, and JJ Ford 2001 Specific staining of Sertoli cell nuclei and evaluation of Sertoli cell number and proliferative activity in Meishan and White Composite boars during the neonatal period. *Biol Reprod* **64** 689-695.

Miller, DJ, AA Wiley, JC Chen, CA Bagnell, and FF Bartol 2013 Nursing for 48 hours from birth supports porcine uterine gland development and endometrial cell-compartment specific gene expression. *Biol Reprod* **88** 4.

Mosinger, B, Z Placer, and O Koldovshy 1959 Passage of insulin through the wall of the gastro-intestinal tract of the infant rat. *Nature* **184** 1245-1246.

Nielsen, M, IB Bogh, M Schmidt, and T Greve 2001 Immunohistochemical localization of estrogen receptor-alpha in sex ducts and gonads of newborn piglets. *Histochem Cell Biol* **115** 521-526.

Nusser, KD, and S Frawley 1997 Depriving neonatal rats of milk from early lactation has long-term consequences on mammotrope development. *Endocrine* **7** 319-323.

Orth, JM 1982 Proliferation of Sertoli cells in fetal and postnatal rats: a quantitative autoradiographic study. *Anat Rec* **203** 485-492.

Pacha, J 2000 Development of Intestinal Transport Function in Mammals. *Physiological Reviews* **80** 1633-1667.

Peaker, M 2002 The mammary gland in mammalian evolution: a brief commentary on some of the concepts. *J Mammary Gland Biol Neoplasia* **7** 347-353.

Philipps, AF, B Dvorak, PJ Kling, JG Grille, and O Koldovsky 2000 Absorption of milk-borne insulin-like growth factor-I into portal blood of suckling rats. *J Pediatr Gastroenterol Nutr* **31** 128-135.

Playford, RJ, CE Macdonald, and WS Johnson 2000 Colostrum and milk-derived peptide growth factors for the treatment of gastrointestinal disorders. *Am J Clin Nutr* **72** 5-14.

Porter, TE, and LS Frawley 1991 Stimulation of prolactin cell differentiation in vitro by a milk-borne peptide. *Endocrinology* **129** 2707-2713.

Ramesh, R, CA Pearl, E At-Taras, JF Roser, and T Berger 2007 Ontogeny of androgen and estrogen receptor expression in porcine testis: effect of reducing testicular estrogen synthesis. *Anim Reprod Sci* **102** 286-299.

Ravelli, AC, JH van Der Meulen, C Osmond, DJ Barker, and OP Bleker 1999 Obesity at the age of 50 y in men and women exposed to famine prenatally. *Am J Clin Nutr* **70** 811-816.

Rooke, JA, and LM Bland 2002 The acquisition of passive immunity in the new-born piglet. *Livestock Production Science* **78** 13-23.

Roseboom, TJ, JH van der Meulen, C Osmond, DJ Barker, AC Ravelli, JM Schroeder-Tanka, GA van Montfrans, RP Michels, and OP Bleker 2000 Coronary heart disease after prenatal exposure to the Dutch famine, 1944-45. *Heart* **84** 595-598.

Saif, LJ, DR Redman, KL Smith, and KW Theil 1983 Passive immunity to bovine rotavirus in newborn calves fed colostrum supplements from immunized or nonimmunized cows. *Infect Immun* **41** 1118-1131.

Samborski, A, A Graf, S Krebs, B Kessler, and S Bauersachs 2013 Deep sequencing of the porcine endometrial transcriptome on day 14 of pregnancy. *Biol Reprod* **88** 84.

Samuel, CS, H Tian, L Zhao, and EP Amento 2003 Relaxin is a key mediator of prostate growth and male reproductive tract development. *Lab Invest* **83** 1055-1067.

Sangild, PT 2003 Uptake of colostral immunoglobulins by the compromised newborn farm animal. *Acta Vet Scand Suppl* **98** 105-122.

Schwarzenberger, F, GS Toole, HL Christie, and JI Raeside 1993 Plasma levels of several androgens and estrogens from birth to puberty in male domestic pigs. *Acta Endocrinol (Copenh)* **128** 173-177.

Simmen, FA, RC Simmen, and G Reinhart 1988 Maternal and neonatal somatomedin C/insulin-like growth factor-I (IGF-I) and IGF binding proteins during early lactation in the pig. *Dev Biol* **130** 16-27.

Simmen, FA, KY Whang, RC Simmen, GA Peterson, MD Bishop, and KM Irvin 1990 Lactational variation and relationship to postnatal growth of insulin-like growth factor-I in mammary secretions from genetically diverse sows. *Domest Anim Endocrinol* **7** 199-206.

Spencer, TE, AA Wiley, and FF Bartol 1993 Neonatal age and period of estrogen exposure affect porcine uterine growth, morphogenesis, and protein synthesis. *Biol Reprod* **48** 741-751.

Steinberger, A, and E Steinberger 1971 Replication pattern of Sertoli cells in maturing rat testis in vivo and in organ culture. *Biol Reprod* **4** 84-87.

Stockard, C 1921 Developmental rate and structural expression: An experimental study of twins, 'double monsters' and single deformities, and the interaction among embryonic organs during their origin and development. *American Journal of Anatomy* **28** 115-277.

Tarleton, BJ, AA Wiley, and FF Bartol 1999 Endometrial development and adenogenesis in the neonatal pig: effects of estradiol valerate and the antiestrogen ICI 182,780. *Biol Reprod* **61** 253-263.

Tarleton, BJ, AA Wiley, and FF Bartol 2001 Neonatal estradiol exposure alters uterine morphology and endometrial transcriptional activity in prepubertal gilts. *Domest Anim Endocrinol* **21** 111-125.

Tarleton, BJ, AA Wiley, TE Spencer, AG Moss, and FF Bartol 1998 Ovary-independent estrogen receptor expression in neonatal porcine endometrium. *Biol Reprod* **58** 1009-1019.

Trott, JF, KJ Simpson, RL Moyle, CM Hearn, G Shaw, KR Nicholas, and MB Renfree 2003 Maternal regulation of milk composition, milk production, and pouch young development during lactation in the tammar wallaby (Macropus eugenii). *Biol Reprod* **68** 929-936.

Vallet, JL, JR Miles, and LA Rempel 2012 A simple novel measure of passive transfer of maternal immunoglobulin is predictive of preweaning mortality in piglets. *Vet J* **195** 91-97.

Van Straaten, HW, and CJ Wensing 1978 Leydig cell development in the testis of the pig. *Biol Reprod* **18** 86-93.

Waddington, CH 1942 The epigenotype. *Int J Epidemiol* **41** 10-13.

Wang, Z, M Gerstein, and M Snyder 2009 RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet* **10** 57-63.

Weaver, LT, MF Laker, and R Nelson 1984 Intestinal permeability in the newborn. *Arch Dis Child* **59** 236-241.

Welter, H, K Wollenhaupt, U Tiemann, and R Einspanier 2003 Regulation of the VEGF-system in the endometrium during steroid-replacement and early pregnancy of pigs. *Exp Clin Endocrinol Diabetes* **111** 33-40.

Wilhelm, BT, and JR Landry 2009 RNA-Seq-quantitative measurement of expression through massively parallel RNA-sequencing. *Methods* **48** 249-257.

Wilson, MR, and J Svendsen 1971 Immunity to Escherichia coli in pigs. The role of milk in protective immunity to e. coli enteritis. *Can J Comp Med* **35** 239-243.

Xu, RJ, DJ Mellor, MJ Birtles, BH Breier, and PD Gluckman 1994 Effects of oral IGF-I or IGF-II on digestive organ growth in newborn piglets. *Biol Neonate* **66** 280-287.

Yan, W, J Chen, AA Wiley, BD Crean-Harris, FF Bartol, and CA Bagnell 2008 Relaxin (RLX) and estrogen affect estrogen receptor alpha, vascular endothelial growth factor, and RLX receptor expression in the neonatal porcine uterus and cervix. *Reproduction* **135** 705-712.

Yan, W, PL Ryan, FF Bartol, and CA Bagnell 2006a Uterotrophic effects of relaxin related to age and estrogen receptor activation in neonatal pigs. *Reproduction* **131** 943-950.

Yan, W, AA Wiley, RA Bathgate, AL Frankshun, S Lasano, BD Crean, BG Steinetz, CA Bagnell, and FF Bartol 2006b Expression of LGR7 and LGR8 by neonatal porcine uterine tissues and transmission of milk-borne relaxin into the neonatal circulation by suckling. *Endocrinology* **147** 4303-4310.

CHAPTER II

Defining the Lactocrine-Sensitive Neonatal Porcine Uterine Transcriptome

ABSTRACT

Milk-borne factors delivered to nursing offspring via a lactocrine mechanism affect development of somatic tissues, including the uterus. Gene expression events associated with porcine endometrial maturation between birth (postnatal day = PND 0) and PND 2 can determine and may define the uterine developmental program. Lactocrine-sensitive elements of the neonatal porcine uterine transcriptome have not been defined during this period. Therefore, the objective here was to determine effects of imposition of the lactocrine-null condition for 48 h from birth on the porcine uterine transcriptome at PND 2 using RNA sequencing (RNAseq). Gilts (n = 4/group) were assigned at birth to be: (1) nursed *ad libitum*, or (2) gavage-fed (30 ml/kg BW/2h) commercial porcine milk-replacer for 48 h. Uteri were obtained at 50 h. Total uterine RNA was extracted and both concentration and integrity were determined. For each uterus, 500 ng of RNA was used for cDNA library preparation. Libraries were sequenced (paired end) at > 90 million reads per sample. After demultiplexing, raw reads were mapped to the latest pig Sscrofa10.2 build using Avadis NGS software. Validation compared RNAseq results to those obtained for targeted genes using quantitative RT-PCR. Gene enrichment and functional analyses were conducted. On PND 2, 896 genes were differentially expressed ($P \leq 0.05$, corrected for false discovery rate) in nursed as compared to milk replacer-fed gilts. Gene enrichment and functional analyses revealed alterations in multiple biological processes and pathways including estrogen receptor-alpha and hedgehog signaling pathways, and the plasminogen activating network. Results can be used to refine and focus hypotheses related to identification of cellular, molecular, and lactocrine mechanisms regulating neonatal uterine and endometrial development.

INTRODUCTION

Colostrum serves as a conduit for delivery of many biologically active molecules to newborn mammals including growth factors, hormones, immunoglobulins, and antimicrobial peptides (Grosvenor 1992, Donovan and Odle 1994, Playford *et al.* 2000). This 'first milk' enables maternal influence on offspring development to continue after birth and into neonatal life. Milk-borne bioactive factors (MbFs), delivered to nursing offspring via a lactocrine mechanism, enter the neonatal circulation and can influence tissue development (Yan *et al.* 2006, Bartol *et al.* 2008). Nursing was reported to support marsupial forestomach development (Kwek *et al.* 2009), differentiation and secretory function of anterior pituitary mammotropes in rats (Nusser and Frawley 1997), and development of the murine hippocampus (Liu *et al.* 2014). Lactocrine signaling also supports development of the neonatal porcine uterus. Imposition of a lactocrine-null state from birth, by feeding porcine milk replacer in lieu of nursing, inhibited development of nascent endometrial glands, reduced epithelial cell proliferation, and was associated with cell compartment-specific changes in expression patterns for estrogen receptor-α (ESR1) and other genes implicated in endometrial adenogenesis (Miller *et al.* 2013). These effects were evident by PND 2 (Miller *et al.* 2013).

The lactocrine-sensitive neonatal uterine transcriptome has yet to be defined. Therefore, the objective of this study was to identify effects of imposition of the lactocrine-null condition from birth on global porcine uterine gene expression patterns on PND 2 using RNA Sequencing (RNAseq) technology.

MATERIALS AND METHODS

Animals and Experimental Design

Gilts (*Sus scrofa domesticus*) were born from an established herd of crossbred pigs and raised at the Swine Unit of the New Jersey Agricultural Experiment Station, Rutgers University. All procedures involving animals were reviewed and approved by the Rutgers Institutional Animal Care and Use Committee and conducted in accordance with the Guide for the Care and Use of Agricultural Animal in Agricultural Research and Teaching (McGlone *et al.* 2010). Care was taken to ensure that sows were nursing litters of similar size.

At birth, gilts were assigned randomly to treatment groups (n = 4/group) to be either: (1) nursed *ad libitum* from birth; or (2) gavage-fed milk replacer (30 mL/kg BW/2h) from birth through 48 h of age. Gilts were euthanized and uterine tissues collected at 50 h of age. The experimental design is depicted in Figure 1. Each uterus was trimmed free of associated tissues and uterine wet weights (mg) were recorded. Uteri were immersed in RNALater and stored at -80ºC until total RNA could be extracted and processed for RNAseq.

Isolation and analysis of uterine RNA

Total RNA was isolated from 50-60 mg of uterine tissue for each sample using the miRNA-easy kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's protocol. Quantity and integrity of total RNA samples were measured using a NanoDrop ND-100 (Thermo Scientific; Waltham, MA, USA), Agilent 2100 Bioanalyzer (Applied Biosystems; Carlsbad, CA), and Qubit® 2.0 Fluorometer (Invitrogen; Carlsbad, CA,

USA). Samples with RNA integrity number (RIN) values of 8.0 or higher were used for RNAseq analyses.

Preparation of RNAseq libraries

Sequencing procedures were conducted by the Levy lab (www.hudsonalpha.org/levy-lab) at the HudsonAlpha Institute for Biotechnology (HAIB; Hunstville, AL, USA). The workflow for RNAseq analyses is depicted in Figure 2. Total RNA (500 ng/sample) was converted into cDNA for library preparation using NEBNext® mRNA Library Prep Reagent Set for Illumina (New England Biolabs Inc., Ipswich, MA, USA) according to manufacturer's protocols. Each cDNA library was individually bar-coded with unique indexed primers and amplified through six cycles of PCR using KAPA HiFi HotStart Ready Mix (Kapa Biosystems Inc., Woburn, MA, USA). The library quality was determined using a Quibit® 2.0 Fluorometer. Each library concentration was estimated using a DNA 1000 chip on an Agilent 2100 Bioanalyzer. Accurate library quantification for sequencing was performed using the qPCR-based KAPA Biosystem Library Quantification kit (Kapa Biosystems Inc., Woburn, MA, USA). Each sample library was then diluted to a final concentration of 12.5 nM and equimolar amounts of each sample were pooled. Pooled samples were then clustered and amplified prior to sequencing.

Sequencing, alignment, and data analysis

RNA sequencing was performed using a 200 cycle TruSeq SBS HSv3 kit on a HiSeq2000 which generated > 90 million reads per sample. Sequenced reads were

demultiplexed using bcl2fastq conversion software v1.8.3 (Illumina Inc., San Diego, CA, USA) with default settings. Post processing of the sequencing reads from RNAseq experiments from each sample was performed. For this purpose, quality control checks on raw sequence data from each sample were performed using FastQC (Babraham Bioinformatics, London, UK). Raw reads were mapped to the latest pig Sscrofa10.2 [National Center for Biotechnology Information (NCBI); Bethesda, MD, USA] build using TopHat v1.4.0 (Langmead *et al.* 2009, Trapnell *et al.* 2009) with two mismatches allowed and other default parameters. Alignment metrics of mapped reads were estimated using SAMtools (Li *et al.* 2009). Aligned reads were then imported onto a commercial data analysis platform, Avadis NGS (Strand Scientifics, CA, USA). After quality inspection, aligned reads were filtered on the basis of read quality metrics where reads with a base quality score less than 30, alignment score less than 95, and mapping quality less than 40 were removed. Remaining reads were then filtered on the basis of their read statistics, where missing mates, translocated, unaligned and flipped reads were removed. The reads list was then filtered to remove duplicates. Samples were grouped with their respective identifiers and quantification of transcript abundance was done on the final read list using Trimmed Means of M-values (TMM) (Robinson and Oshlack) as the normalization method. Differential expression of genes was calculated on the basis of fold change (using default cut-off $\geq \pm 2.0$) observed between treatment groups. The p-value for each differentially expressed gene was estimated by z-score calculations using a false discovery rate (FDR) correction of 0.05 (Benjamini and Hochberg 1995). Human orthologs of unannotated differentially expressed genes were identified by Dr. Anthony K. McNeel [United States Department of Agriculture Agricultural Research Service

(USDA-ARS), Clay Center, NE, USA] using NCBI BLAST (Bethesda, MD) in combination with a custom computer program written in Python (www.python.org) (Haddock and Dunn 2011) and using NCBI modules within BioPython (Cock *et al.* 2009). Principle component analysis (PCA) was conducted to determine clustering patterns for both treatment groups. Additionally, a volcano plot was generated to visualize differential gene expression events between treatment groups. Gene enrichment analysis was performed using Database for Annotation, Visualization, and Integrated Discovery (DAVID) (Huang *et al.* 2009b, a), Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto 2000, Kanehisa *et al.* 2014), Protein Analysis Through Evolutionary Relationships (Panther) (Thomas *et al.* 2003), and Reactome (Joshi-Tope *et al.* 2003, Matthews *et al.* 2007, Vastrik *et al.* 2007, Matthews *et al.* 2008). Functional analyses were accomplished using Ingenuity Pathway Analysis (IPA) software (www.qiagen.com/ingenuity; Red Wood City, CA, USA). Pathways implicated previously in uterine development or function were targeted for IPA. Selected pathways included ESR1, hedgehog signaling, and the plasminogen activating network.

Quantitative RT-PCR

The same RNA used to generate cDNA libraries was also used to validate RNAseq results by quantitative RT-PCR (qPCR). For qPCR, uterine RNA from each animal was combined to produce two pooled treatment group samples. Reverse transcription was performed with 100 ng total RNA per sample using a PTC-200 Peltier Thermal Cycler (Bio-Rad Laboratories Inc.; Hercules, CA, USA) and the SuperScript III First-Strand Synthesis System for RT-PCR following manufacturer's instructions.

Quantitative PCR was performed using the Applied Biosystems StepOne Plus System (Applied Biosystems/Life Technologies; Grand Island, NY, USA), the SYBR Green method, and universal thermal cycling parameters (40 cycles) indicated by the manufacturer. Primers for qPCR were designed using Primer Quest software (Integrated DNA Technologies, Inc.; Coralville, IA, USA) 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 all assays. The quality of primers was evaluated by amplifying serial dilutions of the cDNA template. Dissociation curves for each set of primers were checked to ensure that no amplicon-independent amplification had occurred. Data were analyzed using the ΔΔCt method for quantification of gene expression as described by Applied Biosystems (ABI User Bulletin 2, 2001). The $\Delta\Delta C_T$ method uses C_T values (the cycle number at which the fluorescent signal crosses the threshold) of the samples with a control or calibrator sample (Livak and Schmittgen 2001). The C_T values of the samples and calibrator are then normalized to an appropriate endogenous reference gene, *cyclophilin*, the expression of which was not affected by treatment ($P > 0.05$). The $\Delta\Delta C_T$ method was used since amplification efficiencies determined for target genes were similar to the efficiency determined for *cyclophilin* (ABI User Bulletin 2, 2001). A Pearson correlation coefficient was determined for gene expression fold change of selected genes obtained by RNAseq and the same relationships determined by qPCR.

RESULTS

Effects of treatment on group clustering and differential gene expression

Results of PCA analysis, presented in Figure 3, showed that RNAseq data for nursed and replacer-fed groups clustered independently based on their transcriptomic expression profiles, suggesting unique uterine gene expression signatures. Volcano plot analysis (Figure 4A) revealed 896 differentially expressed genes in nursed as compared to milk replacer-fed gilts on PND 2 ($P \leq 0.05$; Figure 4A). Overall, expression was up-regulated for 291, and down-regulated for 605 of these 896 genes.

RNAseq results validated using qPCR

Results of qPCR analyses for targeted transcripts used in validation of RNAseq data are presented in Figure 4B. Nine genes targeted for this purpose included those which, based on RNAseq results, were: (1) up-regulated more than 2.0 fold [*matrix metalloproteinase 7 (MMP7) and fibrinogen gamma chain (FGG*)]; (2) down-regulated more than 2.0 fold [*transmembrane receptor 126b (TMEM126b), interleukin 5 (IL5), kalirin (KALRN),* and *progesterone receptor (PGR*)]; or that were (3) unchanged [$< \pm$ 2.0 fold change; *follistatin (Foll), glutamate receptor, ionotropic, N-methyl-D-aspartate (GRIN2C), insulin-like growth factor binding protein 5 (IGFBP5)*] in nursed versus replacer-fed gilts. Results obtained using qPCR agreed well and were consistent with expression fold changes determined using RNAseq. The correlation between fold change values determined by RNAseq and qPCR was positive ($r = 0.78$, $P < 0.01$).

Gene enrichment analyses

DAVID analyses identified functional terms for which associated expression events were primarily up- or down-regulated in nursed gilts compared to replacer-fed gilts at PND 2. Results are shown in Table 2. Complementary results obtained using KEGG, Panther, and Reactome analyses are presented in Table 3. Using DAVID, functional terms for which gene expression was primarily up-regulated were associated with "response to wounding", "cell adhesion", and "coagulation". Similarly, results of KEGG, Panther, and Reactome analyses indicated up-regulated gene expression associated with the "cytokine-cytokine receptor interaction", "plasminogen activating cascade", and "coagulation". By contrast, DAVID analysis identified "DNA packaging", "nucleosome organization", and "nucleosome assembly" as functional terms involving transcripts that were primarily down-regulated in uteri of nursed as compared to replacer-fed gilts on PND 2 (Table 2). Results of Panther analysis (Table 3) indicated that uterine gene expression associated with the hedgehog signaling pathway was also down-regulated in nursed as compared to replacer-fed gilts.

Network analyses of lactocrine-sensitive biological pathways

Network IPA of the ESR1 signaling cascade revealed lower expression ($P < 0.01$) of genes including *triiodothyronine receptor auxiliary protein* (*TRAP/media*), *progesterone receptor (PGR)*, and *histone H3* in gilts that nursed compared to those fed milk replacer (Figure 5). Analysis of the hedgehog signaling pathway revealed that expression of *patched (PTCH)* and the *Gli family of transcription factors (GLI)* was also down-regulated ($P < 0.001$) in nursed gilts (Figure 6). Consistently, RNAseq analysis

revealed that uterine *Indian hedgehog (Ihh)* expression was reduced ($P < 0.001$) in nursed gilts. Expression of molecules involved in the plasminogen activating network was higher ($P < 0.001$) in nursed as compared to milk replacer fed gilts. These included *plasmin, plasminogen (PLG)*, *tissue-type plasminogen activator (TPA)*, *serine proteinase inhibitor, clade A, member 1(SERPINA1), fibrinogen (FG), fibrin, von Willebrand factor (vWF), factor XIII (F13), and activated factor XIII (F13a)* (Figure 7).

DISCUSSION

A deep sequencing approach was used to identify lactocrine-sensitive elements of the neonatal porcine uterine transcriptome. Analyses revealed 896 differentially expressed genes in uterine tissues obtained on PND 2 that were associated with imposition of the lactocrine-null condition from birth. Results provide compelling support for the lactocrine hypothesis, indicating global changes in uterine gene expression patterns by PND 2 in gilts fed milk replacer in lieu of nursing.

Since the first RNAseq publication (Bainbridge *et al.* 2006), deep sequencing technology has revolutionized transcriptomics research (McGettigan 2013, Mutz *et al.* 2013). Large datasets generated using RNAseq pose a challenge with respect to defining best practices for analyzing gene expression patterns and identifying differentially expressed genes (McGettigan 2013). Developing strategies and defining protocols for interpretation of such large, complex datasets requires an interdisciplinary approach. Collaborations with experts at the HudsonAlpha Institute for Biotechnology and the USDA-ARS were essential to the success of this RNAseq study. These collaborations provided expertise and guidance concerning experimental and workflow design, gene annotation and bioinformatics necessary to evaluate and interpret this unique dataset.

Use of bioinformatic programs permits researchers to determine how differential gene expression events relate to functional terms describing biological processes. Results of analyses involving DAVID, KEGG, Panther, and Reactome programs revealed functional terms and lactocrine-sensitive biological processes associated with uterine development on PND 2, some of which were expected, and others that were not necessarily intuitive with respect to uterine development.

Not surprisingly, “cell adhesion” was identified as a functional term associated with lactocrine-sensitive uterine development. It is well known that interactions between cells and their extracellular matrix (ECM) support organizational events that determine tissue form and function (Gumbiner 1996). Morphogenesis and cytodifferentiation of uterine tissues are completed postnatally in the pig and it is clear that these events involve both cell-cell and cell-ECM interactions (Bartol *et al.* 1993, Bartol *et al.* 2006).

Up-regulation of elements related to the “cytokine-cytokine receptor interaction” pathway in uteri of nursed animals was identified by KEGG. During early pregnancy in the adult pig, cytokines, such as interferon gamma, originating from the trophoblast, were suggested to affect remodeling of uterine epithelium (Cencic *et al.* 2003, Murphy *et al.* 2009). Interactions between cytokines (such as the interleukins) and their receptors are important for lung morphogenesis (Blackwell *et al.* 2011) and adipogenesis (Annamalai and Clipstone 2013). Milk-borne cytokines were described to act via a lactocrine mechanism in regulation of murine hippocampal development and memory (Liu *et al.* 2014).

DAVID analysis revealed down-regulation of elements related to “DNA packaging”, “nucleosome organization”, and “nucleosome assembly” in uteri of nursed gilts on PND 2. The nucleosome is a basic unit of eukaryotic chromatin structure in which DNA is packaged into the nucleus by wrapping around core histone proteins (Turner 2012). Nucleosomes regulate transcription by controlling access of transcription factors and polymerases to DNA (Turner 2012). *Hox* gene expression occurs in an organized manner that is essential for formation of the body plan (Noordermeer *et al.* 2011). Analysis of the architecture of *Hox* gene loci in murine embryos revealed distinct

three-dimensional (3D) domains were associated with active and inactive *Hox* genes (Noordermeer *et al.* 2011). When *Hox* genes were inactive, they associated into single 3D structures whereas, once transcription began, Hox clusters switched to a bimodal 3D structures (Noordermeer *et al.* 2011). In the neonatal pig, postnatal uterine development involves developmentally and spatially regulated expression of Hox genes (Bartol *et al.* 2006). However, the role of nucleosome modifications in regulation of these or related organizational events in response to lactocrine signaling is presently undefined.

Enrichment analysis of differentially expressed genes also revealed effects on pathways and functional terms that were unanticipated, including "response to wounding" and "blood coagulation". Wound repair involves formation of new ECM that is followed by cellular differentiation and proliferation of endothelium and epithelium (Belacortu and Paricio 2011). Similarly, postnatal adenogenesis involves ECM remodeling, differentiation of glandular epithelium from luminal epithelium, and increased cell proliferation (Gray *et al.* 2001). Thus, the process of wound healing includes events that are similar to those occurring within the developing neonatal porcine uterus (Miller *et al.* 2013). KEGG, Panther, and DAVID analyses indicated that elements of "blood coagulation" were up-regulated in nursed gilts. Coagulation is a process related to wound repair that helps to protect tissue integrity after damage (Furie and Furie 1992). Elements of the coagulation cascade, including plasminogen activators, affect ECM integrity and adult porcine uterine function (Fazleabas *et al.* 1983, Hu *et al.* 2008).

Ingenuity Pathway Analysis is a tool designed for comprehensive analysis of large, complex 'omic' (genomic, transcriptomic, proteomic, operomic) datasets (www.quiagen.com/ingenuity). Here, IPA was used to interrogate data generated via

RNAseq in order to identify and depict elements of biological pathways affected differentially by treatment in the neonatal porcine uterus on PND 2. Targeted pathways known to be lactocrine sensitive included ESR1 signaling (Miller *et al.* 2013). Others pathways explored using IPA, and recognized to be involved in uterine development or function in the adult, included hedgehog signaling (Bartol *et al.* 2006) and the plasminogen activating cascade (Fazleabas *et al.* 1983).

Transformation of the endometrium from histoarchitectural immaturity to a mature state occurs postnatally and is characterized by uterine gland genesis (Bartol *et al.* 1993, Spencer *et al.* 1993). This process is marked and mediated by lactocrine-sensitive ESR1 expression in nascent glandular epithelium (Tarleton *et al.* 1999, Miller *et al.* 2013). Analysis of the ESR1 signaling pathway using IPA identified *PGR* expression in the neonatal porcine uterus that was lower ($P < 0.01$) in nursed gilts. This observation constitutes what may be the first evidence of *PGR* expression in the neonatal porcine uterus.

Expression of PGR was reported in murine and ovine uterine epithelium and stroma shortly after birth (Gray *et al.* 2000, Kurita *et al.* 2000, Franco *et al.* 2012). Based on data for both the neonatal ewe (Bartol *et al.* 1988) and mouse (Bigsby and Cunha 1985, Cooke *et al.* 2012, Filant *et al.* 2012), progestin-sensitive, PGR-mediated signaling was implicated in mechanisms regulating endometrial andenogenesis. In both cases, PGR-mediated signaling is thought to inhibit uterine epithelial proliferation (Bigsby and Cunha 1985, Bartol *et al.* 1988, Cooke *et al.* 2012).

The neonatal porcine uterus is exposed to placental progesterone (P4) *in utero* (Hagen *et al.* 1983). After birth, the neonatal uterus is also exposed to P4 delivered in

colostrum (Grosvenor 1992), a maternal source of this steroid hormone in the neonate. Progesterone is detectable in the circulation of nursed gilts 24 h postnatally (Parker *et al.* 1980). Whether P4 of maternal origin regulates neonatal uterine PGR expression, as described for the adult gilt (Geisert *et al.* 1994), remains to be determined.

Hedgehog proteins are molecules that regulate tissue-patterning events. These proteins elicit effects by binding to patched (PTCH), a cell surface receptor, to initiate the signaling cascade (Cohen 2003). Indian hedgehog (Ihh) is one of three mammalian hedgehog homologs, including Sonic hedgehog (Shh) and Desert hedgehog (Dhh) (Cohen 2003). In mice, P4 action on neonatal uterine development is mediated through PGR and elements of the hedgehog signaling cascade including Ihh, PTCH, and GLI (Simon *et al.* 2009, Franco *et al.* 2012). Expression of Ihh and PTCH was described for the neonatal porcine endometrium shortly after birth (Bartol *et al.* 2006). Here, RNAseq analysis revealed that *Ihh*, *PTCH*, and *GLI* transcripts were, like *PGR*, down-regulated in nursed gilts. Future studies should be conducted to elucidate the roles of these molecules in mediation of lactocrine-sensitive uterine development.

Further IPA-based review of the ESR1 signaling pathway revealed lower *Histone H3* and *TRAP/media* expression in nursed gilts on PND 2. This agreed with DAVID results indicating down-regulated expression of elements associated with "DNA packaging", "nucleosome organization", and "nucleosome assembly". Histone H3 is one of the four core histone proteins that make up the nucleosome. These proteins are subject to post-translational modifications that regulate DNA transcription and chromatin condensation (Hans and Dimitrov 2001). Like Histone H3, TRAP also interacts with DNA through the thyroid hormone response element to stabilize binding of the thyroid

receptor (Beebe *et al.* 1991). While functional roles of these factors in mediation of neonatal uterine development remain unclear, results support the idea that factors affecting basic regulatory mechanisms governing transcription are lactocrine sensitive.

Present results indicated lactocrine effects on the PA network. The PA/plasmin system includes urokinase-PA (Liu *et al.* 2014) and TPA. Both cleave plasminogen to form plasmin. This process is regulated by PA inhibitors (PAIs) (Littlefield 1991). Expression of *TPA* by the neonatal porcine uterus, documented here, is a new observation. However, TPA and TPA activity were identified in rat uterine cells obtained on PND 19 (Peltz *et al.* 1983). Consistent with present data for the neonatal pig, PAI was undetectable in uterine tissues obtained from prepubertal gilts at three to four months of age (Wang-Lee *et al.* 1998). However, the PA/PAI system was implicated in mediation of intrauterine trophoblast invasiveness during the peri-implantation period in pregnant adult gilts (Mullins *et al.* 1980, Fazleabas *et al.* 1983, Menino *et al.* 1997). In that system, endometrial PAIs are thought to limit trophectodermally derived PA, thereby facilitating establishment of a superficial, diffuse, epitheliochorial placenta.

How the PA/PAI system mediates cell-cell interactions or other organizational events associated with neonatal uterine development (Bartol *et al.* 1993, Bartol *et al.* 2006) is not known. However, the PA system is important for regulation of epithelial-mesenchymal interactions associated with development of breast cancer. In an *in vitro* study, Jo and colleagues (2009) showed that up-regulation of the UPA receptor in breast cancer cells decreased E-cadherin protein levels and increased cell-cell junction breakdown. This was reversed if *UPA* expression was silenced (Jo *et al.* 2009). Here, uterine *TPA* expression increased in nursed as compared to replacer-fed animals.

Activation of plasmin by TPA results in: (1) direct breakdown of the ECM; and/or (2) indirect degradation of the ECM through activation of matrix metalloproteinases (MMPs) (Hu *et al.* 2008). In the kidney, TPA-induced MMP9 expression promoted destruction of the basement membrane, thereby facilitating a tubular epithelial-myofibroblast transition and development of fibrotic renal lesions (Yang *et al.* 2002). In the neonatal porcine uterus, MMP9 expression was higher in nursed as compared to replacer-fed gilts by PND 2 (Chen *et al.* 2010). It will be important to determine whether this can be related mechanistically to effects of locally produced uterine TPA.

Analysis of the PA pathway using IPA also indicated expression of *serine proteinase inhibitor, clade A, member 1* (*SERPINA1*). Uterine expression of *SERPINA1* transcripts was higher in nursed gilts on PND 2. SERPINA1 (previously α-1 antitrypsin) is a member of the serpin superfamily, clade A, of extracellular proteins that function primarily in inhibition of proteases (Irving *et al.* 2000). An example of SERPINA1 function comes from a study of ovarian cancer metastasis. Normandin and colleagues (2010) showed that SERPINA1 expression was higher in patients diagnosed with low malignant potential ovarian cancer. Authors proposed that SERPINA1 inhibits proteolytic degradation of the basement membrane, a process that leads to cancer progression (Normandin *et al.*). SERPINA1 was also shown to be produced and secreted by human glandular epithelial cells of pregnancy *in vitro* and was suggested to play a role in limiting endometrial remodeling (Fay *et al.* 1990, Hansen and Liu 1997). Additionally, SERPINA1 was detected in the circulation of pigs from birth through PND 56 (Martin *et al.* 2005). Studies should be conducted to determine if SERPINA1 regulates uterine remodeling in the neonatal gilt.

IPA also revealed additional elements of the plasminogen activating cascade for which expression was higher in nursed gilts, including FG, fibrin, vWf, F13, and F13a. These factors play a role in wound healing and coagulation (Davie *et al.* 1991), processes that DAVID analysis showed were also up-regulated in uteri of nursed gilts reported here. The first step in the wound response involves adhesion of platelets to form a plug that reduces blood loss (Davie *et al.* 1991), a process supported by vWF (Ruggeri 2003). In parallel to platelet activation, conversion of FG to fibrin and activation of F13 (to F13a) forms an insoluble fibrin clot (Davie *et al.* 1991). This clot first stops blood hemorrhage and then serves as a matrix to enable wound healing (Laurens *et al.* 2006). The coagulation cascade is regulated by serine protease inhibitors, such as SERPINA1 (Davie *et al.* 1991). Again, elements of the process of wound healing (Belacortu and Paricio 2011), including ECM remodeling and coagulation pathways, share similarities with postnatal uterine development, including remodeling important for gland genesis initiated shortly after birth in the neonate (Gray *et al.* 2001).

Previous reports (Miller *et al.* 2013) provide compelling evidence to suggest that nursing promotes postnatal reproductive development in pigs. Here, results of RNAseq analysis extended these observations to define the lactocrine-sensitive elements of the neonatal porcine uterine transcriptome at PND 2. Future efforts should focus on elucidating temporospatial expression patterns of identified gene products as well as their functional role in mediating uterine programming and function.

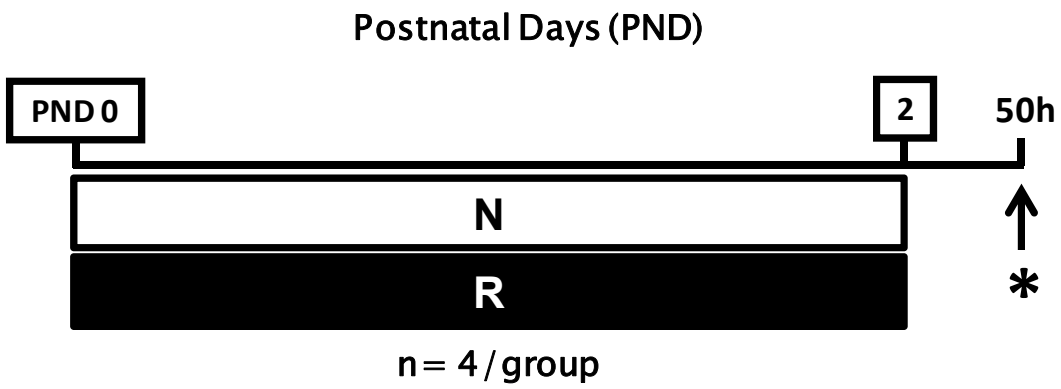

Figure 1. Experimental design. Gilts were assigned at birth (PND 0) to either nurse *ad libitum* (N) or to receive milk replacer by gavage (R). Uteri were collected on PND 2, as indicated by the asterisk.

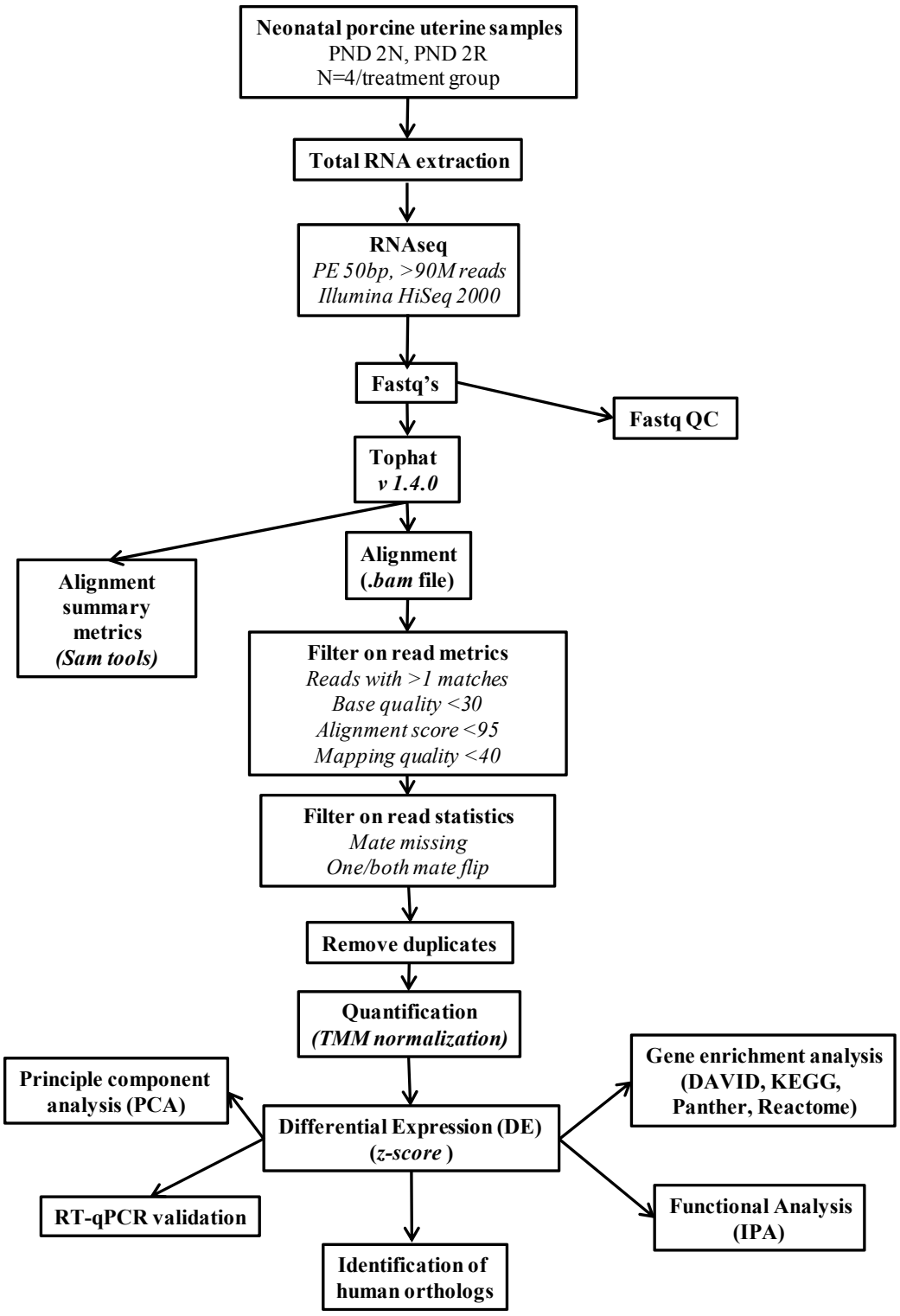

Figure 2. Flow chart depicting all steps conducted in preparing, running, analyzing, and validating the RNAseq data set.

Table 1. Porcine gene symbols, names, accession numbers, and primer sequences for targeted uterine transcripts.

Gene Symbol	Gene Name	Accession #	Forward Primer	Reverse Primer
MMP7	Matrix metalloproteinase 7	NM_214207	CGCCTGCCTATAACTGGAAT	TTTGGCTGGCTTGGGAATAG
GRIN2C	Glutamate receptor, ionotropic, N-methyl D-aspartate	XM_003131237.3	TGTGTACTTGCTGTTCTGTCTC	CACCTCTCTGGGTCTTCATTTC
FGG	Fibrinogen gamma chain	NM_001244524	TCTCCTACTGGAACCACAGA	CCTCCAGCTGCACTCTTAAT
IL5	Interleukin 5	KC_660157	CGTTAGTGCCATTGCTGTAGAA	CATCAAGTTCCCATCGCCTATC
KALRN	Kalirin	EF_443102	GAGGATCCAGAACACTGAAGAC	TCACGTCTCCTCACAGATACT
Foll	Follistatin	AJ_715530	GGCCTATGAGGGAAAGTGTATC	ACAGGCTCCTCAGACTTACT
IGFBP5	Insulin-like growth factor binding protein 5	NM_214099.1	TGAAGAAGGACCGCAGAAAG	CTGCTCAGATTCCTGTCTCATC
TMEM 126b	Transmembrane receptor 126b	NM_001243600	CAGGTAACATAAGCCGGGAA	GTGGCAGTGGAACAGTATGA
PGR	Progesterone receptor	GQ_903679	CCAGCTTGTCGCCTTAGAAA	CGGCATCTAGTGCTCTCATAAC
Cyclo	Cyclophilin	AU_058466	TTATAAAGGTTCCTGCTTTCACAGAA	TGCCATTATGGCGTGTGAAG

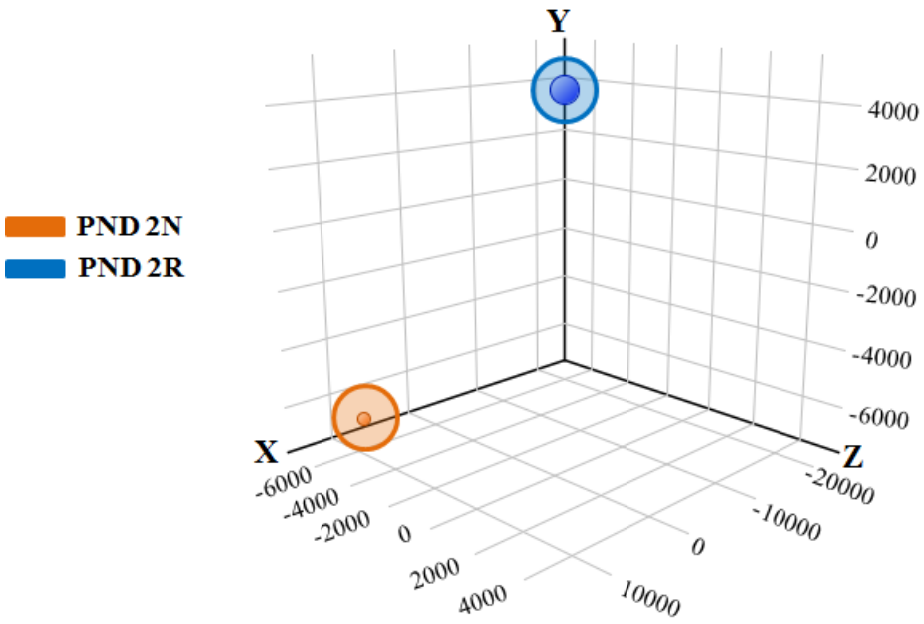

Figure 3. Principal component analysis (PCA) plot of RNAseq data for pooled neonatal porcine uterine samples from PND 2N (orange) and PND 2R (blue). Unique expression profiles were identified for each group.

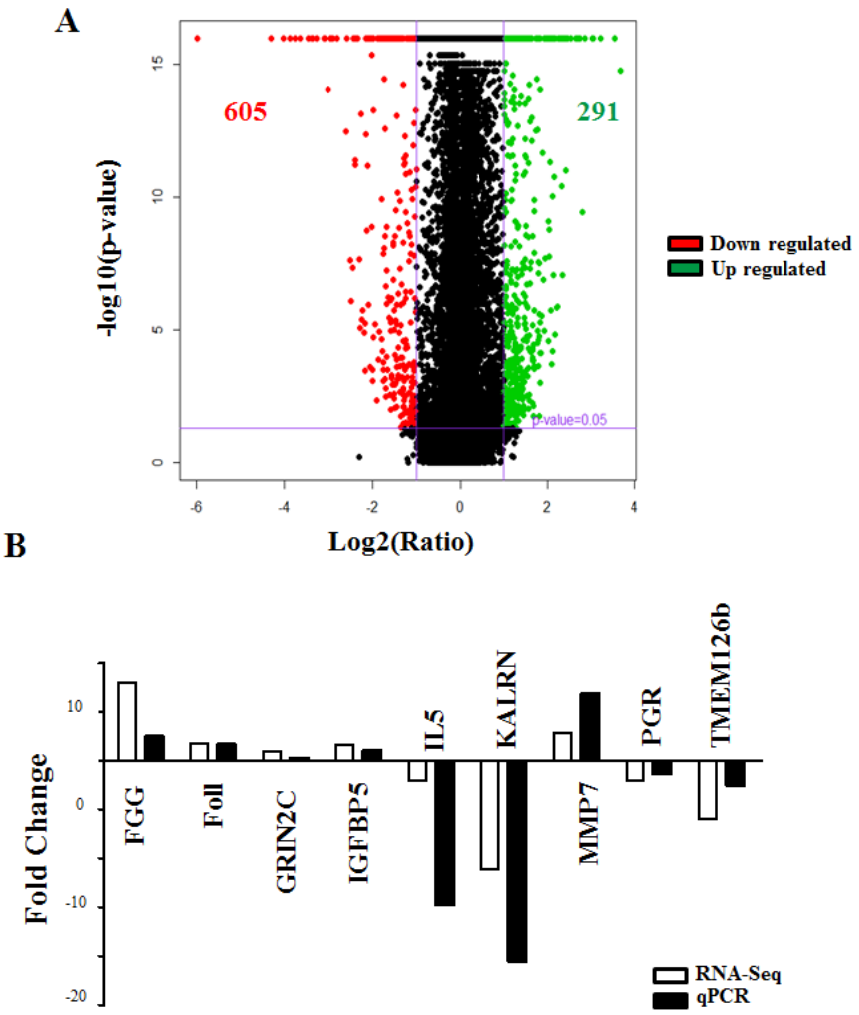

Figure 4. (A) Volcano plot illustrating differences in neonatal uterine gene expression between PND 2N and PND 2R. The purple horizontal line denotes $P = 0.05$; red color denotes gene expression events that were down-regulated (Fold change > 2); green color denotes gene expression events that were up-regulated (Fold change > 2); and black denotes gene expression events for which changes were less than 2-fold. The number of differential gene expression events that were down- (red) and up-regulated (green) are shown. **(B)** Comparison of qPCR results for nine genes determined to be expressed differentially between PND 2N and PND 2R groups based on results of RNAseq (White bars= RNAseq; Black bars= qPCR).

Table 2. Selected results of DAVID functional annotation clustering indicating terms for which associated genes were up- or down-regulated in uteri of gilts nursed versus replacer-fed for two days from birth.

Functional Terms of Over Represented Annotation Clusters[a]	Enrichment Score[b]
Up-regulated	
Response to wounding (39, 2.5)	6.2
Cell adhesion (43, 2.0)	4.9
Biological adhesion (43, 2.0)	4.9
Inflammatory response (25, 2.6)	4.3
Cation transport (33, 2.0)	3.5
Metal ion transport (29, 2.1)	3.4
Ion transport (41, 1.8)	3.4
Coagulation (11, 3.4)	3.0
Blood coagulation (11, 3.6)	3.0
Hemostasis (11, 3.4)	2.8
Down-regulated	
DNA packaging (10, 7.1)	5.0
Nucleosome organization (9, 8.1)	4.8
Nucleosome assembly (8, 8.0)	4.2
Chromatin assembly (8, 7.7)	4.1
Protein-DNA complex assembly (8, 7.3)	4.0
Chromatin assembly or disassembly (8, 5.3)	3.1
Cellular macromolecular complex subunit organization (13, 3.0)	2.9
Cellular macromolecular complex assembly (12, 3.2)	2.8
Phospholipid catabolic process (4, 15.2)	2.7
Cardiac muscle tissue morphogenesis (4, 12.4)	2.4

[a] Values within parentheses indicate the number of genes involved in and fold enrichment score of the corresponding functional term.

[b] Value reflects the degree to which a gene set is overrepresented at the top or bottom of a ranked list of genes. The enrichment score is calculated by taking the geometric mean of the p-values associated with the differentially expressed genes involved in the corresponding annotation cluster ($-\log_{10}$ scale).

Table 3. Selected results of KEGG, Panther, and Reactome analyses listing enriched pathways for which associated genes were up- or down-regulated in uteri of gilts nursed versus replacer-fed for two days from birth.

Bioinformatics Program	Pathways	P-value
	Up-regulated	
KEGG	Complement and coagulation cascades	2.08E-04
	Cytokine-cytokine receptor interaction	0.004
	TGF-beta signaling pathway	0.043
	Androgen and estrogen metabolism	0.048
Panther	Plasminogen activating cascade	1.56E-04
	Blood coagulation	0.001
	Synaptic vesicle trafficking	0.024
Reactome	Hemostasis	0.016
	Integrin cell surface interactions	0.022
	Down-regulated	
KEGG	Systemic lupus erythematous	0.002
Panther	Hedgehog signaling pathway	0.068
Reactome	Telomere maintenance	1.23E-04

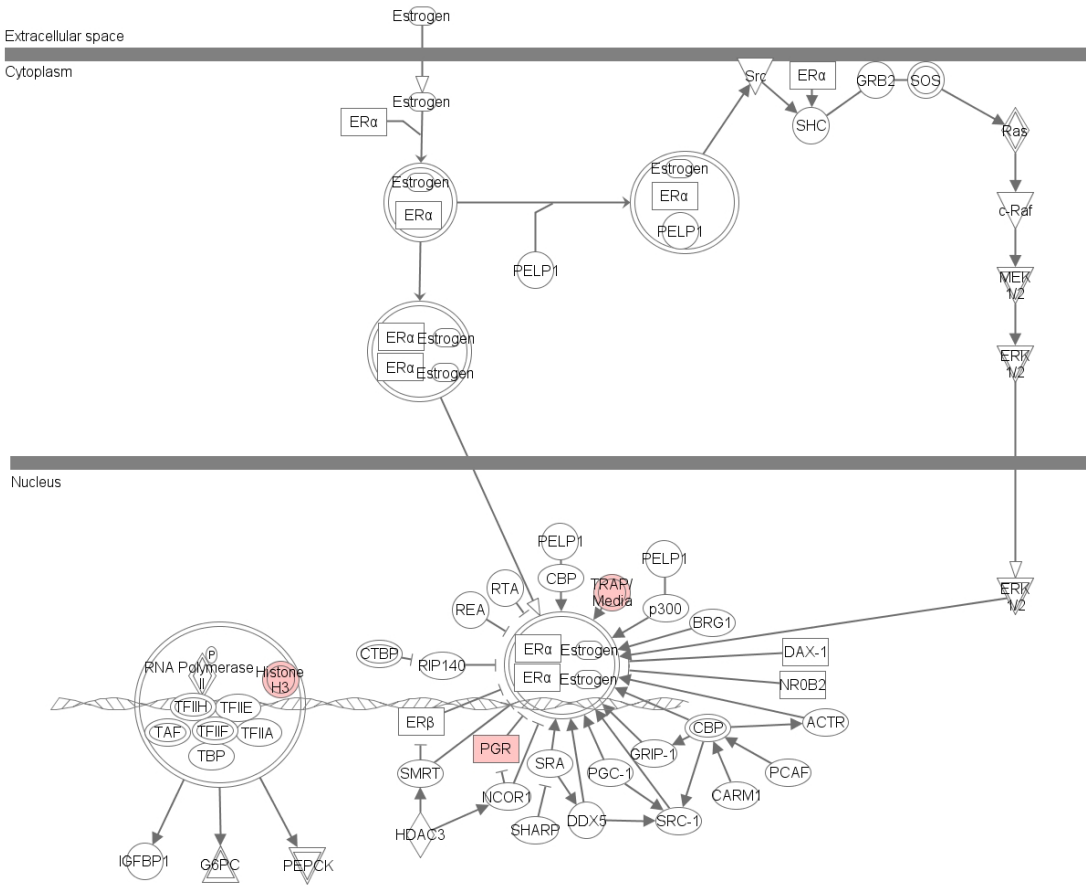

Figure 5. IPA analysis results for estrogen receptor pathway signaling. Effects of the lactocrine-null state on elements of this signaling cascade are shown above. Red color denotes down-regulation ($P < 0.01$) in nursed compared to replacer-fed gilts. Elements of the pathway that are white were not affected by treatment. PGR: progesterone receptor; TRAP/media: triiodothyronine receptor auxiliary protein.

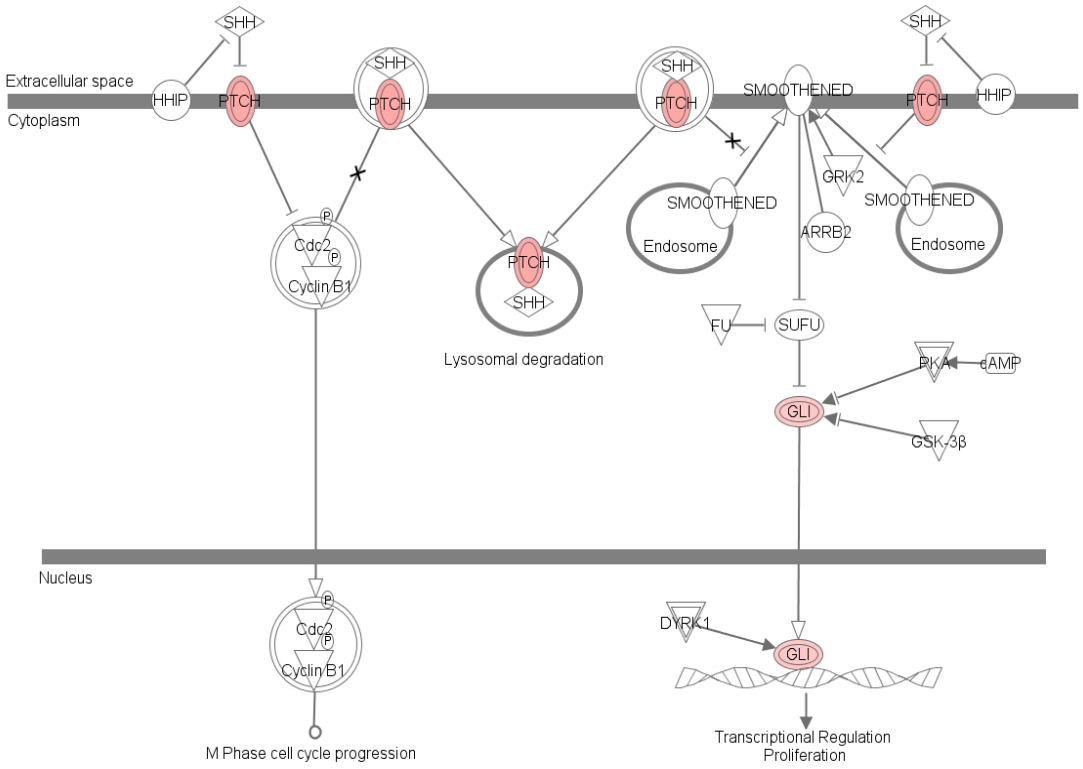

Figure 6. IPA analysis results for hedgehog signaling pathway. Effects of the lactocrine-null state on elements of this signaling cascade are shown above. Red color denotes down-regulation ($P < 0.001$) in nursed compared to replacer-fed gilts. Elements of the pathway that are white were not affected by treatment. PTCH: patched; GLI: Gli family of transcription factors.

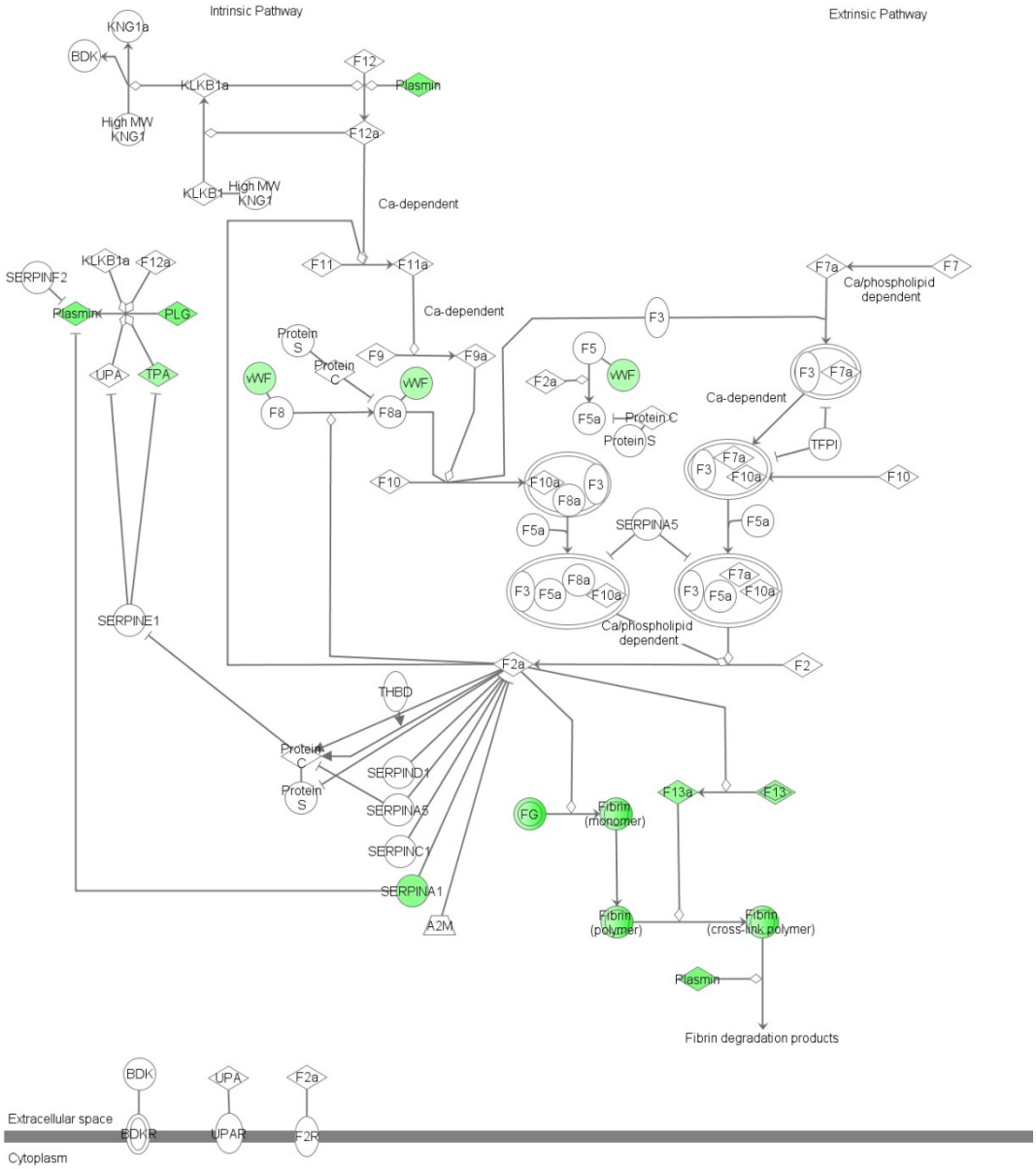

Figure 7. IPA analysis results for the plasminogen activating cascade. Effects of the lactocrine-null state on elements of this pathway are shown. Green color denotes up-regulation ($P < 0.001$) in nursed compared to replacer-fed gilts. Elements of the pathway that are white were not affected by treatment. TPA: tissue-type plasminogen activator; PLG: plasminogen; SERPINA1: serine proteinase inhibitor, clade A, member 1; FG: fibrinogen; vWF: von Willebrand factor; F13: factor XIII; F13a: activated factor XIII.

REFERENCES

Annamalai, D, and NA Clipstone 2013 Prostaglandin F2alpha Inhibits Adipogenesis Via an Autocrine-Mediated Interleukin-11/Glycoprotein 130/STAT1-Dependent Signaling Cascade. *J Cell Biochem*.

Bainbridge, MN, RL Warren, M Hirst, T Romanuik, T Zeng, A Go, A Delaney, M Griffith, M Hickenbotham, V Magrini, ER Mardis, MD Sadar, AS Siddiqui, MA Marra, and SJ Jones 2006 Analysis of the prostate cancer cell line LNCaP transcriptome using a sequencing-by-synthesis approach. *BMC Genomics* **7** 246.

Bartol, FF, AA Wiley, and CA Bagnell 2006 Uterine development and endometrial programming. *Soc Reprod Fertil Suppl* **62** 113-130.

Bartol, FF, AA Wiley, and CA Bagnell 2008 Epigenetic programming of porcine endometrial function and the lactocrine hypothesis. *Reprod Domest Anim* **43 Suppl 2** 273-279.

Bartol, FF, AA Wiley, DA Coleman, DF Wolfe, and MG Riddell 1988 Ovine uterine morphogenesis: effects of age and progestin administration and withdrawal on neonatal endometrial development and DNA synthesis. *J Anim Sci* **66** 3000-3009.

Bartol, FF, AA Wiley, TE Spencer, JL Vallet, and RK Christenson 1993 Early uterine development in pigs. *J Reprod Fertil Suppl* **48** 99-116.

Beebe, JS, DS Darling, and WW Chin 1991 3,5,3'-triiodothyronine receptor auxiliary protein (TRAP) enhances receptor binding by interactions within the thyroid hormone response element. *Mol Endocrinol* **5** 85-93.

Belacortu, Y, and N Paricio 2011 Drosophila as a model of wound healing and tissue regeneration in vertebrates. *Dev Dyn* **240** 2379-2404.

Benjamini, Y, and Y Hochberg 1995 Controlling the False Discovery Rate: a Practical and Powerful Approach to Multiple Testing. *J. R. Statist. Soc. B* **57** 289-300.

Bigsby, RM, and GR Cunha 1985 Effects of progestins and glucocorticoids on deoxyribonucleic acid synthesis in the uterus of the neonatal mouse. *Endocrinology* **117** 2520-2526.

Blackwell, TS, AN Hipps, Y Yamamoto, W Han, WJ Barham, MC Ostrowski, FE Yull, and LS Prince 2011 NF-kappaB signaling in fetal lung macrophages disrupts airway morphogenesis. *J Immunol* **187** 2740-2747.

Cencic, A, M Guillomot, S Koren, and C La Bonnardiere 2003 Trophoblastic interferons: do they modulate uterine cellular markers at the time of conceptus attachment in the pig? *Placenta* **24** 862-869.

Chen, X, C Gao, H Li, L Huang, Q Sun, Y Dong, C Tian, S Gao, H Dong, D Guan, X Hu, S Zhao, L Li, L Zhu, Q Yan, J Zhang, K Zen, and CY Zhang 2010 Identification and characterization of microRNAs in raw milk during different periods of lactation, commercial fluid, and powdered milk products. *Cell Res* **20** 1128-1137.

Cock, PJ, T Antao, JT Chang, BA Chapman, CJ Cox, A Dalke, I Friedberg, T Hamelryck, F Kauff, B Wilczynski, and MJ de Hoon 2009 Biopython: freely available Python tools for computational molecular biology and bioinformatics. *Bioinformatics* **25** 1422-1423.

Cohen, MM, Jr. 2003 The hedgehog signaling network. *Am J Med Genet A* **123A** 5-28.

Cooke, PS, GC Ekman, J Kaur, J Davila, IC Bagchi, SG Clark, PJ Dziuk, K Hayashi, and FF Bartol 2012 Brief exposure to progesterone during a critical neonatal window prevents uterine gland formation in mice. *Biol Reprod* **86** 63.

Davie, EW, K Fujikawa, and W Kisiel 1991 The coagulation cascade: initiation, maintenance, and regulation. *Biochemistry* **30** 10363-10370.

Donovan, SM, and J Odle 1994 Growth factors in milk as mediators of infant development. *Annu Rev Nutr* **14** 147-167.

Fay, TN, S Lindenberg, B Teisner, LG Westergaard, JG Westergaard, and JG Grudzinskas 1990 Identification of specific serum proteins synthesized de novo by monolayer cultures of glandular cells of gestational endometrium. *Hum Reprod* **5** 14-18.

Fazleabas, AT, RD Geisert, FW Bazer, and RM Roberts 1983 Relationship between release of plasminogen activator and estrogen by blastocysts and secretion of plasmin inhibitor by uterine endometrium in the pregnant pig. *Biol Reprod* **29** 225-238.

Filant, J, H Zhou, and TE Spencer 2012 Progesterone inhibits uterine gland development in the neonatal mouse uterus. *Biol Reprod* **86** 146, 141-149.

Franco, HL, CA Rubel, MJ Large, M Wetendorf, R Fernandez-Valdivia, JW Jeong, TE Spencer, RR Behringer, JP Lydon, and FJ Demayo 2012 Epithelial progesterone receptor exhibits pleiotropic roles in uterine development and function. *Faseb J* **26** 1218-1227.

Furie, B, and BC Furie 1992 Molecular and cellular biology of blood coagulation. *N Engl J Med* **326** 800-806.

Geisert, RD, TN Pratt, FW Bazer, JS Mayes, and GH Watson 1994 Immunocytochemical localization and changes in endometrial progestin receptor protein during the porcine oestrous cycle and early pregnancy. *Reprod Fertil Dev* **6** 749-760.

Gray, CA, FF Bartol, BJ Tarleton, AA Wiley, GA Johnson, FW Bazer, and TE Spencer 2001 Developmental biology of uterine glands. *Biol Reprod* **65** 1311-1323.

Gray, CA, KM Taylor, FW Bazer, and TE Spencer 2000 Mechanisms regulating norgestomet inhibition of endometrial gland morphogenesis in the neonatal ovine uterus. *Mol Reprod Dev* **57** 67-78.

Grosvenor, CE, Picciano, M.R., and Baumrucker, C.R. 1992 Hormones and Growth Factors in Milk. *Endocrine Reviews* **14** 710-728.

Gumbiner, BM 1996 Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* **84** 345-357.

Haddock, and Dunn 2011 Practical computing for Biologists: Sinauer and Associates.

Hagen, DR, SK Houtz, LC Griel, Jr., and JF Kavanaugh 1983 Concentrations of progesterone in arterial and venous plasma of fetal pigs and their dams in late gestation. *Biol Reprod* **29** 919-923.

Hans, F, and S Dimitrov 2001 Histone H3 phosphorylation and cell division. *Oncogene* **20** 3021-3027.

Hansen, PJ, and WJ Liu 1997 Biology of progesterone-induced uterine serpins. *Adv Exp Med Biol* **425** 143-154.

Hu, K, WM Mars, and Y Liu 2008 Novel actions of tissue-type plasminogen activator in chronic kidney disease. *Front Biosci* **13** 5174-5186.

Huang, D, B Sherman, and R Lempicki 2009a Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res.* **37** 1-13.

Huang, D, B Sherman, and R Lempicki 2009b Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources. *Nature Protoc.* **4** 44-57.

Irving, JA, RN Pike, AM Lesk, and JC Whisstock 2000 Phylogeny of the serpin superfamily: implications of patterns of amino acid conservation for structure and function. *Genome Res* **10** 1845-1864.

Jo, M, RD Lester, V Montel, B Eastman, S Takimoto, and SL Gonias 2009 Reversibility of epithelial-mesenchymal transition (EMT) induced in breast cancer cells by activation of urokinase receptor-dependent cell signaling. *J Biol Chem* **284** 22825-22833.

Joshi-Tope, G, I Vastrik, G Gopinathrao, L Matthews, E Schmidt, M Gillespie, P D'Eustachio, B Jassal, S Lewis, G Wu, E Birney, and L Stein 2003 The Genome Knowledgebase: A Resource for Biologists and Bioinformatics. *Cold Spring Harb Symp Quant Biol* **68** 237-243.

Kanehisa, M, and S Goto 2000 KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res.* **28** 27-30.

Kanehisa, M, S Goto, Y Sato, M Kawashima, M Furumichi, and M Tanabe 2014 Data, information, knowledge and principle: back to metabolism in KEGG. *Nucleic Acids Res.* **42** D199-D205.

Kurita, T, KJ Lee, PS Cooke, JP Lydon, and GR Cunha 2000 Paracrine regulation of epithelial progesterone receptor and lactoferrin by progesterone in the mouse uterus. *Biol Reprod* **62** 831-838.

Kwek, JH, RD Iongh, MR Digby, MB Renfree, KR Nicholas, and M Familari 2009 Cross-fostering of the tammar wallaby (Macropus eugenii) pouch young accelerates fore-stomach maturation. *Mech Dev* **126** 449-463.

Langmead, B, C Trapnell, M Pop, and SL Salzberg 2009 Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol* **10** R25.

Laurens, N, P Koolwijk, and MP de Maat 2006 Fibrin structure and wound healing. *J Thromb Haemost* **4** 932-939.

Li, H, B Handsaker, A Wysoker, T Fennell, J Ruan, N Homer, G Marth, G Abecasis, and R Durbin 2009 The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25** 2078-2079.

Littlefield, B 1991 Plasminogen activators in endometrial physiology and embryo implantation: A Review. *The Primate Endometrium* **622** 167-175.

Liu, B, B Zupan, E Laird, S Klein, G Gleason, M Bozinoski, J Gal Toth, and M Toth 2014 Maternal hematopoietic TNF, via milk chemokines, programs hippocampal development and memory. *Nat Neurosci* **17** 97-105.

Livak, KJ, and TD Schmittgen 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25** 402-408.

Martin, M, MA Tesouro, N Gonz Am, A Pi, and F Lampreave 2005 Major plasma proteins in pig serum during postnatal development. *Reprod Fertil Dev* **17** 439-445.

Matthews, L, P D'Eustachio, M Gillespie, D Croft, B de Bono, G Gopinath, B Jassal, S Lewis, E Schmidt, I Vastrik, G Wu, E Birney, and L Stein 2007 An Introduction to the Reactome Knowledgebase of Human Biological Pathways and Processes. *Bioinformatics Primer*.

Matthews, L, G Gopinath, M Gillespie, M Caudy, D Croft, B de Bono, P Garapati, J Hemish, H Hermjakob, B Jassal, A Kanapin, S Lewis, S Mahajan, B May, E Schmidt, I Vastrik, G Wu, E Birney, L Stein, and P D'Eustachio 2008 Reactome knowledgebase of biological pathways and processes. *Nucleic Acids Res.* **2008**.

McGettigan, PA 2013 Transcriptomics in the RNA-seq era. *Curr Opin Chem Biol* **17** 4-11.

McGlone, J, S Ford, F Mitloehner, T Grandin, P Ruegg, C Stull, G Lewis, J Swanson, W Underwood, J Mench, T Mader, S Eicher, P Hester, and J Salak-Johnson 2010 Guide for the Care and Use of Agricultural Animals in Resaerch and Teaching, edn 3. Federation of Animal Science Societies.

Menino, AR, Jr., A Hogan, GA Schultz, S Novak, W Dixon, and GH Foxcroft 1997 Expression of proteinases and proteinase inhibitors during embryo-uterine contact in the pig. *Dev Genet* **21** 68-74.

Miller, DJ, AA Wiley, JC Chen, CA Bagnell, and FF Bartol 2013 Nursing for 48 hours from birth supports porcine uterine gland development and endometrial cell-compartment specific gene expression. *Biol Reprod* **88** 4.

Mullins, DE, FW Bazer, and RM Roberts 1980 Secretion of a progesterone-induced inhibitor of plasminogen activator by the porcine uterus. *Cell* **20** 865-872.

Murphy, SP, C Tayade, AA Ashkar, K Hatta, J Zhang, and BA Croy 2009 Interferon gamma in successful pregnancies. *Biol Reprod* **80** 848-859.

Mutz, KO, A Heilkenbrinker, M Lonne, JG Walter, and F Stahl 2013 Transcriptome analysis using next-generation sequencing. *Curr Opin Biotechnol* **24** 22-30.

Noordermeer, D, M Leleu, E Splinter, J Rougemont, W De Laat, and D Duboule 2011 The dynamic architecture of Hox gene clusters. *Science* **334** 222-225.

Normandin, K, B Peant, C Le Page, M de Ladurantaye, V Ouellet, PN Tonin, DM Provencher, and AM Mes-Masson Protease inhibitor SERPINA1 expression in epithelial ovarian cancer. *Clin Exp Metastasis* **27** 55-69.

Nusser, KD, and S Frawley 1997 Depriving neonatal rats of milk from early lactation has long-term consequences on mammotrope development. *Endocrine* **7** 319-323.

Parker, R, T Dunn, and C Kaltenbach 1980 Disappearance of progesterone from the serum of neonatal pigs. *Can. J. Anim. Sci* **60** 807-809.

Peltz, SW, BS Katzenellenbogen, MA Kneifel, and WF Mangel 1983 Plasminogen activators in tissues of the immature and estrogen-stimulated rat uterus and in uterine luminal fluid: characterization and properties. *Endocrinology* **112** 890-897.

Playford, RJ, CE Macdonald, and WS Johnson 2000 Colostrum and milk-derived peptide growth factors for the treatment of gastrointestinal disorders. *Am J Clin Nutr* **72** 5-14.

Robinson, MD, and A Oshlack A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biol* **11** R25.

Ruggeri, ZM 2003 Von Willebrand factor, platelets and endothelial cell interactions. *J Thromb Haemost* **1** 1335-1342.

Saif, LJ, DR Redman, KL Smith, and KW Theil 1983 Passive immunity to bovine rotavirus in newborn calves fed colostrum supplements from immunized or nonimmunized cows. *Infect Immun* **41** 1118-1131.

Simon, L, KA Spiewak, GC Ekman, J Kim, JP Lydon, MK Bagchi, IC Bagchi, FJ DeMayo, and PS Cooke 2009 Stromal progesterone receptors mediate induction of Indian Hedgehog (IHH) in uterine epithelium and its downstream targets in uterine stroma. *Endocrinology* **150** 3871-3876.

Spencer, TE, FF Bartol, AA Wiley, DA Coleman, and DF Wolfe 1993 Neonatal porcine endometrial development involves coordinated changes in DNA synthesis, glycosaminoglycan distribution, and 3H-glucosamine labeling. *Biol Reprod* **48** 729-740.

Tarleton, BJ, AA Wiley, and FF Bartol 1999 Endometrial development and adenogenesis in the neonatal pig: effects of estradiol valerate and the antiestrogen ICI 182,780. *Biol Reprod* **61** 253-263.

Thomas, P, M Campbell, A Kejariwal, M Huaiyu, B Karlak, R Daverman, K Diemer, A Muruganujan, and A Narechania 2003 PANTHER: a library of protein families and subfamilies indexed by function. *Genome Res.* **13** 2129-2141.

Trapnell, C, L Pachter, and SL Salzberg 2009 TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics* **25** 1105-1111.

Turner, BM 2012 The adjustable nucleosome: an epigenetic signaling module. *Trends Genet* **28** 436-444.

Vastrik, I, P D'Eustachio, E Schmidt, G Gopinath, D Croft, B de Bono, M Gillespie, B Jassal, S Lewis, L Matthews, G Wu, E Birney, and L Stein 2007 Reactome: a knowledge base of biologic pathways and processes. *Genome Biol* **8** R39.

Wang-Lee, JL, JA Lenhart, KM Ohleth, PL Ryan, and CA Bagnell 1998 Regulation of urokinase- and tissue-type plasminogen activator by relaxin in the uterus and cervix of the prepubertal gilt. *J Reprod Fertil* **114** 119-125.

Yan, W, AA Wiley, RA Bathgate, AL Frankshun, S Lasano, BD Crean, BG Steinetz, CA Bagnell, and FF Bartol 2006 Expression of LGR7 and LGR8 by neonatal porcine uterine tissues and transmission of milk-borne relaxin into the neonatal circulation by suckling. *Endocrinology* **147** 4303-4310.

Yang, J, RW Shultz, WM Mars, RE Wegner, Y Li, C Dai, K Nejak, and Y Liu 2002 Disruption of tissue-type plasminogen activator gene in mice reduces renal interstitial fibrosis in obstructive nephropathy. *J Clin Invest* **110** 1525-1538.

CHAPTER III

Colostrum-Feeding and Oral IGF-I Support Neonatal Porcine Uterine Cell Proliferation

ABSTRACT

Delivery of milk-borne bioactive factors (MbFs), including relaxin, to nursing offspring via a lactocrine mechanism can affect development of somatic tissues, including the uterus. The extent to which other MbFs, such as insulin-like growth factor (IGF)-I, affect uterine development is unknown. Here, the objective was to determine how feeding colostrum with or without IGF-I supplementation supports uterine cell proliferation at 12 h of age. At birth, gilts were randomly assigned to receive a single dose (15 ml) of: (1) colostrum collected at 0 hour (0 h) lactation; (2) colostrum (0 h) supplemented with IGF-I (1μg/ml); (3) porcine milk replacer alone; or (4) porcine milk replacer supplemented with IGF-I (1μg/ml). Following the initial feeding, all gilts received milk replacer (30ml/kg BW/2h) alone until 10 h of age. Uterine tissues were collected at 12 h and subjected to immunostaining for proliferating cell nuclear antigen (PCNA) to determine cell proliferation patterns and labeling indices (LI) for epithelial and stromal cell compartments. PCNA immunostaining was detected in all animals. Compared to gilts fed a dose of colostrum at birth, supplementation of colostrum with IGF-I did not affect uterine epithelial or stromal PCNA LI. In contrast, feeding milk replacer alone reduced ($P < 0.01$) both epithelial and stromal PCNA LI compared to animals fed a single dose of colostrum. However, as compared to gilts fed milk replacer alone, supplementation of replacer with IGF-I increased ($P < 0.01$) PCNA LI in both epithelium and stroma. Feeding IGF-I supplemented milk replacer at birth resulted in uterine PCNA LI values similar to those observed for gilts fed colostrum. Results provide the basis for an efficient neonatal porcine bioassay for detection of lactocrine activity in a single dose of colostrum

by 12 h postnatal. Results also suggest that IGF-I is a potentially lactocrine-active factor in the neonatal pig.

INTRODUCTION

Lactation, a defining characteristic of mammals, extends the period of maternal influence on developing offspring beyond gestation. First milk (colostrum) serves as a conduit for both nutritive and non-nutritive, milk-borne bioactive factors (MbFs) that can affect neonatal development as proposed by the lactocrine hypothesis (Yan *et al.* 2006, Bartol *et al.* 2008). Transient permeability of the neonatal intestinal epithelium, the duration of which is species-specific (Pacha 2000), allows MbFs, including proteins and peptides, to enter into the peripheral circulation intact (Sangild 2003). Evidence that MbFs are important for development comes from studies showing that nursing supports mammotrope development and secretory function in rats (Nusser and Frawley 1997), hippocampal development and memory in mice (Liu *et al.* 2014), gastric development in marsupials (Kwek *et al.* 2009), and immune system maturation in humans (Andersson *et al.* 2009). Studies of the neonatal porcine uterus (Miller *et al.* 2013) indicate that nursing from birth supports normal patterns of uterine gland development, cell proliferation, and endometrial cell compartment-specific gene expression events, evident by as early as postnatal day (PND) 2.

A member of the insulin-like family of hormones, relaxin (RLX) is present in colostrum (Eddie *et al.* 1989, Goldsmith *et al.* 1994, Frankshun *et al.* 2010) and is important for promoting growth and remodeling of reproductive tissues (Yan *et al.* 2008). Detectable in the peripheral circulation of nursing pigs at 12 h postnatal, RLX was undetectable in serum of neonates fed RLX-free porcine milk replacer from birth (Yan *et al.* 2006). These results were interpreted to indicate that RLX was delivered from mother to offspring in colostrum via a lactocrine mechanism (Yan *et al.* 2006). Evidence to

support RLX as a protypical, lactocrine-active hormone included: (1) identification of a natural source of bioactive RLX in colostrum (Frankshun *et al.* 2010); (2) data indicating detectable concentrations of RLX in the neonatal porcine circulation after nursing; (3) expression of RLX receptors in the neonatal porcine uterus (Yan *et al.* 2006); and (4) data indicating neonatal uterine responsiveness to exogenous RLX (Yan *et al.* 2008).

Like RLX, insulin-like growth factor (IGF)-I, a 7 kDa mitogenic peptide, is present in the milk of many species including humans (Baxter *et al.* 1984), rats (Donovan *et al.* 1991), cows (Francis *et al.* 1986), goats (Faulkner 1999), and pigs (Simmen *et al.* 1988). Additionally, IGF-I is present at higher concentrations in colostrum than in mature milk (Burrin 1997). Orally administered IGF-I is bioactive in the neonatal pig and acts locally to promote increased villus growth and cell proliferation in the small intestine (Burrin *et al.* 1996, Houle *et al.* 1997). Whether orally administered IGF-I affects development of neonatal somatic tissues beyond the small intestine remains to be determined.

Since lactocrine-mediated effects on neonatal uterine development and related cell compartment-specific events associated with endometrial morphogenesis in the neonatal pig are evident by PND 2 (Miller *et al.* 2013), it is likely that such effects could be detected within 48h of birth. If so, basic elements of a more efficient bioassay for identification of potentially lactocrine-active factors would be in hand. Thus, objectives of this study were to determine: (1) if a single oral dose of 0 h lactation colostrum would affect compartment-specific patterns of uterine endometrial cell proliferation at 12 h postnatal; and (2) whether such effects would be altered by co-administration of IGF-I, a potentially lactocrine-active factor.

MATERIALS AND METHODS

Animals

Gilts (*Sus scrofa domesticus*) were born from an established herd of crossbred (Duroc, Hampshire, Yorkshire, and Landrace) pigs and raised at the Swine Unit of the New Jersey Agricultural Experiment Station, Rutgers University. All procedures involving animals were reviewed and approved by the Rutgers University Institutional Animal Care and Use Committee and conducted in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (McGlone *et al.* 2010). Care was taken to ensure that treatments were balanced for potential effects of litter (n = 6), and that sows were nursing litters of similar size. Based on a study suggesting that pigs of lower birth weight display below average postnatal development (Rehfeldt and Kuhn 2006), gilts were included in the experiment if birth weight was 1.3 kg or greater. Pigs were fed a nutritionally complete commercial pig milk replacer (Advance Liqui-Wean MSC Specialty Nutrition; Carpentersville, IL, USA) based on body weight and housed in a pen adjacent to the sow and littermates.

Experimental design and tissue collection

At birth, crossbred gilts were randomly assigned to treatment groups (n = 4-6/group) and gavage-fed (15 ml/kg BW) a single dose of either: (1) colostrum collected at the onset of parturition [0 hour (0 h) lactation]; (2) colostrum (0h) supplemented with recombinant human IGF-I (1 μg/ml; Peprotech; Rocky Hill, NJ, USA); (3) milk replacer alone; or (4) milk replacer supplemented with IGF-I (1 μg/ml), as illustrated in Figure 1. The dose of IGF-I was based on results of a study that measured IGF-I in porcine

colostrum within 12 h of birth (Simmen et al., 1990). Gilts in all treatment groups were then fed milk replacer alone (30 ml/kg BW/2h) until 10 h of age. At 12 h postnatal, animals were euthanized and uteri were collected. Uteri were trimmed of associated tissues and uterine wet weights (mg) were recorded. Tissues were fixed in Xpress Universal Molecular fixative (Sakura; Torrance, CA, USA).

Histology and immunohistochemistry

Porcine uteri were embedded in Paraplast Plus (VWR; Radnor, PA, USA) and sectioned at 6 μm. Tissue sections from each animal were affixed to SuperFrost plus slides (Thermo Scientific; Waltham, MA, USA). Sections were deparaffinized, rehydrated, and subjected to staining for proliferating cell nuclear antigen (PCNA). Sections were then incubated overnight at 4°C with mouse anti-rat PCNA antibody (1:500; clone PC10; Santa Cruz Biotechnology; Santa Cruz, CA, USA). Sections representing all animals from each treatment group were processed together using the Vecta-Stain ABC elite kit (Vector Laboratories; Burlingame, CA, USA). Following incubation with secondary antibody and ABC reagent, color was developed using 0.1% 3,3'-Diaminobenzidine substrate (Santa Cruz Biotechnology; Santa Cruz, CA, USA). Negative controls included substitution of mouse primary antibody isotype IgG (for PCNA; Zymed; Carlsbad, CA, USA) for primary antibodies as appropriate.

PCNA labeling index

Following PCNA immunolocalization, four to six images were taken from two non-sequential immunostained uterine sections from 3-4 gilts per treatment group.

Images were obtained using a QImaging camera (QImaging, Surrey, Canada) and converted to 8-bit grayscale in Adobe Photoshop. Grayscale images were analyzed using ImageJ software (National Institutes of Health; http://rsbweb.nih.gov/ij/) as described elsewhere (Masters et al., 2007). Briefly, PCNA-positive nuclei were identified when staining intensity values generated by image analysis were within 25% of the highest possible intensity value. PCNA LI, expressed as a percentage, was determined for epithelium and stroma in tissues obtained at 12 h of age for all four treatment groups. A minimum of 800 epithelial and 900 stromal cells (labeled and unlabeled) were counted for each gilt. The number of labeled cells was divided by the total number of cells counted and multiplied by 100 to generate PCNA LI values as a percentage of total cells counted. Grayscale images were converted to pseudocolor using procedures previously described (Masters et al., 2007). Colors were assigned based on relative staining intensity of the grayscale images. For images presented here, the red-yellow color denotes highest staining intensity whereas the blue-black color denotes areas where little to no staining occurred.

Statistical analysis

Data were subjected to ANOVA using general linear model procedures available with SAS (SAS 2009-2010). Statistical models for all quantitative data used preplanned contrasts and considered main effects of a single dose of colostrum or milk replacer with or without oral IGF-I (colostrum alone versus replacer alone; colostrum versus colostrum + IGF-I; replacer alone versus replacer + IGF-I). Uterine wet weights and whole body weights, as well as PCNA LI values for the uterine epithelium and stroma, were tested for

normality using the Shapiro-Wilks test (Henderson, 2006) and found to be normally distributed. Error terms were identified based upon expectations of the mean squares for error. Results are expressed as least squares means (LSM) with SEM.

RESULTS

Effects of a single oral dose of colostrum or milk replacer at birth, with or without IGF-I, on uterine weight, PCNA immunostaining, and PCNA LI

As depicted in Figure 2, uterine weights at 12 h postnatal were similar in gilts fed a single dose of colostrum at birth, with or without oral IGF-I, or milk replacer alone (163 $\pm$ 12 mg/kg). However, mean uterine weight was greater ($P < 0.05$) in replacer-fed gilts supplemented with IGF-I (270 $\pm$ 12 mg/kg), as compared to gilts fed replacer alone. Body weights at 12 h postnatal were not affected by treatment (1.8 $\pm$ 0.08 kg).

Photomicrographs depicting effects of a single oral dose of colostrum or replacer at birth, with or without supplemental IGF-I, on endometrial PCNA immunostaining patterns are shown in Figures 3 and 4. Figure 3 depicts original grayscale images used for calculation of PCNA LI for epithelial and stromal compartments. Those images were converted to pseudocolor, as illustrated in Figure 4. In both figures, nuclear, PCNA-positive staining was observed in epithelial and stromal compartments across all treatment groups. Uterine immunostaining was more intense in gilts fed colostrum, colostrum with IGF-I, and replacer with IGF-I as compared to gilts fed replacer alone.

Histograms depicting treatment effects on PCNA LI are shown in Figure 5. At 12 h postnatal, PCNA LI was reduced ($P < 0.01$) in both cell epithelial and stromal compartments for gilts fed replacer as compared to gilts fed a single dose of colostrum. Addition of IGF-1 to colostrum did not affect PCNA LI in either compartment. In contrast, PCNA LI increased ($P < 0.01$) in both cell compartments for gilts fed replacer supplemented with IGF-I at birth as compared to those fed replacer alone.

DISCUSSION

Studies in the pig showed that effects of nursing on uterine wall development are detectable by 48 h from birth, providing support for a two day neonatal porcine bioassay for detection of lactocrine activity (Miller *et al.* 2013). Imposition of the lactocrine-null state, by feeding milk replacer in lieu of colostrum from birth, reduced PCNA LI in both luminal epithelium (LE) and GE at PND 2 when compared to gilts that nursed over the same period (Miller *et al.* 2013). Results presented here support and extend these observations with evidence that, when compared to replacer-fed gilts, a single dose (15 ml) of colostrum at birth increased endometrial cell proliferation at 12 h postnatal. These results provide the basis for a more efficient bioassay for detection of colostral lactocrine activity in the neonatal pig. Furthermore, present data indicate that oral IGF-1 increased endometrial cell PCNA LI when co-administered with replacer, but not with colostrum. Thus, in the neonatal pig, bioactivity of oral IGF-I, as defined by effects on uterine cell proliferation, is dependent on the mode of delivery (colostrum versus replacer).

The fact that body weights were similar whether gilts were fed a single dose of colostrum or milk replacer at birth, with or without IGF-1, indicates that uterine responses to treatments at 12 h postnatal were not biased by body weight. These results are consistent with evidence that replacer-feeding does not compromise growth of neonatal pigs within 48 h of birth (Chen *et al.* 2011). The increase in uterine weight observed for gilts fed replacer supplemented with IGF-I is consistent with previous findings that oral IGF-I can increase organ weights. For example, pigs fed formula with supplemental IGF-I had increased pancreatic weights at 24 h postnatal (Xu *et al.* 1994),

and neonatal rats fed a milk substitute containing IGF-I displayed increased brain and liver weights compared to controls (Philipps *et al.* 1997).

In this study, all piglets were fed by orogastric gavage using a feeding tube. Consequently, none of the piglets in this study were allowed to nurse or engage in sucking behavior. Few studies address the question of whether method of feeding from birth affects developmental outcomes in pigs. Widowski and colleagues (2005) understood that newborn pigs are highly motivated to engage in specific nursing behaviors, particularly sucking. They observed that, in contrast to littermates fed replacer from a trough, pigs allowed to ingest milk replacer from baby-bottle nipples mounted to a wall displayed typical nursing behaviors (i.e., sucking limited to nipples and sleeping between feedings) (Widowski *et al.* 2005). Present results indicate that colostrum, delivered by orogastric gavage, was effective in promoting uterine cell proliferation when compared to gilts gavage-fed replacer alone. Thus, results suggest, but do not confirm, that it is colostrum, not nursing behavior, that is important for lactocrine signaling in pigs at 12 h postnatal.

In order to determine if an MbF acts via a lactocrine mechanism, several criteria should be met including the following: (1) MbF detection in milk; (2) higher MbF levels in the neonatal circulation post nursing; (3) expression of ligand-specific receptors in target tissues; and (4) evidence of biological activity of the MbF in the neonate (Peaker 2002, Yan *et al.* 2006). Results from previous reports suggested that IGF-I meets the first criterion in that it was identified in colostrum of several species (Baxter *et al.* 1984, Francis *et al.* 1986, Donovan *et al.* 1991, Faulkner 1999) including the pig (Simmen *et al.* 1988). However, whether oral IGF-I can be absorbed into the circulation following

ingestion remains controversial. For example, supplementation of formula with rhIGF-I for four days from birth did not affect plasma IGF-I levels as determined by radio immunoassay (Burrin *et al.* 1996). In contrast, when neonatal rats (PND 10-12) were given oral rh^{125} I-IGF-I, the highest concentrations of this ^{125}I-labeled ligand were identified in portal blood at 20 and 30 minutes following ingestion (Philipps *et al.* 2000). Whether orally administered IGF-I is transmitted into the circulation of neonatal pigs and, therefore, meets the second criterion of a lactocrine-active MbF remains to be determined.

Receptors for IGF-I have been reported in uterine GE of cyclic and pregnant baboons (Hild-Petito *et al.* 1994), myometrium of immature and cyclic rats (Ghahary *et al.* 1990), and both LE and GE of cyclic mice (Baker *et al.* 1996). Although IGF-I receptors (IGF-IR) were detected in the adult porcine uterus (Hofig *et al.* 1991), expression has not been demonstrated for the neonatal pig. However, *IGFIR* expression was reported for the developing neonatal ovine uterus from PND 1 to PND 56, and localized to developing LE and GE (Taylor *et al.* 2001). Similar data have yet to be reported for the neonatal pig, leaving the third criterion implicating IGF-I as a porcine MbF unsatisfied as well.

Results of this study indicate that IGF-I, when fed orally in milk replacer, is biologically active in the neonatal porcine uterus, as reflected by positive effects on uterine PCNA LI. Others reported that feeding formula supplemented with IGF-I from birth increased intestinal weight, protein, and DNA content, as well as villus height in neonatal pigs (Burrin *et al.* 1996). Similarly, newborn pigs fed formula with rhIGF-I exhibited increased intestinal crypt depth and enzyme activity (Houle *et al.* 1997, Houle

et al. 2000). Xu and colleagues (1994) reported that feeding formula supplemented with IGF-I for 24 h after birth increased neonatal porcine pancreatic weight and DNA content (Xu *et al.* 1994). Why, in the present experiment, supplemental IGF-I had no effect on uterine cell proliferation when administered in colostrum is unclear. One explanation for this result may be that factors in colostrum, including IGF binding proteins (IGFBPs) (Gibson *et al.* 1999, Blum and Baumrucker 2008) bind IGF-I and prevent it from associating with the IGF-IR (Ferry *et al.* 1999). Indeed, the majority of IGF-I in porcine colostrum is associated with carrier proteins (Simmen *et al.* 1988). Caseins can also bind IGF-I (Xian *et al.* 1995). However, the extent to which such proteins inhibit the ability of IGF-I to interact with the IGF-IR is not known. Whether it is IGFBPs or other colostral proteins, absent in milk replacer, that affect IGF-I bioavailability remains to be determined.

Data provided here and elsewhere (Miller *et al.* 2013) show that lactocrine signaling supports neonatal porcine uterine development. Present results provide the basis for a more efficient neonatal bioassay with which to identify lactocrine-active porcine MbFs. Results also implicate IGF-I as a potential lactocrine-active factor in the neonatal pig. The stage is now set for additional experiments designed to determine if IGF-I can be added to the list of lactocrine-active molecules affecting uterine development in the neonatal pig.

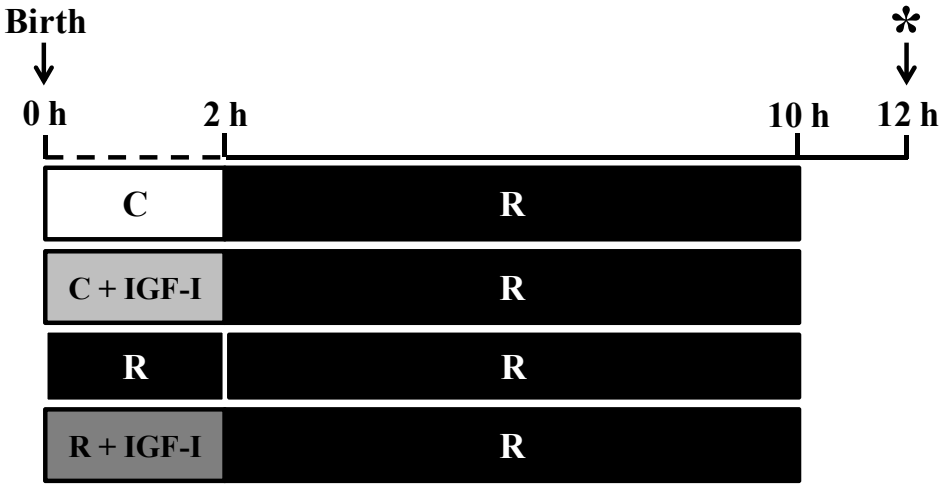

Figure 1. Experimental design. Gilts were assigned at birth (0 h) to treatment groups and gavage-fed (15 ml) a single dose of one of the following: (1) colostrum (C) collected at 0h lactation; (2) colostrum with oral IGF-I (1 μg/ml; C + IGF-I); (3) milk replacer alone (R); or (4) milk replacer with IGF-I (1 μg/ml; R + IGF-I). All gilts were then gavage-fed replacer through 10 h postnatal. Uteri were collected at 12 h postnatal (asterisk).

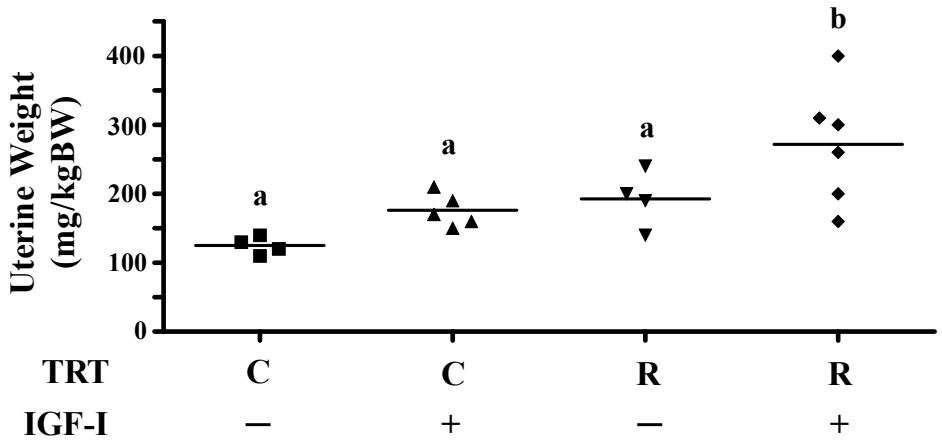

Figure 2. Effects of feeding a single dose of colostrum (C) or milk replacer (R) alone (-) or supplemented with IGF-I (+) on neonatal porcine uterine weight (mg/kg) at 12 h postnatal. Individual weights are presented and horizontal bars indicate LSM. Different letters denote differences at $P < 0.05$.

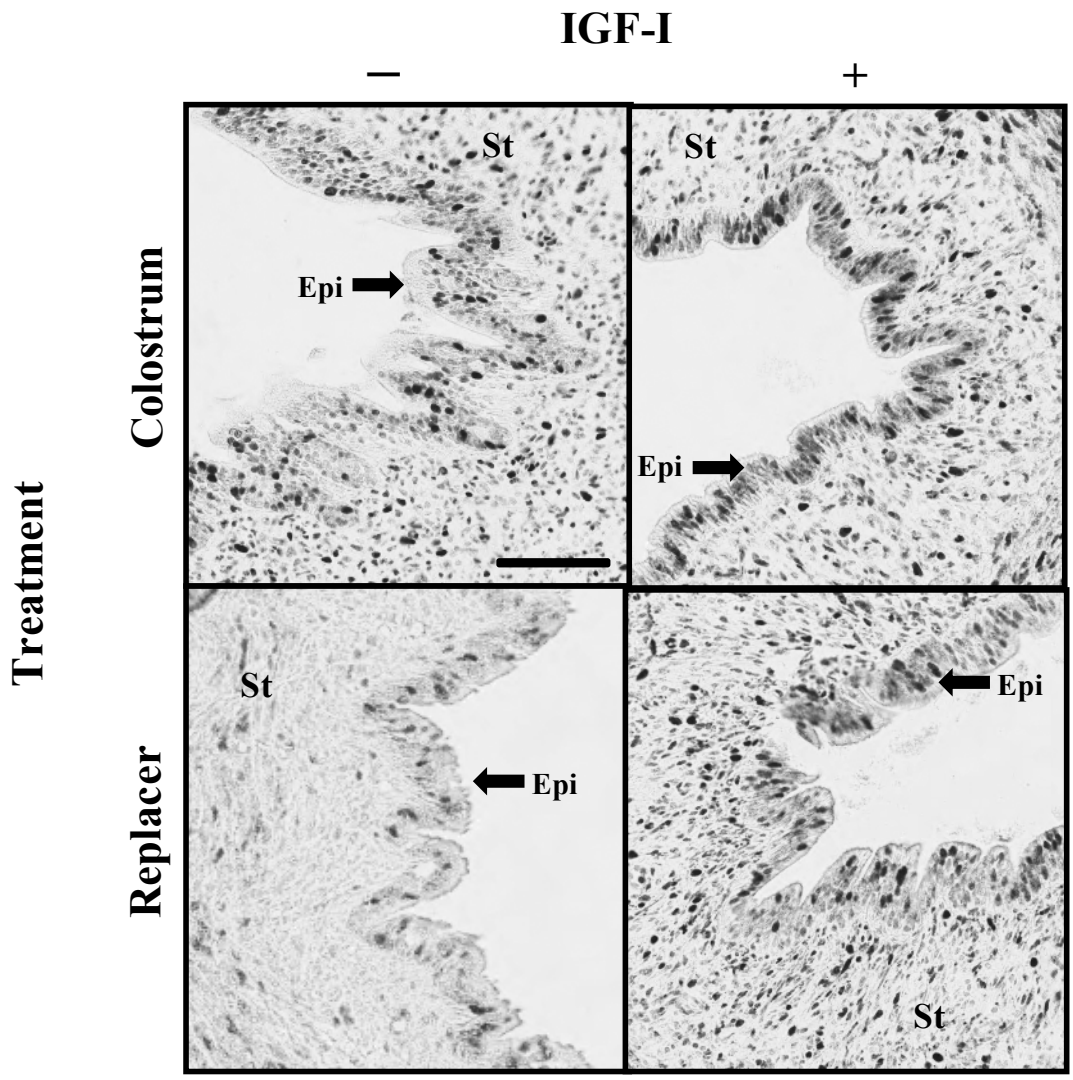

Figure 3. Photomicrographs depicting endometrial hisology and effects of a single dose of colostrum or replacer at birth, with or without oral IGF-I, on PCNA immunostaining patterns for stroma (St) and epithelium (Epi) at 12 h postnatal. Nuclei of cells stained positively appear black. Original magnification 10X. Bars indicate 50 μm.

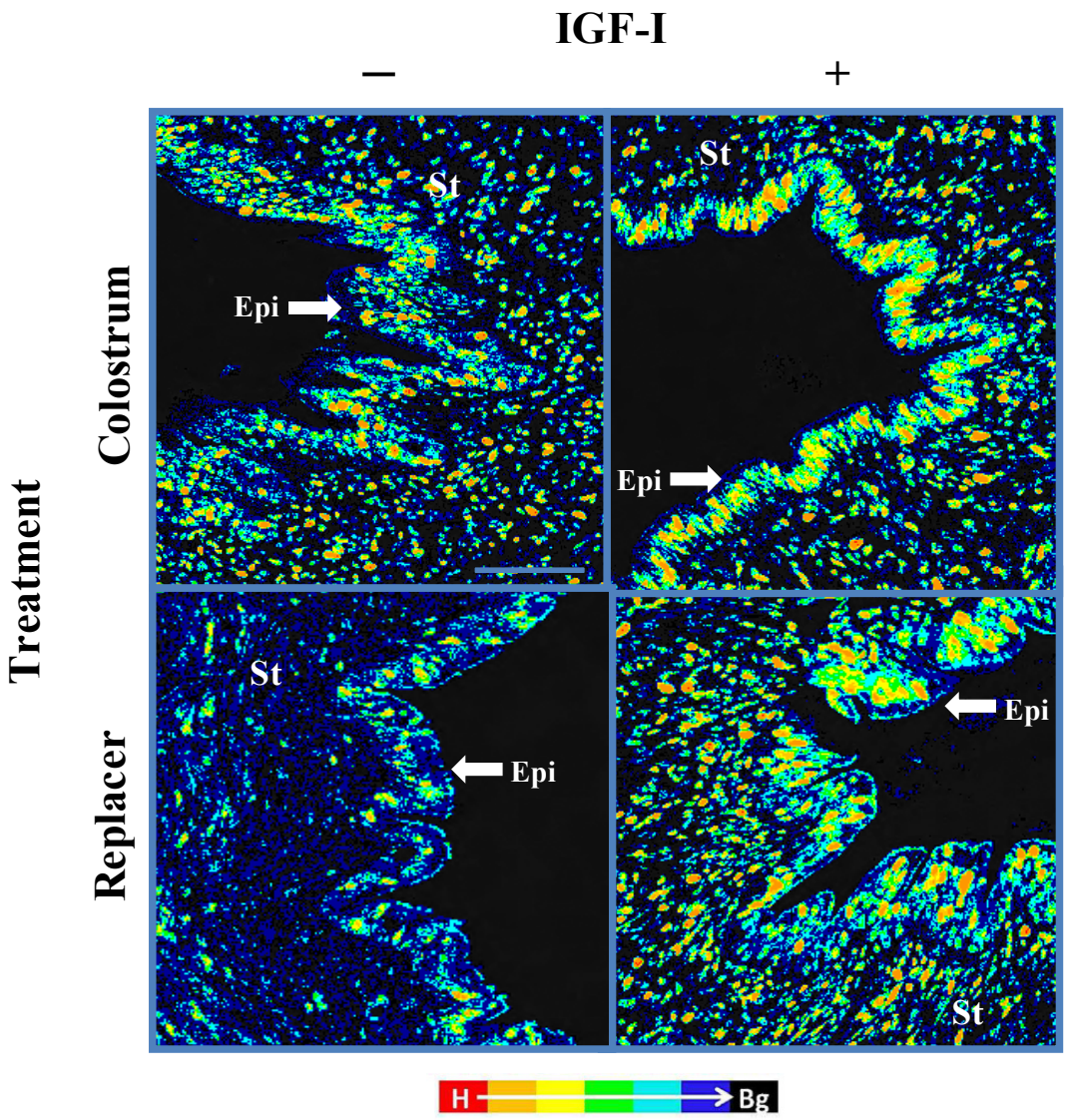

Figure 4. Photomicrographs depicting effects of feeding a single dose of colostrum or replacer with or without oral IGF-I on PCNA immunostaining patterns of the uterine epithelium (Epi) or stroma (St) at 12 h postnatal. Nuclei of cells stained positively appear red-yellow. Blue-black color denotes background. Signal intensity is indicated by color (bottom legend: H: high to Bg: background). Original magnification 10X. Bars indicate 50 μm.

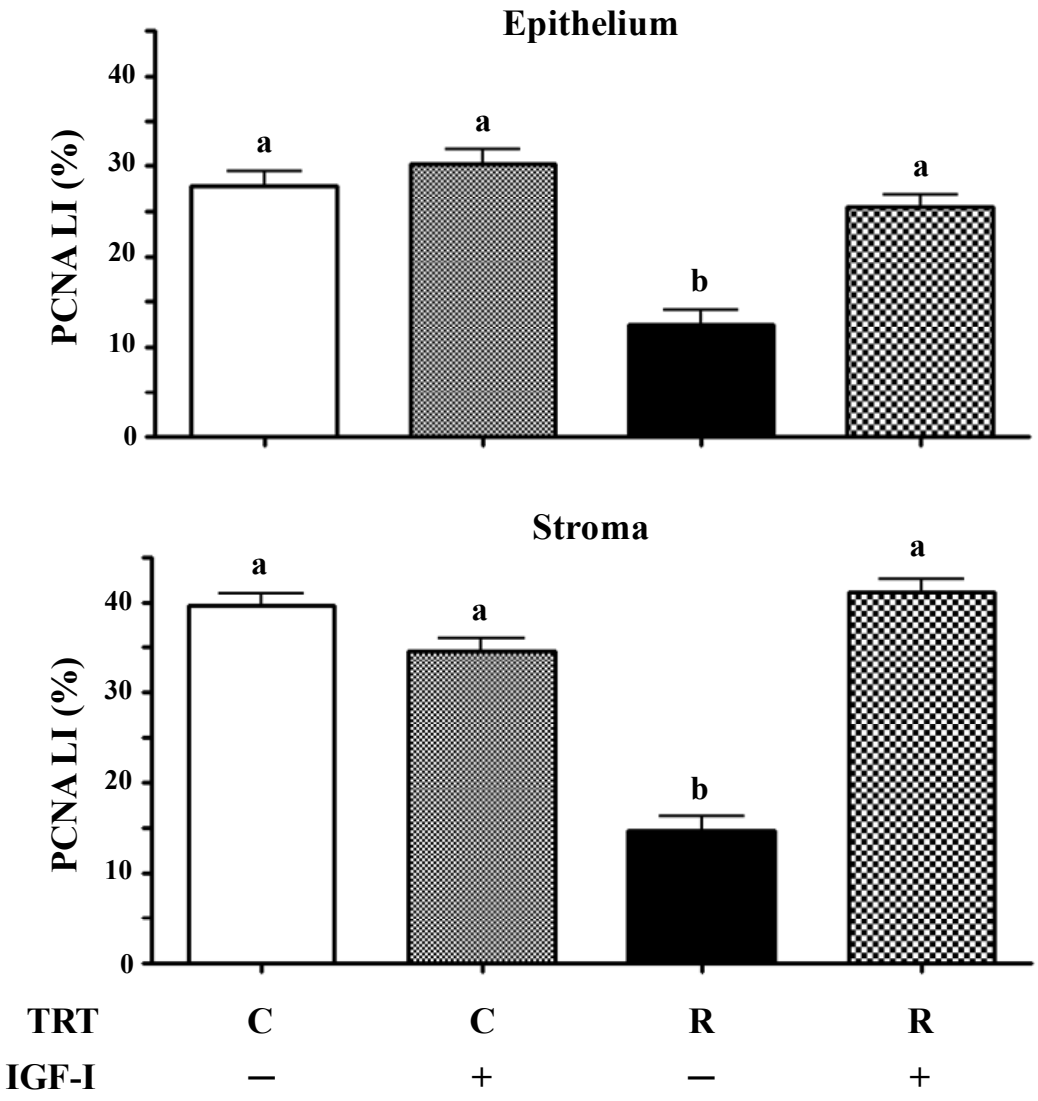

Figure 5. Effects of feeding a single dose of colostrum (C) or milk replacer (R) alone (-) or supplemented with IGF-I (+) on the PCNA LI for epithelial (top) and stromal (bottom) compartments of the neonatal porcine uterus at 12 h postnatal. PCNA LI data are presented as LSM $\pm$ SEM. Different letters denote differences at $P < 0.01$.

REFERENCES

Andersson, Y, ML Hammarstrom, B Lonnerdal, G Graverholt, H Falt, and O Hernell 2009 Formula feeding skews immune cell composition toward adaptive immunity compared to breastfeeding. *J Immunol* **183** 4322-4328.

Baker, J, MP Hardy, J Zhou, C Bondy, F Lupu, AR Bellve, and A Efstratiadis 1996 Effects of an Igf1 gene null mutation on mouse reproduction. *Mol Endocrinol* **10** 903-918.

Bartol, FF, AA Wiley, and CA Bagnell 2008 Epigenetic programming of porcine endometrial function and the lactocrine hypothesis. *Reprod Domest Anim* **43 Suppl 2** 273-279.

Baxter, RC, Z Zaltsman, and JR Turtle 1984 Immunoreactive somatomedin-C/insulin-like growth factor I and its binding protein in human milk. *J Clin Endocrinol Metab* **58** 955-959.

Blum, JW, and CR Baumrucker 2008 Insulin-like growth factors (IGFs), IGF binding proteins, and other endocrine factors in milk: role in the newborn. *Adv Exp Med Biol* **606** 397-422.

Burrin, DG 1997 Is milk-borne insulin-like growth factor-I essential for neonatal development? *J Nutr* **127** 975S-979S.

Burrin, DG, TJ Wester, TA Davis, S Amick, and JP Heath 1996 Orally administered IGF-I increases intestinal mucosal growth in formula-fed neonatal pigs. *Am J Physiol* **270** R1085-1091.

Chen, JC, AL Frankshun, AA Wiley, DJ Miller, KA Welch, TY Ho, FF Bartol, and CA Bagnell 2011 Milk-borne lactocrine-acting factors affect gene expression patterns in the developing neonatal porcine uterus. *Reproduction* **141** 675-683.

Donovan, SM, RL Hintz, DM Wilson, and RG Rosenfeld 1991 Insulin-like growth factors I and II and their binding proteins in rat milk. *Pediatr Res* **29** 50-55.

Eddie, LW, B Sutton, S Fitzgerald, RJ Bell, PD Johnston, and GW Tregear 1989 Relaxin in paired samples of serum and milk from women after term and preterm delivery. *Am J Obstet Gynecol* **161** 970-973.

Faulkner, A 1999 Changes in plasma and milk concentrations of glucose and IGF-1 in response to exogenous growth hormone in lactating goats. *J Dairy Res* **66** 207-214.

Ferry, RJ, Jr., LE Katz, A Grimberg, P Cohen, and SA Weinzimer 1999 Cellular actions of insulin-like growth factor binding proteins. *Horm Metab Res* **31** 192-202.

Francis, GL, LC Read, FJ Ballard, CJ Bagley, FM Upton, PM Gravestock, and JC Wallace 1986 Purification and partial sequence analysis of insulin-like growth factor-1 from bovine colostrum. *Biochem J* **233** 207-213.

Frankshun, AL, TY Ho, DC Reimer, J Chen, S Lasano, BG Steinetz, FF Bartol, and CA Bagnell 2010 Characterization and biological activity of relaxin in porcine milk. *Reproduction* **141** 373-380.

Ghahary, A, S Chakrabarti, and LJ Murphy 1990 Localization of the sites of synthesis and action of insulin-like growth factor-I in the rat uterus. *Mol Endocrinol* **4** 191-195.

Gibson, CA, MD Staley, and CR Baumrucker 1999 Identification of IGF binding proteins in bovine milk and the demonstration of IGFBP-3 synthesis and release by bovine mammary epithelial cells. *J Anim Sci* **77** 1547-1557.

Goldsmith, LT, G Lust, and BG Steinetz 1994 Transmission of relaxin from lactating bitches to their offspring via suckling. *Biol Reprod* **50** 258-265.

Hild-Petito, S, HG Verhage, and AT Fazleabas 1994 Characterization, localization, and regulation of receptors for insulin-like growth factor I in the baboon uterus during the cycle and pregnancy. *Biol Reprod* **50** 791-801.

Hofig, A, FJ Michel, FA Simmen, and RC Simmen 1991 Constitutive expression of uterine receptors for insulin-like growth factor-I during the peri-implantation period in the pig. *Biol Reprod* **45** 533-539.

Houle, VM, YK Park, SC Laswell, GG Freund, MA Dudley, and SM Donovan 2000 Investigation of three doses of oral insulin-like growth factor-I on jejunal lactase phlorizin hydrolase activity and gene expression and enterocyte proliferation and migration in piglets. *Pediatr Res* **48** 497-503.

Houle, VM, EA Schroeder, J Odle, and SM Donovan 1997 Small intestinal disaccharidase activity and ileal villus height are increased in piglets consuming formula containing recombinant human insulin-like growth factor-I. *Pediatr Res* **42** 78-86.

Kwek, JH, RD Iongh, MR Digby, MB Renfree, KR Nicholas, and M Familari 2009 Cross-fostering of the tammar wallaby (Macropus eugenii) pouch young accelerates fore-stomach maturation. *Mech Dev* **126** 449-463.

Liu, B, B Zupan, E Laird, S Klein, G Gleason, M Bozinoski, J Gal Toth, and M Toth 2014 Maternal hematopoietic TNF, via milk chemokines, programs hippocampal development and memory. *Nat Neurosci* **17** 97-105.

McGlone, J, S Ford, F Mitloehner, T Grandin, P Ruegg, C Stull, G Lewis, J Swanson, W Underwood, J Mench, T Mader, S Eicher, P Hester, and J Salak-Johnson 2010 Guide for the Care and Use of Agricultural Animals in Resaerch and Teaching, edn 3. Federation of Animal Science Societies.

Miller, DJ, AA Wiley, JC Chen, CA Bagnell, and FF Bartol 2013 Nursing for 48 hours from birth supports porcine uterine gland development and endometrial cell-compartment specific gene expression. *Biol Reprod* **88** 4.

Nusser, KD, and S Frawley 1997 Depriving neonatal rats of milk from early lactation has long-term consequences on mammotrope development. *Endocrine* **7** 319-323.

Pacha, J 2000 Development of Intestinal Transport Function in Mammals. *Physiological Reviews* **80** 1633-1667.

Peaker, M 2002 The mammary gland in mammalian evolution: a brief commentary on some of the concepts. *J Mammary Gland Biol Neoplasia* **7** 347-353.

Philipps, AF, GG Anderson, B Dvorak, CS Williams, M Lake, AV Lebouton, and O Koldovsky 1997 Growth of artificially fed infant rats: effect of supplementation with insulin-like growth factor I. *Am J Physiol* **272** R1532-1539.

Philipps, AF, B Dvorak, PJ Kling, JG Grille, and O Koldovsky 2000 Absorption of milk-borne insulin-like growth factor-I into portal blood of suckling rats. *J Pediatr Gastroenterol Nutr* **31** 128-135.

Rehfeldt, C, and G Kuhn 2006 Consequences of birth weight for postnatal growth performance and carcass quality in pigs as related to myogenesis. *J Anim Sci* **84 Suppl** E113-123.

Sangild, PT 2003 Uptake of colostral immunoglobulins by the compromised newborn farm animal. *Acta Vet Scand Suppl* **98** 105-122.

SAS 2009-2010 *SAS/STAT User's Guide*. *Cary, NC: SAS Institute Inc.*

Simmen, FA, RC Simmen, and G Reinhart 1988 Maternal and neonatal somatomedin C/insulin-like growth factor-I (IGF-I) and IGF binding proteins during early lactation in the pig. *Dev Biol* **130** 16-27.

Taylor, KM, C Chen, CA Gray, FW Bazer, and TE Spencer 2001 Expression of messenger ribonucleic acids for fibroblast growth factors 7 and 10, hepatocyte growth factor, and insulin-like growth factors and their receptors in the neonatal ovine uterus. *Biol Reprod* **64** 1236-1246.

Widowski, TM, Y Yuan, and JM Gardner 2005 Effect of accommodating sucking and nosing on the behaviour of artificially reared piglets. *Lab Anim* **39** 240-250.

Xian, CJ, CA Shoubridge, and LC Read 1995 Degradation of IGF-I in the adult rat gastrointestinal tract is limited by a specific antiserum or the dietary protein casein. *J Endocrinol* **146** 215-225.

Xu, RJ, DJ Mellor, MJ Birtles, BH Breier, and PD Gluckman 1994 Effects of oral IGF-I or IGF-II on digestive organ growth in newborn piglets. *Biol Neonate* **66** 280-287.

Yan, W, J Chen, AA Wiley, BD Crean-Harris, FF Bartol, and CA Bagnell 2008 Relaxin (RLX) and estrogen affect estrogen receptor alpha, vascular endothelial growth factor, and RLX receptor expression in the neonatal porcine uterus and cervix. *Reproduction* **135** 705-712.

Yan, W, AA Wiley, RA Bathgate, AL Frankshun, S Lasano, BD Crean, BG Steinetz, CA Bagnell, and FF Bartol 2006 Expression of LGR7 and LGR8 by neonatal porcine uterine tissues and transmission of milk-borne relaxin into the neonatal circulation by suckling. *Endocrinology* **147** 4303-4310.

CHAPTER IV

Nursing Supports Neonatal Porcine Testicular Development

ABSTRACT

The lactocrine hypothesis suggests a mechanism whereby milk-borne bioactive factors delivered to nursing offspring and affect development of neonatal tissues. The objective of this study was to assess whether nursing affects testicular development in neonatal boars as reflected by: (1) Sertoli cell number and proliferation measured by GATA-4 expression and proliferating cell nuclear antigen (PCNA) immunostaining patterns; (2) Leydig cell development and steroidogenic activity as reflected by *insulin-like factor 3 (INSL3)* and P450 side chain cleavage (scc) enzyme expression; and (3) expression of *estrogen receptor-alpha* (*ESR1)*, *vascular endothelial growth factor* (*VEGF) A*, and *relaxin family peptide receptor* (*RXFP) 1*. At birth, boars were randomly assigned (n = 6 - 7/group) to nurse *ad libitum* or to be pan fed porcine milk replacer for 48 h. Testes were collected from boars at birth, prior to nursing, and from nursed and replacer-fed boars at 50 h on postnatal day (PND) 2. Sertoli cell PCNA labeling index increased ($P < 0.01$) from birth to PND 2 in nursed, but not in replacer-fed boars. Sertoli cell number and testicular GATA-4 protein levels increased ($P < 0.01$) from PND 0 to PND 2 only in nursed boars. Neither age nor nursing affected testicular *INSL3*, P450scc, *ESR1,* or *VEGFA* levels. However, testicular *RXFP1* levels increased ($P < 0.01$) with age and were greater in replacer-fed boars on PND 2. Results suggest that nursing supports neonatal porcine testicular development and provide additional evidence for the importance of lactocrine signaling in pigs.

INTRODUCTION

Maternal effects on offspring development do not end at birth (postnatal day = PND 0) (Bartol *et al.* 2013), but continue thereafter, when biologically active factors present in milk, such as growth factors, antimicrobial peptides and hormones (Playford *et al.* 2000) are communicated to neonates via nursing (Yan *et al.* 2006, Bartol *et al.* 2008). Thus, the lactocrine hypothesis was proposed to describe the mechanism by which milk-borne bioactive factors, exemplified by the hormone relaxin (RLX) in the pig, might affect development of neonatal somatic tissues (Yan *et al.* 2006). It is known that nursing promotes differentiation and function of anterior pituitary mammotropes in rats (Nusser and Frawley 1997), forestomach development in marsupials (Kwek *et al.* 2009), and immune system maturation and function in humans (Andersson *et al.* 2009). In the pig, nursing for 2 d from birth supports endometrial development, including cell compartment-specific expression of *estrogen receptor-α* (*ESR1)*, *vascular endothelial growth factor A* (*VEGFA*), and *relaxin family peptide receptor 1* (*RXFP1)* (Miller *et al.* 2013). Further, imposition of the lactocrine-null condition for 2 d from birth by feeding milk replacer in lieu of colostrum altered patterns of uterine gene expression at transcriptional and translational levels by PND 2 (Miller *et al.* 2013). These effects persisted to PND 14, even after replacer-fed gilts were returned to nursing on PND 2, such that uterine gland development was markedly reduced by PND 14 (Miller *et al.* 2013). Thus, effects of lactocrine signaling on the reproductive tract, evident as early as PND 2, can indicate changes in the organizational program that affect tissue developmental trajectory (Bartol *et al.* 2013).

In the boar, Sertoli cell number, established during the prepubertal period in pigs (Franca *et al.* 2000), determines adult testis size and capacity for sperm production (McCoard *et al.* 2001a). The first phase of Sertoli cell proliferation occurs during the first 2 wk of porcine neonatal life (Franca *et al.* 2000, McCoard *et al.* 2001a). Leydig cell development, reflected by insulin-like factor 3 (INSL3) expression (Ivell *et al.* 2013), is also significant during the first mo of neonatal life in the pig (Franca *et al.* 2000). Accordingly, testicular steroidogenesis, marked by P450 side-chain cleavage (scc) enzyme expression (Choi *et al.* 2009), increases markedly during this period (Raeside *et al.* 1997).

Lactocrine-sensitive endometrial gene products, including *ESR1*, *VEGFA*, and *RXFP1* (Miller *et al.* 2013), also affect testicular development, structure, and function. Localized to Sertoli, Leydig, peritubular myoid, and germ cells in the neonatal boar (Ramesh *et al.* 2007), ESR1 is required to sustain germ cells and stabilize seminiferous tubule structure (Hess 2003, Gould *et al.* 2007). Similarly, VEGFA is important for seminiferous tubule development and vascularization, as well as for maintenance of germ cells in developing testes (Bott *et al.* 2006, Caires *et al.* 2009). Signaling through RXFP1, localized in Sertoli, Leydig and germ cells of neonatal boars (Kato *et al.* 2010), supports sperm maturation and fertility (Samuel *et al.* 2003, Krajnc-Franken *et al.* 2004). Factors affecting neonatal porcine testis development, including Sertoli cell proliferation, Leydig cell development and associated steroidogenic activity, can determine reproductive capacity in adulthood.

Whether lactocrine signaling is required to: (1) establish the neonatal testicular developmental program; or (2) determine testicular developmental trajectory has yet to be

determined. To address the first of these questions, objectives were to determine effects of neonatal age and nursing from birth on: (1) Sertoli cell number and proliferation as reflected by GATA-4 expression (McCoard *et al.* 2001b) and proliferating cell nuclear antigen (PCNA) immunostaining patterns (Iatropoulos and Williams 1996, Nolte *et al.* 2005); (2) Leydig cell development and steroidogenic activity indicated by *INSL3* and P450scc expression; and (3) *ESR1*, *VEGFA*, and *RXFP1* expression in testes collected from boars immediately after birth and on PND 2.

MATERIALS AND METHODS

Animals

Boars (*Sus scrofa domesticus*) were born from an established herd of crossbred (Duroc, Hampshire, Yorkshire, and Landrace) sows and raised at the Swine Unit of the New Jersey Agricultural Experiment Station, Rutgers University. All procedures involving animals were reviewed and approved by the Rutgers Institutional Animal Care and Use Committee (Protocol #88-709) and conducted in accordance with the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (McGlone and Swanson 2010). Care was taken to ensure that treatments were balanced for potential effects of litter (n=16) by placing littermates into each of the treatment groups and insuring that sows were nursing litters of similar size. Based on a study suggesting that pigs of lower birth weight display below average postnatal development (Rehfeldt and Kuhn 2006), boars were weighed at birth and included in the experiment if birth weight was 1.3 kg or greater. Body weights were recorded daily through PND 2. Pigs were randomly selected to be fed a nutritionally complete commercial pig milk replacer (Advance Liqui-Wean MSC Specialty Nutrition; Carpentersville, IL, USA) and housed in a pen adjacent to the sow and littermates.

Experimental design and tissue collection

At birth, crossbred boars were randomly assigned to treatment groups in which pigs were either: (1) euthanized for tissue collection at birth, prior to nursing (PND 0; n = 7); (2) nursed *ad libitum* for 48 h (PND 2N; n = 6); or (3) pan-fed milk replacer *ad libitum* (PND 2R; n = 6) for 48 h. Figure 1 depicts the experimental design. Boars were

euthanized and testes were obtained on either PND 0, immediately after birth, or on PND 2 at 50 h of age after nursing or replacer-feeding. Testes were trimmed of associated tissues and testicular wet weights (mg) were recorded. Each testis was cut in half and halves were immersed in either RNALater and stored at –80ºC, or fixed in Xpress Universal Molecular fixative (Sakura; Torrance, CA, USA).

Protein extraction and evaluation of P450scc and GATA-4 levels

Whole testicular tissue cross-sections (20 - 50 mg) were homogenized in 200 µL 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 centrifuged (12,000 g, 4ºC) for 15 min. Supernatant was removed and stored at –80ºC. Protein concentration of supernatant was determined using the DC Protein Assay kit (Bio-Rad Laboratories; Hercules, CA, USA). To evaluate relative tissue levels of targeted proteins, testicular proteins (20 - 30 µg) were resolved on 12.0% total monomer, Bis-Tris-HCl-buffered polyacrylamide gels under reducing conditions and transferred onto nitrocellulose membranes (Bio-Rad Laboratories; Hercules, CA, USA). In addition to sample testicular protein from neonatal boars representative of each day and treatment, protein isolated from adult porcine ovary was included on each gel/blot as a positive control for GATA-4 (Gillio-Meina *et al.* 2003) and P450scc (Kozlowska *et al.* 2009). After blocking in 10.0% non-fat dry milk in Tris-buffered saline containing Tween-20 (TBST; 25 mM Tris (pH 7.5), 0.14 mM NaCl, 3 mM KCl, and 0.05% Tween-20), membranes were probed with either goat anti-mouse GATA-4 antibody (1:500; sc-1237; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-rat P450scc antibody (1:2,000; Ab-1244; Millipore, Darmstadt, Germany), or

goat anti-human actin antibody (1:1,000; sc-1615; Santa Cruz Biotechnology; Santa Cruz, CA, USA) overnight at 4ºC. After washing with TBST, blots were incubated with either HRP-conjugated rabbit anti-goat secondary antibody (GATA-4/actin; 1:2,000; Zymed; Carlsbad, CA, USA) or goat anti-rabbit antibody (P450scc; 1:2,000; Life Technologies; Grand Island; NY; USA) for 1 h at room temperature. Bound antibodies were detected by chemiluminescence using Renaissance Western Blot Chemiluminescence Reagent Plus kit (Perkin Elmer Life Sciences; Waltham, MA, USA). For both GATA-4 and P450scc, antibody specificity was evaluated by omission of the primary antibodies in western blot analyses. In both cases, substitution of appropriate secondary antibody (goat anti-rabbit for P450scc; rabbit anti-goat for GATA-4) for primary antibodies failed to reveal a signal at 53 kDa, indicative of targeted proteins. Inclusion of primary antibodies produced strong, target-specific signal. Protein loading was monitored using actin as a reference since the relative abundance of this protein was not affected by treatment. Chemiluminescence signals were quantified densitometrically from film using Scion Image for Windows (Scion Corporation, Frederick, MD, USA).

Histology and immunohistochemistry

Porcine testicular tissues fixed in Xpress Universal Molecular fixative were embedded in Paraplast Plus (VWR; Radnor, PA, USA) and sectioned at 5 μm. Three sections from each animal per treatment group were affixed to SuperFrost plus slides (Thermo Scientific; Waltham, MA, USA). Sections were deparaffinized, rehydrated, and subjected to staining with Harris hematoxylin (20 s; Sigma-Aldrich; St. Louis, MO, USA) and eosin (H&E) solutions (2 min; Thermo Fisher Scientific; Waltham, MA,

USA). Following H&E staining, all images were captured digitally at 10X and 30X magnification using an Olympus FSX100 Digital Microscope.

For PCNA and GATA-4 immunohistochemistry, sections were deparaffinized, rehydrated, and subjected to heat-induced epitope retrieval. Sections were then incubated overnight at 4°C with either mouse anti-rat PCNA antibody (1:500; clone PC10; Santa Cruz Biotechnology; Santa Cruz, CA, USA) or goat anti-mouse GATA-4 antibody (1:200; sc-1237; Santa Cruz Biotechnology; Santa Cruz, CA, USA). Sections representing all animals from each treatment group were processed together using Vecta-Stain ABC elite kits (Vector Laboratories; Burlingame, CA, USA). Following incubation with secondary antibody and ABC reagent, color was developed using 0.1% 3,3'-Diaminobenzidine substrate (Santa Cruz Biotechnology; Santa Cruz, CA, USA). Negative controls included substitution of mouse primary antibody isotype IgG (for PCNA; Zymed; Carlsbad, CA, USA) or goat primary antibody isotype IgG (for GATA-4; Life Technologies; Grand Island, NY, USA) for primary antibodies as appropriate. All slides were lightly counterstained using ImmunoMaster hematoxylin (American MasterTech; Lodi, CA, USA) to allow for identification of Sertoli and germ cell nuclei within seminiferous tubules.

PCNA labeling index

Following PCNA immunolocalization, two images were taken from two non-sequential immunostained sections for both testes for every boar (n = 6 - 7 boars/group). All images were captured digitally at 20X magnification using an Olympus FSX100 Digital Microscope. Images were converted to 8-bit grayscale in Adobe Photoshop and

analyzed using ImageJ software (National Institutes of Health; http://rsbweb.nih.gov/ij/) as described elsewhere (Masters *et al.* 2007). Briefly, nuclei were defined as PCNA-positive when staining intensity values generated by image analysis were determined to be within 25% of the highest possible intensity value. PCNA labeling index (LI), expressed as a percentage, was determined for Sertoli cells in tissues obtained on PND 0 and on PND 2 for both nursed and replacer-fed groups. A minimum of 3000 Sertoli cells (labeled and unlabeled) were counted for each boar. The number of labeled cells counted was divided by the total number of cells counted and multiplied by 100 to generate PCNA LI values. Germ cells, also located within the seminiferous tubules, and Leydig cells, present in the interstitial compartment, exhibit negligible proliferation during the period immediately after birth in swine (Franca *et al.* 2000, McCoard *et al.* 2001a). Consequently, data for Sertoli cell-specific PCNA LI could be obtained confidently.

GATA-4 immunostaining and Sertoli cell number

Following immunolocalization of GATA-4 in Sertoli cell nuclei, two images were taken from two nonsequential sections for both testes of every boar. These images were captured digitally at 20X magnification using an Olympus FSX Digital Microscope. Nuclear diameter values were determined at 70X magnification using the measurement tool of the Bio-Imaging Navigator software (Olympus; Center Valley, PA, USA). Crude Sertoli cell counts were obtained based on methods outlined elsewhere (Berndtson *et al.* 1987, Wreford 1995) using ImageJ software. Briefly, GATA-4-stained Sertoli cell nuclei with visible nucleoli were enumerated from a minimum of 120 round seminiferous tubular (ST) cross sections for each treatment group to obtain a crude count. Average

nuclear diameter was determined by measuring a minimum of 60 round Sertoli cell nuclei from each group. Both values were used to calculate true Sertoli cell counts. This was accomplished by multiplying the crude count by the section thickness and dividing this value by the section thickness plus the average nuclear diameter (Abercrombie 1946, Wreford 1995).

RNA isolation, cDNA generation, and quantitative RT-PCR

Total RNA was isolated from 50 - 60 mg of testicular tissue for each sample using TRI Reagent and the RNeasy Mini kit. Traces of DNA were removed using the RNase-free DNase Set. The concentration and purity of RNA (260/280 absorbance ratio) were evaluated by using a Nanodrop spectrophotometer. 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.; Hercules, CA, USA) and SuperScript III First-Strand Synthesis System for RT-PCR. All procedures were carried out following manufacturer's instructions. Quantitative RT-PCR (qPCR) was performed using an Applied Biosystems StepOne Plus System (Applied Biosystems/Life Technologies; Grand Island, NY, USA) with the SYBR Green method and universal thermal cycling parameters (40 cycles) indicated by the manufacturer. Primers for qPCR were designed using Primer Express Software (Applied Biosystems/Life Technologies; Grand Island, NY, USA) 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 assays. The quality of 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.

Data were analyzed using the $\Delta\Delta C_T$ method for quantification of gene expression for *ESR1*, *VEGFA*, and *RXFP1* as described by Applied Biosystems (ABI User Bulletin 2, 2001). The $\Delta\Delta C_T$ method of analysis uses the C_T values (the cycle number at which the fluorescent signal crosses the threshold) of the samples with a control or calibrator sample (Livak and Schmittgen 2001). The C_T values of the samples and calibrator are then normalized to an appropriate endogenous reference gene (here *cyclophilin*). The $\Delta\Delta C_T$ method was used since the amplification efficiencies for the target genes (*ESR1*, *VEGFA*, and *RXFP1*) were similar to the efficiency determined for *cyclophilin*, the endogenous control (ABI User Bulletin 2, 2001). Data for *INSL3* were analyzed following the standard curve method for quantification of gene expression as described by Applied Biosystems (ABI User Bulletin 2, 2001). This method of analysis was used because *INSL3* amplification efficiency differed from that determined for *cyclophilin* (ABI User Bulletin 2, 2001). Data from all qPCR analyses are presented as relative mRNA units.

Statistical analysis

Data were subjected to ANOVA using general linear model procedures available with SAS (SAS 2009-2010). Statistical models for all quantitative data, generated by immunoblotting, immunohistochemistry, and qPCR, used preplanned contrasts and considered main effects of treatment (nursing versus replacer) and postnatal age (PND 0

versus PND 2 nursed boars) using a two-way ANOVA. Error terms were identified based upon expectations of the mean squares for error. Data were analyzed for normality using the Shapiro-Wilks test. In any instance where data were determined not to be normally distributed, the non-parametric Kruskal-Wallis test was employed. In all cases, identical results were obtained regardless of statistical procedures used (ANOVA vs non-parametric). Pearson correlation coefficients were determined for data obtained on PND 2. Graphs of raw data for PND 2 supported correlation relationships presented below. Results are expressed as least squares means (LSM) with S.E.M.

RESULTS

Testicular weights and histology

Testicular weights were similar for all treatment groups (PND 0: 674 mg/kg BW; PND 2N: 566 mg/kg BW; PND 2R: 563 mg/kg BW, ± 90 mg/kg BW). Testes appeared normal histologically, with seminiferous tubules, interstitium, Sertoli, Leydig and germ cells all readily identifiable. No differences in testicular histology associated with age or nursing were observed (data not shown). Images depicting a typical testicular cross section obtained from a nursed boar on PND 2 are shown (Figure 2A, B).

Effects of age and nursing on PCNA *LI in neonatal porcine Sertoli cells*

Photomicrographs depicting effects of age and nursing on patterns of PCNA immunostaining and related data for Sertoli cell PCNA LI are shown in Figure 3. Nuclear, PCNA-positive staining was observed in Sertoli cells across all age and treatment groups (Figure 3A). Sertoli cell PCNA LI increased ($P < 0.01$) from birth to PND 2 in nursed, but not in replacer-fed boars (Figure 3B).

Effects of age and nursing on neonatal testicular GATA-4 *protein and Sertoli cell number*

Results of protein expression analyses for testicular GATA-4 are presented in Figure 4. An immunoreactive 53 kDa protein band, corresponding in size to GATA-4 (Gillio-Meina *et al.* 2003, Bosse *et al.* 2006) and identified in positive control tissue extract (adult porcine ovary), was observed in testes from all categories (Figure 4A). The GATA-4 signal increased ($P < 0.01$) from birth to PND 2 in nursed, but not in replacer-

fed boars (Figure 4B). Typical Sertoli cell immunostaining for GATA-4 and data for Sertoli cell number are presented in Figure 5. Nuclear immunostaining for GATA-4 was localized to Sertoli cells (Figure 5A). Consistent with results for GATA-4 expression (Figure 4), the number of Sertoli cells per seminiferous tubule cross section increased ($P < 0.01$) from birth to PND 2 only in nursed boars (Figure 5B). The PCNA LI was positively correlated with both testicular GATA-4 levels ($r = 0.84$, $P < 0.01$) and Sertoli cell number ($r = 0.59$, $P < 0.04$) on PND 2.

Effect of age and nursing on neonatal testicular INSL3 and P450scc *levels*

Results of gene and protein expression analyses indicative of Leydig cell development and activity are presented in Figure 6. Testicular *INSL3* expression was detected in all three treatment groups (Figure 6A). Relative testicular *INSL3* expression was not affected by age or nursing. On PND 2, *INSL3* levels were positively correlated with Sertoli cell number ($r = 0.62$, $P < 0.03$). Results of protein expression analyses for P450scc are presented in Figure 6B,C. Signal indicative of testicular P450scc (53 kDa) was detected in positive control tissue (adult porcine ovary) and in tissues from all boars regardless of age or nursing status. On PND 2, P450scc levels were positively correlated with GATA-4 levels ($r = 0.69$, $P < 0.01$) and PCNA LI ($r = 0.68$, $P < 0.02$).

Effects of nursing on neonatal testicular ESR1, VEGFA, and RXFP1 expression

Results of qPCR analyses for testicular *ESR1, VEGFA*, and *RXFP1* expression are depicted in Figure 7. Data are expressed in relative mRNA units. Testicular *ESR1* and *VEGFA* expression was unaffected by age or nursing status (Figure 7A, B). In contrast,

RXFP1 expression increased ($P < 0.01$) markedly from birth to PND 2 only in replacer-fed boars (Figure 7C). On PND 2, a negative correlation was identified between *RXFP1* levels and Sertoli cell number ($r = -0.67$, $P < 0.02$).

DISCUSSION

Results of the current study suggest that lactocrine-mediated events occurring shortly after birth support the porcine testicular developmental program by PND 2. More specifically, aspects of Sertoli cell development may be influenced by nursing as indicated by treatment effects on PCNA LI, GATA-4 protein expression, and Sertoli cell counts. Additionally, results suggest that testicular *RXFP1* expression is lactocrine-sensitive. Thus, results suggest that testicular development in the neonatal boar is influenced by nursing from birth.

Data indicating that testicular wet weights were unaffected by either age or nursing on PND 2 supports the idea that porcine testicular growth is negligible within 50 h of birth. Testicular histology was not affected overtly by age or treatment by PND 2 (data not shown) and resembled that described in other reports (Avelar *et al.* 2010, Lee *et al.* 2013).

Data for PCNA LI, indicative of cell proliferation (Iatropoulos and Williams 1996), revealed both age and treatment effects on Sertoli cells at PND 2. In contrast to reports for rats (Orth 1982) and mice (Kluin *et al.* 1984), in which Sertoli cell proliferation is maximal during the late fetal period, proliferation of Sertoli cells in pigs is greatest between birth and PND 14 (McCoard *et al.* 2001a). Patterns of Sertoli cell proliferation during this period can define testicular size in adult pigs (McCoard *et al.* 2001a). Whether nursing from birth affects Sertoli cell proliferation positively beyond PND 2 remains to be determined.

Trophic effects of both age and nursing on Sertoli cell number were identified. The GATA family of peptides are zinc finger transcription factors that regulate gene

expression, differentiation, and cell proliferation (Orkin 1992). Used here as a marker for enumeration of Sertoli cells (McCoard *et al.* 2001a), GATA-4 may also affect Sertoli cell development (Orth 1982, Yagi *et al.* 2007). Expression of GATA-4 is hormone sensitive. Reducing endogenous estrogen synthesis increased GATA-4 expression and Sertoli cell number at 2 mo of age in boars (At-Taras *et al.* 2006). Present results indicate that *ESR1* expression in the neonatal porcine testis is not influenced by age or nursing. Positive relationships documented here between Sertoli cell number, PCNA LI, and testicular GATA-4 levels are consistent with the idea that GATA-4 expression could support as well as reflect development of neonatal porcine seminiferous epithelium.

In contrast to observations for rodents, Leydig cells in pigs and humans undergo three distinct phases of development (Van Straaten and Wensing 1978, Bay *et al.* 2007). In humans, Leydig cell development is characterized by high levels of INSL3 expression (Bay *et al.* 2007), indicative of both cell differentiation and cell number (Ivell *et al.* 2013). Recently, INSL3 was localized to porcine Leydig cells (Minagawa *et al.* 2012). Present results indicate that neonatal testicular *INSL3* expression is not sensitive to age or nursing effects. However, Leydig cell growth, number, and function can be affected by signals originating from Sertoli cells (Russell *et al.* 2001). Thus, given the effects of nursing on Sertoli cells reported here, disruption of lactocrine signaling may also alter Sertoli-Leydig cell interactions that are not apparent until later in life. The positive correlation ($r = 0.62$, $P < 0.03$) identified between *INSL3* expression and Sertoli cell number is broadly consistent with this hypothesis.

Leydig cells, located in the interstitial space, are the primary site of expression of steroidogenic enzymes and serve as the main source of testicular androgen and estrogen

production (Choi *et al.* 2009). Steroidogenesis begins with the conversion of cholesterol to pregnenolone, a process catalyzed by P450scc (Choi *et al.* 2009). Here, testicular P450scc was detected, although relative levels of the protein were not affected by age or nursing. However, the fact that both Sertoli cell proliferation and cell numbers were reduced on PND 2 in replacer-fed boars, and that neonatal seminiferous epithelial development is steroid hormone sensitive (At-Taras *et al.* 2006, Ramesh *et al.* 2007), suggests that closer evaluation of lactocrine effects on the expression of enzymes regulating testicular steroidogenesis is warranted. Positive correlations identified between P450scc, PCNA LI, and GATA-4 levels are consistent with the idea that steroidogenic activity supports development of the seminiferous epithelium.

The lactocrine hypothesis was proposed to describe a feed-forward mechanism in which milk-borne bioactive factors, exemplified by the hormone relaxin (RLX), might support expression of mediators of porcine reproductive tract development including ESR1, VEGFA, and RXFP1 (Bartol *et al.* 2009). Although ESR1 and VEGFA support testicular development and adult fertility (Hess 2003, Bott *et al.* 2006, Gould *et al.* 2007, Caires *et al.* 2009), neither age nor nursing affected their expression at PND 2. Still, effects induced by feeding replacer from birth may be cryptic and observed later in neonatal life, as reported for the porcine endometrium (Miller *et al.* 2013). In contrast, testicular *RXFP1* expression was increased in replacer-fed boars on PND 2. This may be explained, in part, by the fact that RLX is present in porcine colostrum and in the circulation of nursed but not in that of replacer-fed gilts (Yan *et al.* 2006, Frankshun *et al.* 2010). Relaxin decreased *RXFP1* expression in porcine cervix and uterus (Yan *et al.* 2008, Chen *et al.* 2011). Thus, eliminating milk-borne RLX via replacer-feeding may

remove an inhibitor of *RXFP1* expression in the neonatal porcine testis and compromise development of seminiferous epithelium. Consistently, a negative correlation ($r = -0.67$, $P < 0.02$) was identified between *RXFP1* levels and Sertoli cell number. This observation, taken together with evidence indicating that RLX increases Sertoli cell proliferation *in vitro* (Cardoso *et al.* 2010), supports present results indicating increased Sertoli cell proliferation and cell numbers in nursed as compared to replacer-fed boars on PND 2.

The idea that maternal programming of development in the neonate involves the transmission of information from mother to offspring in milk is receiving increased attention (Neville *et al.* 2012). Evidence that, as compared to breast-fed human infants, formula-feeding is associated with reduced testis volume by four months of age (Gilchrist *et al.* 2010) suggests that nursing supports testis development in humans. Here, data reinforce this idea with evidence implicating a lactocrine-driven mechanism that may support establishment of a normal testicular developmental program in pigs. Although present results suggest that nursing supports neonatal testicular development, other factors should be considered. These include potential effects of stress associated with removal of piglets from their litters and dams, and/or pan-feeding. The extent to which such behavioral and environmental factors may affect lactocrine-sensitive developmental events remains to be determined.

Gene	Accession #	Forward primer	Reverse primer
INSL3	X68369.1	TACAGTGGCTGGAAGG ACAA	TGGTGGTGATGGCC AGAG
ESR1	AF035775	AGGGAGAGGAGTTTGT GTG	TCTCCAGCAGCAGG TCATAG
VEGFA	AF318502	AAGATCCGCAGACGTG TAAA	CACATCTGCAAGTA CGTTCG
RXFP1	CA994862	GCATCACTTTGAGGCA GAGACA	CCTCGGCAAAGACA TTGCAT
cyclophilin	AU058466	TTATAAAGGTTCCTGCT TTCACAGAA	TGCCATTATGGCGT GTGAAG

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

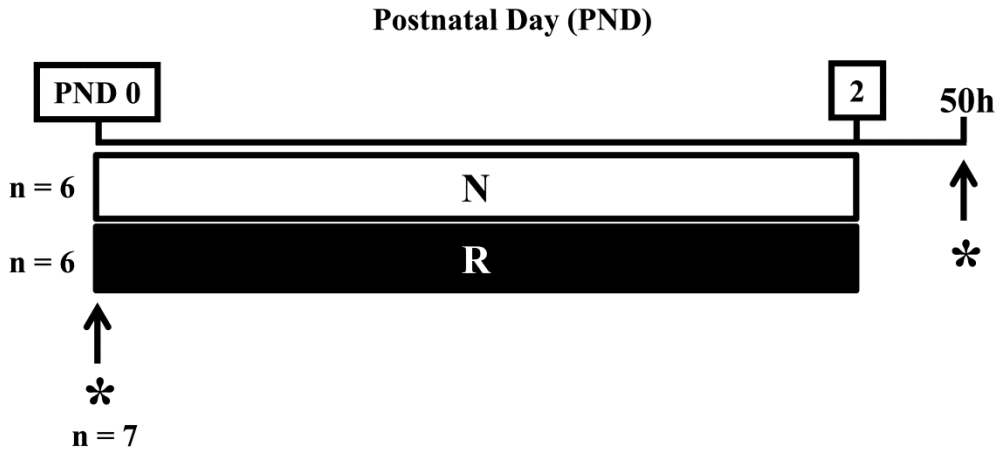

Figure 1. Experimental design. Boars were assigned at birth (PND 0) to either nurse *ad libitum* (N) or to receive milk replacer by pan-feeding (R). Testes were collected on PND 0 or PND 2, as indicated by asterisks.

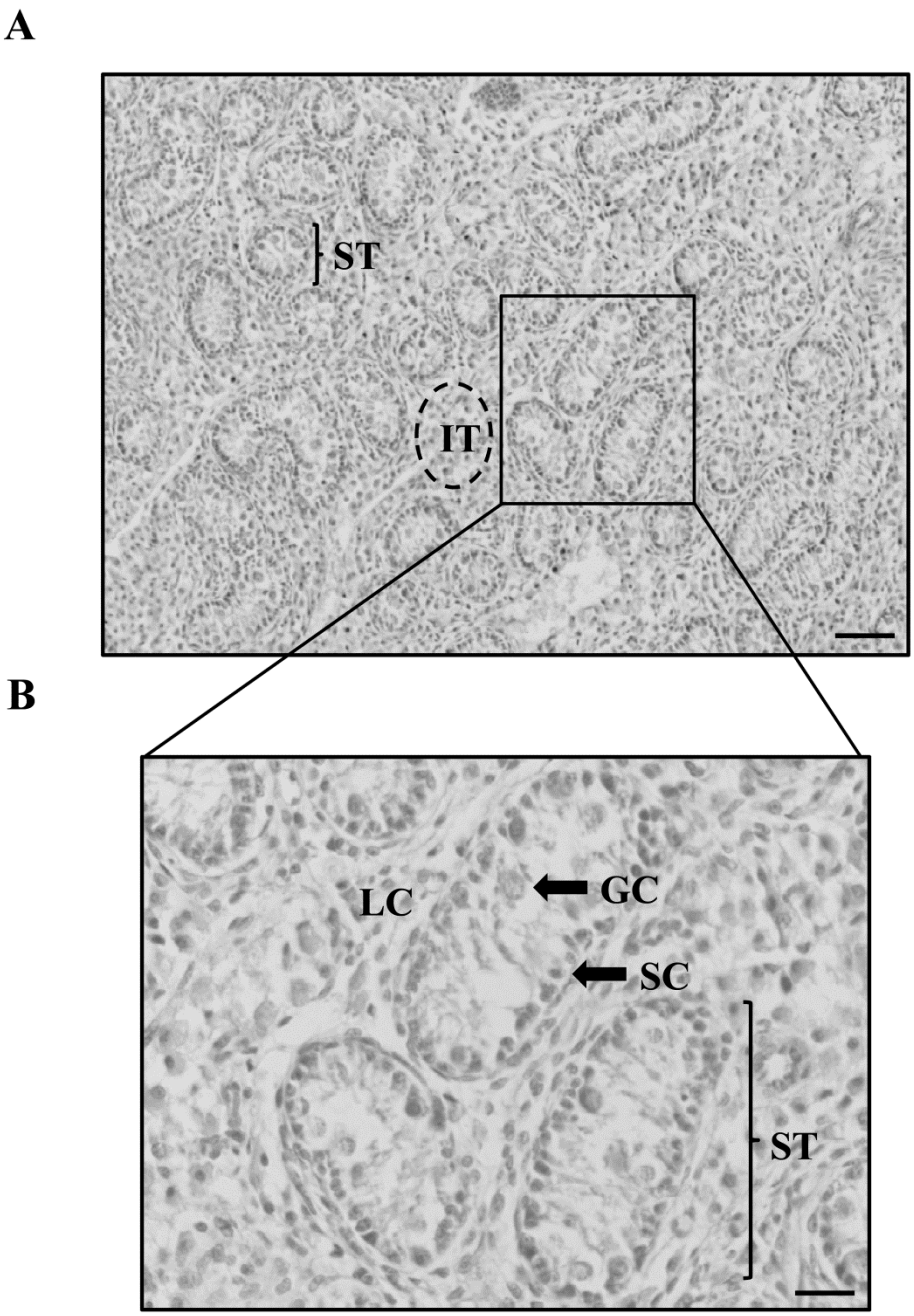

Figure 2. Neonatal porcine testicular histology at PND 2. (A) Low-maginification image of H&E stained testis identifying seminiferous tubules (ST) and interstitial tissue (IT). Bar indicates 64 μm. (B) High-magnification image of H&E stained testis identifying seminiferous tubules (ST), germ cells (GC), Sertoli cells (SC), and Leydig cells (LC). Bar indicates 21 μm.

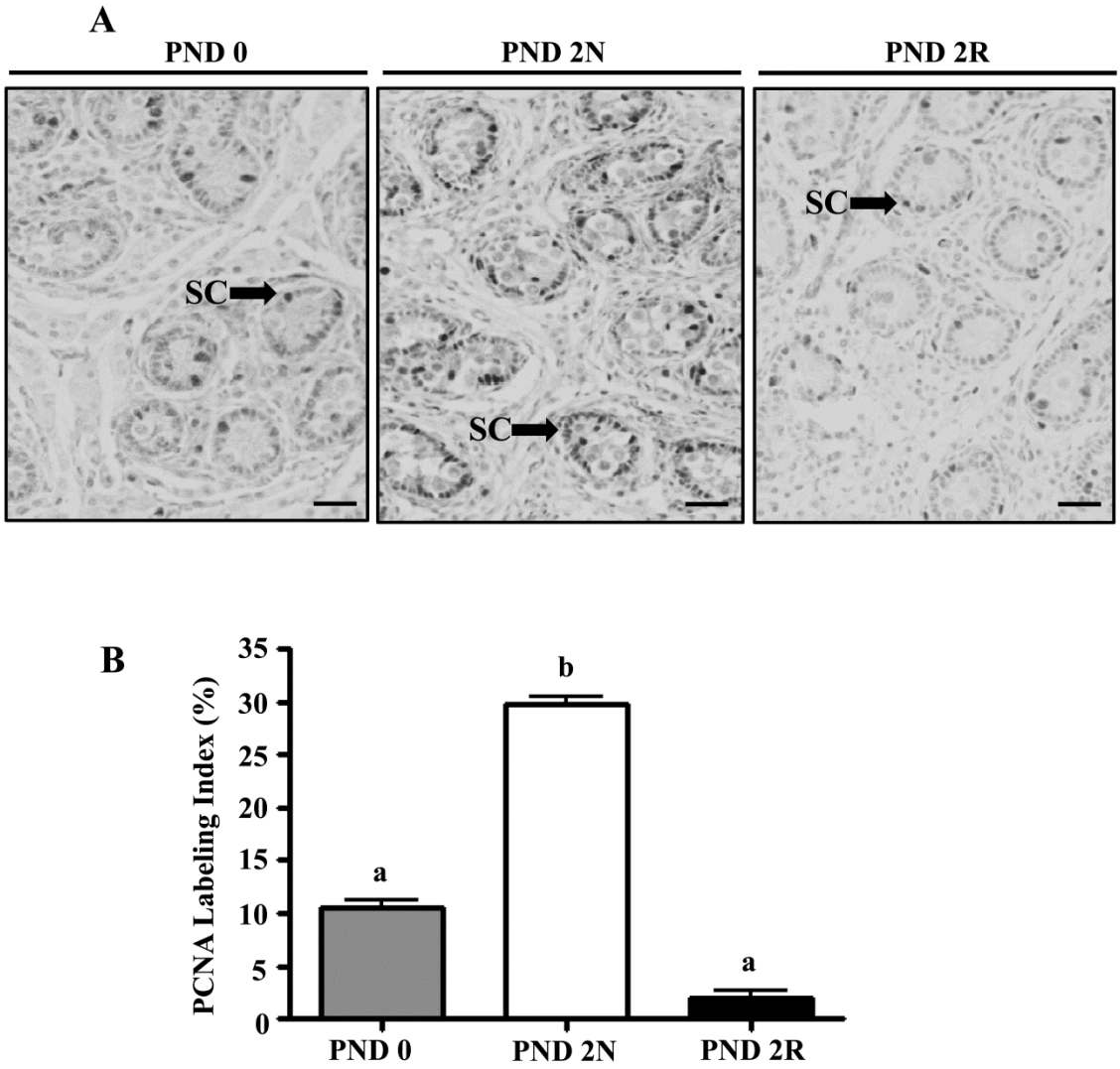

Figure 3. Effects of age and nursing on neonatal Sertoli cell PCNA immunostaining and PCNA LI. (A) Representative PCNA immunostaing of testicular sections from PND 0, PND 2N (nursed) and PND 2R (replacer-fed) are shown. Bars indicate 32 μm. SC= Sertoli cell. (B) PCNA LI (%) data are presented as LSM $\pm$ S.E.M. Different letters indicate differences at $P < 0.01$.

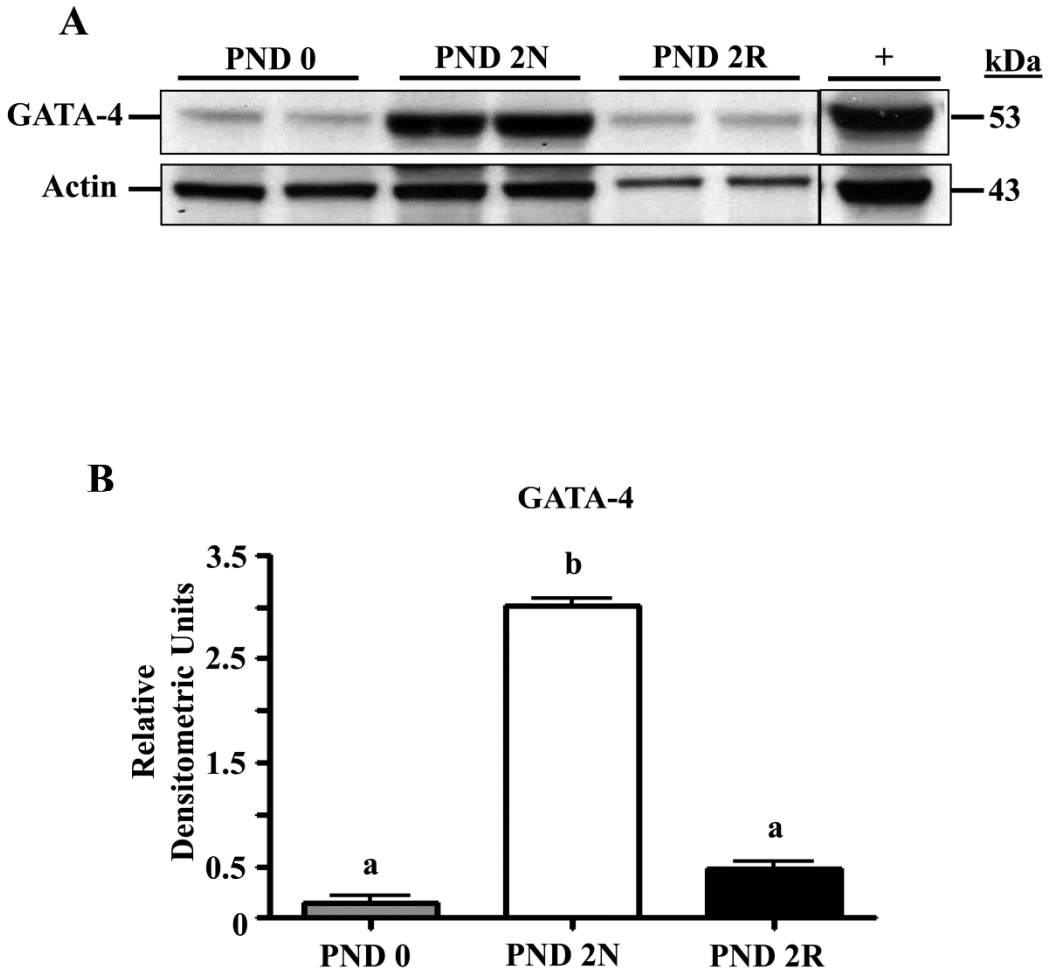

Figure 4. Effects of age and nursing on neonatal porcine testicular GATA-4 protein expression and Sertoli cell number. (A) Representative immunoblot for GATA-4 on PND 0, PND 2 N (nursed) and PND 2 R (replacer-fed) testicular protein. Positive control (+), adult pig ovary. (B) Densitometric data for relative expression of GATA-4 protein in relation to actin presented as LSM $\pm$ S.E.M. Different letters indicate differences at $P < 0.01$.

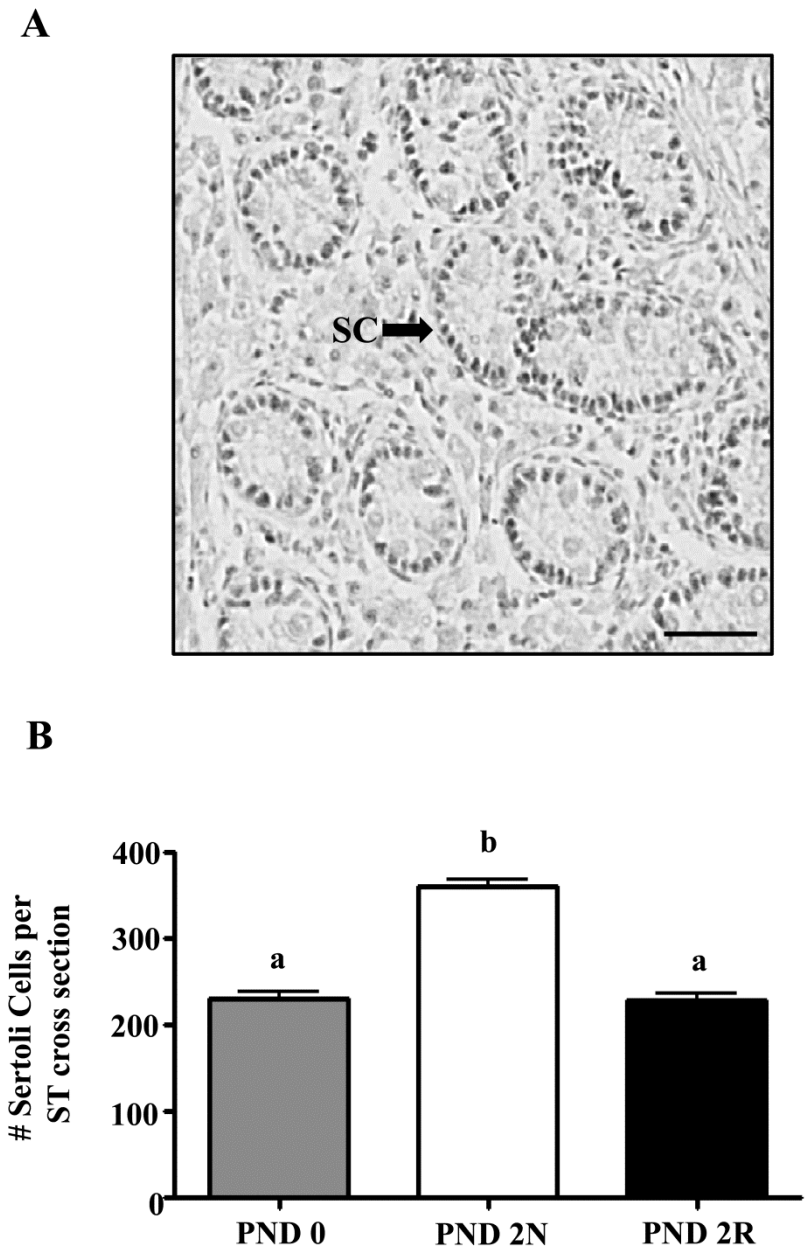

Figure 5. Effects of age and nursing on neonatal porcine Sertoli cell number. (A) Image showing GATA-4 immunostaining of Sertoli cell nuclei. Bar indicates 43 μm. SC= Sertoli cell. (B) Number of Sertoli cells per seminiferous tubule (ST) cross section presented as LSM $\pm$ S.E.M. Different letters indicate differences at $P < 0.01$.

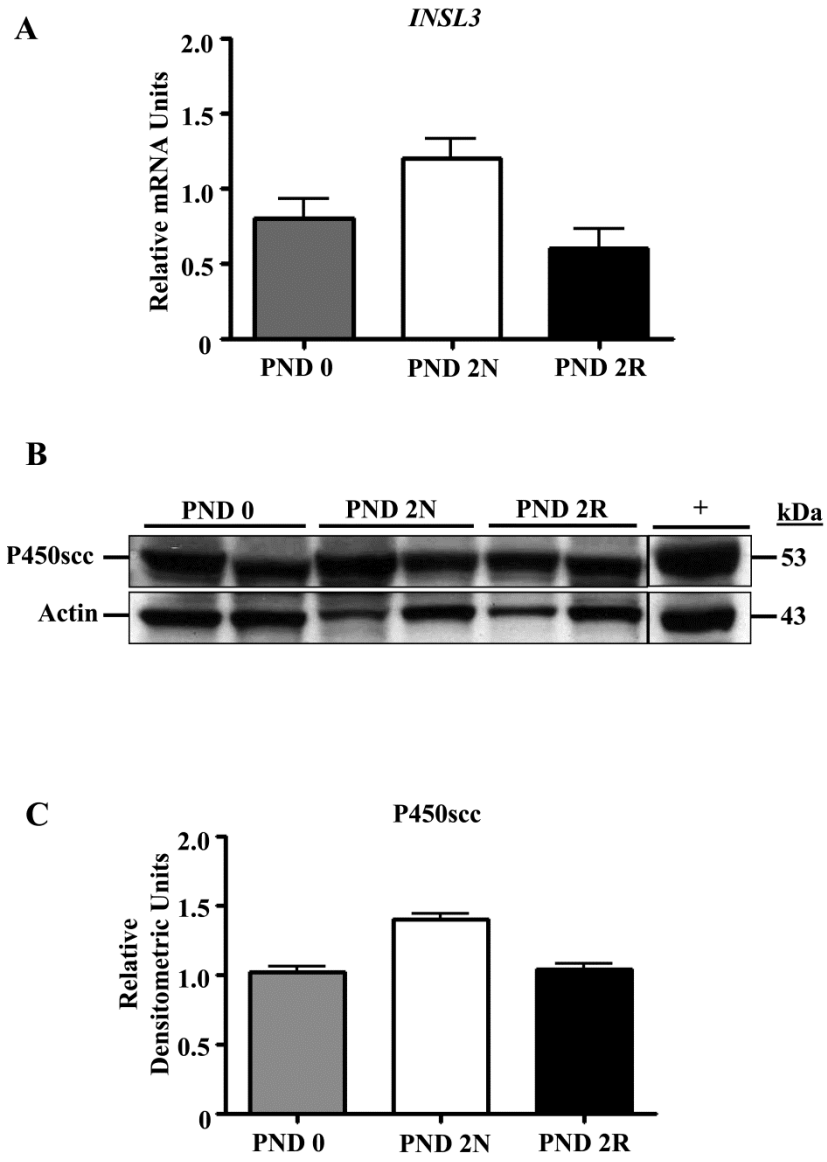

Figure 6. Effects of age and nursing on porcine testicular *INSL3* expression and P450scc protein. (A) Data for *INSL3* were normalized to *cyclophilin* expression and presented as LSM ± S.E.M. (B) Representative immunoblot for expression of P450scc is shown. Positive control (+), adult pig ovary. (C) Densitometric data for relative expression of P450scc in relation to actin presented as LSM ± S.E.M. No effects of age or nursing were identified for *INSL3* or P450scc expression.

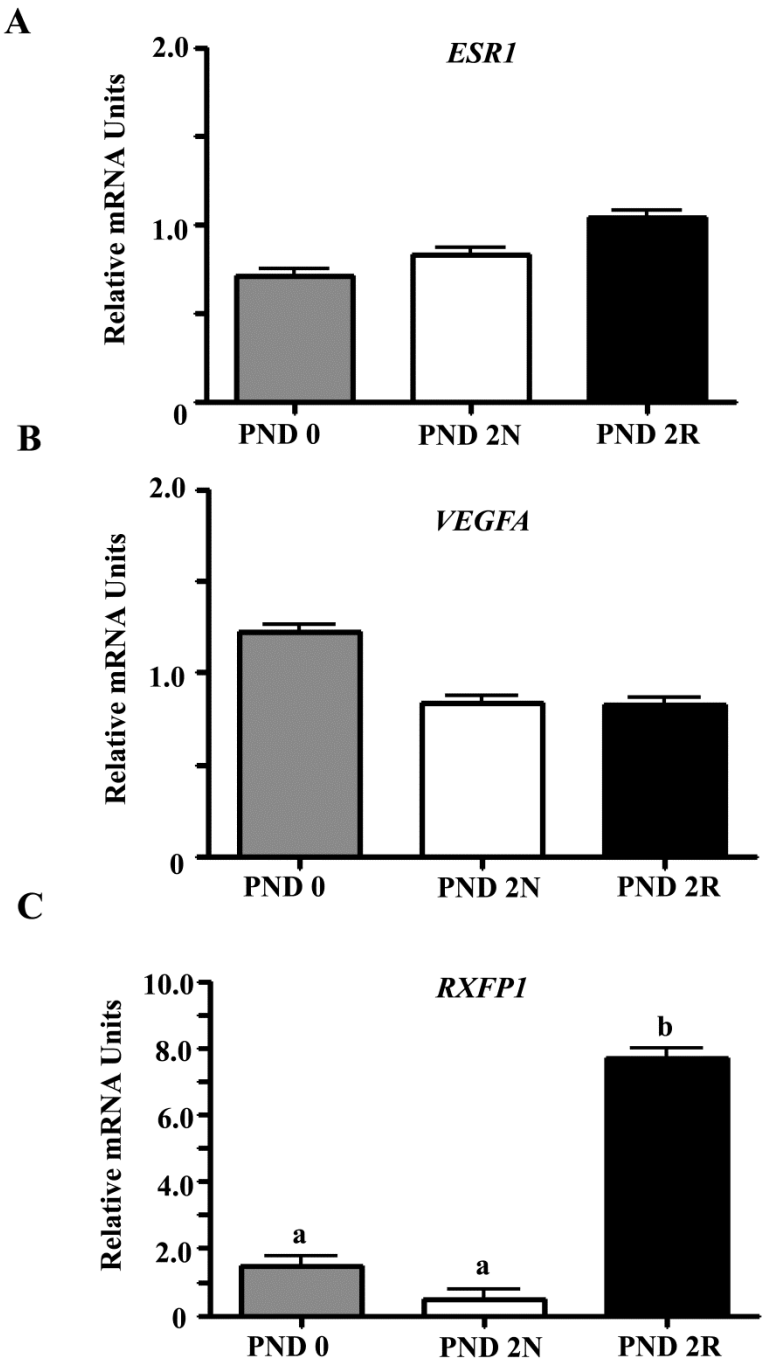

Figure 7. Effects of age and nursing on porcine testicular expression of: (A) *ESR1*, (B) *VEGFA*, and (C) *RXFP1* transcripts as determined by qPCR. Data were normalized to *cyclophilin* expression and are presented as LSM $\pm$ S.E.M. Different letters indicate differences at $P < 0.01$.

REFERENCES

Abercrombie, M 1946 Estimation of nuclear population from microtome sections. *Anat Rec* **94** 239-247.

Andersson, Y, ML Hammarstrom, B Lonnerdal, G Graverholt, H Falt, and O Hernell 2009 Formula feeding skews immune cell composition toward adaptive immunity compared to breastfeeding. *J Immunol* **183** 4322-4328.

At-Taras, EE, T Berger, MJ McCarthy, AJ Conley, BJ Nitta-Oda, and JF Roser 2006 Reducing estrogen synthesis in developing boars increases testis size and total sperm production. *J Androl* **27** 552-559.

Avelar, GF, CF Oliveira, JM Soares, IJ Silva, I Dobrinski, RA Hess, and LR Franca 2010 Postnatal somatic cell proliferation and seminiferous tubule maturation in pigs: a non-random event. *Theriogenology* **74** 11-23.

Bartol, FF, AA Wiley, and CA Bagnell 2008 Epigenetic programming of porcine endometrial function and the lactocrine hypothesis. *Reprod Domest Anim* **43 Suppl 2** 273-279.

Bartol, FF, AA Wiley, and CA Bagnell 2009 Relaxin and maternal lactocrine programming of neonatal uterine development. *Ann N Y Acad Sci* **1160** 158-163.

Bartol, FF, AA Wiley, DJ Miller, AJ Silva, KE Roberts, ML Davolt, JC Chen, AL Frankshun, ME Camp, KM Rahman, JL Vallet, and CA Bagnell 2013 Lactation Biology Symposium: lactocrine signaling and developmental programming. *J Anim Sci* **91** 696-705.

Bay, K, HE Virtanen, S Hartung, R Ivell, KM Main, NE Skakkebaek, AM Andersson, and J Toppari 2007 Insulin-like factor 3 levels in cord blood and serum from children: effects of age, postnatal hypothalamic-pituitary-gonadal axis activation, and cryptorchidism. *J Clin Endocrinol Metab* **92** 4020-4027.

Berndtson, WE, G Igboeli, and WG Parker 1987 The numbers of Sertoli cells in mature Holstein bulls and their relationship to quantitative aspects of spermatogenesis. *Biol Reprod* **37** 60-67.

Bosse, T, CM Piaseckyj, E Burghard, JJ Fialkovich, S Rajagopal, WT Pu, and SD Krasinski 2006 Gata4 is essential for the maintenance of jejunal-ileal identities in the adult mouse small intestine. *Mol Cell Biol* **26** 9060-9070.

Bott, RC, RM McFee, DT Clopton, C Toombs, and AS Cupp 2006 Vascular endothelial growth factor and kinase domain region receptor are involved in both seminiferous cord formation and vascular development during testis morphogenesis in the rat. *Biol Reprod* **75** 56-67.

Caires, KC, J de Avila, and DJ McLean 2009 Vascular endothelial growth factor regulates germ cell survival during establishment of spermatogenesis in the bovine testis. *Reproduction* **138** 667-677.

Cardoso, LC, AR Nascimento, C Royer, CS Porto, and MF Lazari 2010 Locally produced relaxin may affect testis and vas deferens function in rats. *Reproduction* **139** 185-196.

Chen, JC, AL Frankshun, AA Wiley, DJ Miller, KA Welch, TY Ho, FF Bartol, and CA Bagnell 2011 Milk-borne lactocrine-acting factors affect gene expression patterns in the developing neonatal porcine uterus. *Reproduction* **141** 675-683.

Choi, I, JY Kim, EJ Lee, YY Kim, CS Chung, J Chang, NJ Choi, HJ Chung, and KH Lee 2009 Ontogeny of expression and localization of steroidogenic enzymes in the neonatal and prepubertal pig testes. *J Androl* **30** 57-74.

Franca, LR, VA Silva, Jr., H Chiarini-Garcia, SK Garcia, and L Debeljuk 2000 Cell proliferation and hormonal changes during postnatal development of the testis in the pig. *Biol Reprod* **63** 1629-1636.

Frankshun, AL, TY Ho, DC Reimer, J Chen, S Lasano, BG Steinetz, FF Bartol, and CA Bagnell 2010 Characterization and biological activity of relaxin in porcine milk. *Reproduction* **141** 373-380.

Gilchrist, JM, MB Moore, A Andres, JA Estroff, and TM Badger 2010 Ultrasonographic patterns of reproductive organs in infants fed soy formula: comparisons to infants fed breast milk and milk formula. *J Pediatr* **156** 215-220.

Gillio-Meina, C, YY Hui, and HA LaVoie 2003 GATA-4 and GATA-6 transcription factors: expression, immunohistochemical localization, and possible function in the porcine ovary. *Biol Reprod* **68** 412-422.

Gould, ML, PR Hurst, and HD Nicholson 2007 The effects of oestrogen receptors alpha and beta on testicular cell number and steroidogenesis in mice. *Reproduction* **134** 271-279.

Hess, RA 2003 Estrogen in the adult male reproductive tract: a review. *Reprod Biol Endocrinol* **1** 52.

Iatropoulos, MJ, and GM Williams 1996 Proliferation markers. *Exp Toxicol Pathol* **48** 175-181.

Ivell, R, JD Wade, and R Anand-Ivell 2013 INSL3 as a biomarker of Leydig cell functionality. *Biol Reprod* **88** 147.

Kato, S, Siqin, I Minagawa, T Aoshima, D Sagata, H Konishi, K Yogo, T Kawarasaki, H Sasada, H Tomogane, and T Kohsaka 2010 Evidence for expression of relaxin hormone-receptor system in the boar testis. *J Endocrinol* **207** 135-149.

Kluin, PM, MF Kramer, and DG de Rooij 1984 Proliferation of spermatogonia and Sertoli cells in maturing mice. *Anat Embryol (Berl)* **169** 73-78.

Kozlowska, A, M Majewski, and B Jana 2009 Expression of steroidogenic enzymes in porcine polycystic ovaries. *Folia Histochem Cytobiol* **47** 257-264.

Krajnc-Franken, MA, AJ van Disseldorp, JE Koenders, S Mosselman, M van Duin, and JA Gossen 2004 Impaired nipple development and parturition in LGR7 knockout mice. *Mol Cell Biol* **24** 687-696.

Kwek, JH, RD Iongh, MR Digby, MB Renfree, KR Nicholas, and M Familari 2009 Cross-fostering of the tammar wallaby (Macropus eugenii) pouch young accelerates fore-stomach maturation. *Mech Dev* **126** 449-463.

Lee, K, W Lee, J Kim, M Yoon, N Kim, J Kim, S Uhm, D Kim, H Chung, and H Song 2013 Characterization of GFRalpha-1-Positive and GFRalpha-1-Negative Spermatogonia in Neonatal Pig Testis. *Reprod Domest Anim*.

Livak, KJ, and TD Schmittgen 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25** 402-408.

Masters, RA, BD Crean, W Yan, AG Moss, PL Ryan, AA Wiley, CA Bagnell, and FF Bartol 2007 Neonatal porcine endometrial development and epithelial proliferation affected by age and exposure to estrogen and relaxin. *Domest Anim Endocrinol* **33** 335-346.

McCoard, SA, DD Lunstra, TH Wise, and JJ Ford 2001a Specific staining of Sertoli cell nuclei and evaluation of Sertoli cell number and proliferative activity in Meishan and White Composite boars during the neonatal period. *Biol Reprod* **64** 689-695.

McCoard, SA, TH Wise, SC Fahrenkrug, and JJ Ford 2001b Temporal and spatial localization patterns of Gata4 during porcine gonadogenesis. *Biol Reprod* **65** 366-374.

McGlone, J, and J Swanson 2010 Guide for the Care and Use of Agricultural Animals in Research and Teaching. edn 3rd. Champaign, IL: Federation of Animal Science Societies.

Miller, DJ, AA Wiley, JC Chen, CA Bagnell, and FF Bartol 2013 Nursing for 48 hours from birth supports porcine uterine gland development and endometrial cell-compartment specific gene expression. *Biol Reprod* **88** 4.

Minagawa, I, M Fukuda, H Ishige, H Kohriki, M Shibata, EY Park, T Kawarasaki, and T Kohsaka 2012 Relaxin-like factor (RLF)/insulin-like peptide 3 (INSL3) is secreted from testicular Leydig cells as a monomeric protein comprising three domains B-C-A with full biological activity in boars. *Biochem J* **441** 265-273.

Neville, MC, SM Anderson, JL McManaman, TM Badger, M Bunik, N Contractor, T Crume, D Dabelea, SM Donovan, N Forman, DN Frank, JE Friedman, JB German, A Goldman, D Hadsell, M Hambidge, K Hinde, ND Horseman, RC Hovey, E Janoff, NF Krebs, CB Lebrilla, DG Lemay, PS MacLean, P Meier, AL Morrow, J Neu, LA Nommsen-Rivers, DJ Raiten, M Rijnkels, V Seewaldt, BD Shur, J VanHouten, and P Williamson 2012 Lactation and

neonatal nutrition: defining and refining the critical questions. *J Mammary Gland Biol Neoplasia* **17** 167-188.

Nolte, T, W Kaufmann, F Schorsch, T Soames, and E Weber 2005 Standardized assessment of cell proliferation: the approach of the RITA-CEPA working group. *Exp Toxicol Pathol* **57** 91-103.

Nusser, KD, and S Frawley 1997 Depriving neonatal rats of milk from early lactation has long-term consequences on mammotrope development. *Endocrine* **7** 319-323.

Orkin, SH 1992 GATA-binding transcription factors in hematopoietic cells. *Blood* **80** 575-581.

Orth, JM 1982 Proliferation of Sertoli cells in fetal and postnatal rats: a quantitative autoradiographic study. *Anat Rec* **203** 485-492.

Playford, RJ, CE Macdonald, and WS Johnson 2000 Colostrum and milk-derived peptide growth factors for the treatment of gastrointestinal disorders. *Am J Clin Nutr* **72** 5-14.

Raeside, JI, RM Friendship, and OE Vrablic 1997 Effects of castration on early postnatal development of male accessory sex glands in the domestic pig. *Eur J Endocrinol* **137** 287-292.

Ramesh, R, CA Pearl, E At-Taras, JF Roser, and T Berger 2007 Ontogeny of androgen and estrogen receptor expression in porcine testis: effect of reducing testicular estrogen synthesis. *Anim Reprod Sci* **102** 286-299.

Rehfeldt, C, and G Kuhn 2006 Consequences of birth weight for postnatal growth performance and carcass quality in pigs as related to myogenesis. *J Anim Sci* **84 Suppl** E113-123.

Russell, LD, J Warren, L Debeljuk, LL Richardson, PL Mahar, KG Waymire, SP Amy, AJ Ross, and GR MacGregor 2001 Spermatogenesis in Bclw-deficient mice. *Biol Reprod* **65** 318-332.

Samuel, CS, H Tian, L Zhao, and EP Amento 2003 Relaxin is a key mediator of prostate growth and male reproductive tract development. *Lab Invest* **83** 1055-1067.

SAS 2009-2010 *SAS/STAT User's Guide*. *Cary, NC: SAS Institute Inc.*

Van Straaten, HW, and CJ Wensing 1978 Leydig cell development in the testis of the pig. *Biol Reprod* **18** 86-93.

Wreford, NG 1995 Theory and practice of stereological techniques applied to the estimation of cell number and nuclear volume in the testis. *Microsc Res Tech* **32** 423-436.

Yagi, M, M Takenaka, K Suzuki, and H Suzuki 2007 Reduced mitotic activity and increased apoptosis of fetal sertoli cells in rat hypogonadic (hgn/hgn) testes. *J Reprod Dev* **53** 581-589.

Yan, W, J Chen, AA Wiley, BD Crean-Harris, FF Bartol, and CA Bagnell 2008 Relaxin (RLX) and estrogen affect estrogen receptor alpha, vascular endothelial

growth factor, and RLX receptor expression in the neonatal porcine uterus and cervix. *Reproduction* **135** 705-712.

Yan, W, AA Wiley, RA Bathgate, AL Frankshun, S Lasano, BD Crean, BG Steinetz, CA Bagnell, and FF Bartol 2006 Expression of LGR7 and LGR8 by neonatal porcine uterine tissues and transmission of milk-borne relaxin into the neonatal circulation by suckling. *Endocrinology* **147** 4303-4310.

CHAPTER V

DISSERTATION CONCLUSIONS

DISSERTATION CONCLUSIONS

Milk-borne bioactive factors are delivered to nursing offspring via a lactocrine mechanism to affect development of somatic tissues, including the neonatal porcine uterus (Miller *et al.* 2013). Results of experiments described in this dissertation extend the scope of the lactocrine hypothesis to: (1) include effects of the lactocrine-null state on global uterine gene expression; (2) provide preliminary evidence implicating IGF-I as a lactocrine-acting factor; and (3) include effects of the lactocrine-null state on aspects of neonatal porcine testicular development. Results provide a foundation upon which future studies aimed at testing the lactocrine hypothesis can be built.

Miller and colleagues (2013) reported that imposition of the lactocrine-null state from birth was associated with cell compartment-specific changes in expression patterns for ESR1 and other genes implicated in endometrial adenogenesis that were evident as early as PND 2. Results of the study outlined in Chapter II provide support for the lactocrine hypothesis and revealed hundreds of novel, differentially expressed genes and pathways that may prove to be important regulators of neonatal porcine uterine development. Further, data provide compelling evidence that maternal effects on tissue development extend into neonatal life via a lactocrine mechanism. The role of early nutrition and its effects on developmental programming is receiving increasing attention (Khan *et al.* 2005, Neville *et al.* 2012, Liu *et al.* 2014). Data presented here provide the first evidence of global, lactocrine-driven effects on the neonatal uterine transcriptome. Results will call attention to the importance of both early nutrition and lactocrine signaling on developmental programming of neonatal tissues.

Previous studies established RLX as a prototypical, lactocrine-acting hormone in the neonatal pig (Yan *et al.* 2006, Frankshun *et al.* 2010). However, it is well known that many other potentially bioactive factors are found in milk (Grosvenor 1992, Donovan and Odle 1994, Playford *et al.* 2000), including IGF-I (Simmen *et al.* 1988). Data presented in Chapter III showed that positive effects of a single dose of 0 h lactation colostrum on uterine epithelial and stromal cell proliferation were detectable at 12 h after birth. This observation is important because it established that lactocrine-effects on neonatal uterine development occur rapidly. Practically, this single observation can be used to frame a bioassay protocol for identification of lactocrine-active MbFs that can be completed within 12 h.

Results indicating that oral IGF-I increased endometrial cell proliferation only when administered in milk replacer was both unexpected and intriguing. This observation indicates that MbF bioavailability and/or bioactivity may be regulated by factors unique to colostrum that are yet to be identified. Amendments to human infant formulas (Hernell 2011), designed to enhance development of the newborn (Tai *et al.* 2013), include addition of bioactive factors such as docosahexaenic acid (Bentley *et al.* 2008) and arachidonic acid. Such additives may be particularly important, especially in premature infants. This is supported by evidence suggesting that DHA supplementation in formula fed premature infants improved neurologic development (Lapillonne *et al.* 2013). Present results support the idea that composition of the liquid medium (formula) should be considered carefully when designing infant formulas in order to optimize bioavailability of important MbFs.

To date, studies testing the lactocrine hypothesis have focused on female reproductive tract development (Chen *et al.* 2010, Frankshun *et al.* 2012, Miller *et al.* 2013). However, whether male reproductive tissues are affected by nursing had not been investigated until now. Research presented in Chapter IV provides evidence indicating that neonatal porcine testicular development is sensitive to lactocrine signaling by PND 2. More specifically, Sertoli cell proliferation and number were increased in nursed compared to newborn and replacer-fed boars. This is particularly important considering that Sertoli cell number dictates testis size and capacity to produce sperm later in life (McCoard *et al.* 2001). If these effects persist into adulthood, it may well be that nursed boars demonstrate greater reproductive capacity compared to replacer-fed littermates. Given that the commercial swine industry is substantially dependent upon artificial insemination (Robinson and Buhr 2005), results of research described here have great potential to impact commercial pig production. Currently, boars are selected for growth and carcass characteristics, however it is semen quantity and quality that affect the number of inseminations that can be obtained from a single ejaculate (Flowers 2008). Present results indicate that colostrum intake has the potential to be an important effector of semen quality and breeding soundness later in life. It will be important to determine the extent to which colostrum consumption affects semen quality, and whether husbandry guidelines for development of male breeding stock should include assessment of neonatal colostrum consumption.

Future studies should focus on analysis of lactocrine-sensitive biological processes and pathways affected by nursing in the neonatal porcine uterus as revealed using RNAseq. Results described herein also implicate IGF-I as a potential lactocrine-

active factor affecting uterine development. To confirm this, additional studies should be designed to determine if IGF-I: (1) is detectable in the circulation of nursed gilts at higher concentrations compared to animals fed IGF-I free milk replacer; (2) has receptors present in the neonatal porcine uterus; and (3) is lactocrine active in the neonate. The shorter, more efficient 12 h postnatal bioassay establish here should facilitate evaluation of different fractions of colostrum in order to identify MbFs that support porcine neonatal reproductive tract development. Finally, whether the alterations in testicular development identified at PND 2 in lactocrine-null piglets persist into adulthood and affect breeding soundness in boars is unknown and deserves attention.

Taken together, results presented in this dissertation provide additional support for the lactocrine hypothesis, providing clear evidence of maternal lactocrine-driven effects on reproductive development in the neonatal pig. Results indicate that nursing from birth has a global effect on the neonatal porcine uterine transcriptome by PND 2 and set the stage for additional studies designed to define which MbFs are responsible for developmental changes observed in response to nursing. Furthermore, results have expanded the scope of the lactocrine hypothesis to include males as well as females. Considering that all mammals nurse their young these and future studies will serve to underscore the importance and elucidate the mechanism of lactocrine signaling in support of reproductive development.

REFERENCES

Bentley, DR, S Balasubramanian, HP Swerdlow, GP Smith, J Milton, CG Brown, KP Hall, DJ Evers, CL Barnes, HR Bignell, JM Boutell, J Bryant, RJ Carter, R Keira Cheetham, AJ Cox, DJ Ellis, MR Flatbush, NA Gormley, SJ Humphray, LJ Irving, MS Karbelashvili, SM Kirk, H Li, X Liu, KS Maisinger, LJ Murray, B Obradovic, T Ost, ML Parkinson, MR Pratt, IM Rasolonjatovo, MT Reed, R Rigatti, C Rodighiero, MT Ross, A Sabot, SV Sankar, A Scally, GP Schroth, ME Smith, VP Smith, A Spiridou, PE Torrance, SS Tzonev, EH Vermaas, K Walter, X Wu, L Zhang, MD Alam, C Anastasi, IC Aniebo, DM Bailey, IR Bancarz, S Banerjee, SG Barbour, PA Baybayan, VA Benoit, KF Benson, C Bevis, PJ Black, A Boodhun, JS Brennan, JA Bridgham, RC Brown, AA Brown, DH Buermann, AA Bundu, JC Burrows, NP Carter, N Castillo, ECM Chiara, S Chang, R Neil Cooley, NR Crake, OO Dada, KD Diakoumakos, B Dominguez-Fernandez, DJ Earnshaw, UC Egbujor, DW Elmore, SS Etchin, MR Ewan, M Fedurco, LJ Fraser, KV Fuentes Fajardo, W Scott Furey, D George, KJ Gietzen, CP Goddard, GS Golda, PA Granieri, DE Green, DL Gustafson, NF Hansen, K Harnish, CD Haudenschild, NI Heyer, MM Hims, JT Ho, AM Horgan, et al. 2008 Accurate whole human genome sequencing using reversible terminator chemistry. *Nature* **456** 53-59.

Chen, JC, A-L Frankshun, KA Welch, KM Ferio, AA Wiley, T-Y Ho, FF Bartol, and CA Bagnell 2010 Lactocrine Signaling Supports Matrix Metalloproteinase (MMP) 9 Expression in the Neonatal Porcine Uterus. *Biology of Reproduction* **83** 169.

Donovan, SM, and J Odle 1994 Growth factors in milk as mediators of infant development. *Annu Rev Nutr* **14** 147-167.

Flowers, WL 2008 Genetic and phenotypic variation in reproductive traits of AI boars. *Theriogenology* **70** 1297-1303.

Frankshun, AL, J Chen, LA Barron, TY Ho, DJ Miller, KM Rahman, FF Bartol, and CA Bagnell 2012 Nursing during the first two days of life is essential for the expression of proteins important for growth and remodeling of the neonatal porcine cervix. *Endocrinology* **153** 4511-4521.

Frankshun, AL, TY Ho, DC Reimer, J Chen, S Lasano, BG Steinetz, FF Bartol, and CA Bagnell 2010 Characterization and biological activity of relaxin in porcine milk. *Reproduction* **141** 373-380.

Grosvenor, CE, Picciano, M.R., and Baumrucker, C.R. 1992 Hormones and Growth Factors in Milk. *Endocrine Reviews* **14** 710-728.

Hernell, O 2011 Human milk vs. cow's milk and the evolution of infant formulas. *Nestle Nutr Workshop Ser Pediatr Program* **67** 17-28.

Khan, IY, V Dekou, G Douglas, R Jensen, MA Hanson, L Poston, and PD Taylor 2005 A high-fat diet during rat pregnancy or suckling induces cardiovascular dysfunction in adult offspring. *Am J Physiol Regul Integr Comp Physiol* **288** R127-133.

Lapillonne, A, S Groh-Wargo, CH Gonzalez, and R Uauy 2013 Lipid needs of preterm infants: updated recommendations. *J Pediatr* **162** S37-47.

Liu, B, B Zupan, E Laird, S Klein, G Gleason, M Bozinoski, J Gal Toth, and M Toth 2014 Maternal hematopoietic TNF, via milk chemokines, programs hippocampal development and memory. *Nat Neurosci* **17** 97-105.

McCoard, SA, DD Lunstra, TH Wise, and JJ Ford 2001 Specific staining of Sertoli cell nuclei and evaluation of Sertoli cell number and proliferative activity in Meishan and White Composite boars during the neonatal period. *Biol Reprod* **64** 689-695.

Miller, DJ, AA Wiley, JC Chen, CA Bagnell, and FF Bartol 2013 Nursing for 48 hours from birth supports porcine uterine gland development and endometrial cell-compartment specific gene expression. *Biol Reprod* **88** 4.

Neville, MC, SM Anderson, JL McManaman, TM Badger, M Bunik, N Contractor, T Crume, D Dabelea, SM Donovan, N Forman, DN Frank, JE Friedman, JB German, A Goldman, D Hadsell, M Hambidge, K Hinde, ND Horseman, RC Hovey, E Janoff, NF Krebs, CB Lebrilla, DG Lemay, PS MacLean, P Meier, AL Morrow, J Neu, LA Nommsen-Rivers, DJ Raiten, M Rijnkels, V Seewaldt, BD Shur, J VanHouten, and P Williamson 2012 Lactation and neonatal nutrition: defining and refining the critical questions. *J Mammary Gland Biol Neoplasia* **17** 167-188.

Playford, RJ, CE Macdonald, and WS Johnson 2000 Colostrum and milk-derived peptide growth factors for the treatment of gastrointestinal disorders. *Am J Clin Nutr* **72** 5-14.

Robinson, JA, and MM Buhr 2005 Impact of genetic selection on management of boar replacement. *Theriogenology* **63** 668-678.

Simmen, FA, RC Simmen, and G Reinhart 1988 Maternal and neonatal somatomedin C/insulin-like growth factor-I (IGF-I) and IGF binding proteins during early lactation in the pig. *Dev Biol* **130** 16-27.

Tai, EK, XB Wang, and ZY Chen 2013 An update on adding docosahexaenoic acid (DHA) and arachidonic acid (AA) to baby formula. *Food Funct* **4** 1767-1775.

Yan, W, AA Wiley, RA Bathgate, AL Frankshun, S Lasano, BD Crean, BG Steinetz, CA Bagnell, and FF Bartol 2006 Expression of LGR7 and LGR8 by neonatal porcine uterine tissues and transmission of milk-borne relaxin into the neonatal circulation by suckling. *Endocrinology* **147** 4303-4310.