LACTOCRINE SIGNALING AND NEONATAL PORCINE REPRODUCTIVE TRACT DEVELOPMENT

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ABSTRACT OF THE DISSERTATION

Lactocrine Signaling and Neonatal Porcine

Reproductive Tract Development

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Nursing supports neonatal porcine reproductive tract development by delivering milk-borne bioactive factors (MbFs) from mother to offspring as proposed in the lactocrine hypothesis. In pigs, studies showed that age and nursing support postnatal uterine and testicular development between birth [postnatal day (PND) 0] and PND 2 via a lactocrine mechanism and define the developmental program. However, age-sensitive events associated with development of the neonatal porcine uterine transcriptome have not been defined during this period. Additionally, the extent to which nursing affects the development of the porcine cervix is unknown. Furthermore, the extent to which MbFs, particularly insulin-like growth factor-1 (IGF1), affect development of the porcine cervix remains to be determined. Research aims were to (1) define the age-sensitive uterine transcriptome in gilts at birth compared to animals at PND 2; (2) determine the effects of age and nursing on porcine cervical histoarchitecture and cell proliferation; and (3) determine whether a single feeding of colostrum or milk replacer at birth, with or without oral IGF1 supplementation, supports cervical cell proliferation and development using a

new 12 h neonatal pig bioassay. Results showed that in uteri of PND 2 as compared to PND 0 gilts, 3283 genes were differentially expressed and multiple age-sensitive biological processes and pathways, including 'immune response' and 'Wnt-β-catenin signaling', were affected. Additionally, results indicated that both age and nursing supported cervical development histologically by PND 14 and cell proliferation by PND 2 and 14. Furthermore, oral IGF1 increased cervical cell proliferation when administered in milk replacer, but not with colostrum. However, IGF1 supplementation in either colostrum or milk replacer increased cervical IGF1 signaling cascade markers B-cell lymphoma 2 and phospho-AKT compared to gilts fed colostrum or milk replacer alone. In conclusion, the global changes in uterine gene expression identified here within the first 48 h after birth provide a foundation for future studies to better understand the mechanisms and pathways governing FRT development. Collectively, results presented here reinforce and extend previous findings that both age and lactocrine signaling are effectors of organizationally critical structural changes in developing female reproductive tissues with potential to determine reproductive capacity in adulthood.

DEDICATION

To My Family…

"Families are the compass that guides us.

They are the inspiration to reach great heights,

and our comfort when we occasionally falter."

I dedicate this work to you.

Thank you for your love, encouragement, and support.

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TABLE OF CONTENTS

LIST OF TABLES

Chapter II

LIST OF FIGURES

CHAPTER I

Review of Literature

A. The purpose and scope of the literature review

The aim of this research was to understand the effects of lactocrine signaling on neonatal porcine reproductive tract development. Colostrum, or first milk, provides nutrition, immunoglobulins, hormones, and other milk-borne bioactive factors (MbFs) that have the ability to influence and define the developmental program and trajectory of neonatal tissues. The importance of colostrum for neonatal nutrition and passive immunity is well defined. However, the influence of nursing and delivery of non-nutritive bioactive factors by way of colostrum on neonatal development, including the reproductive tract, is unclear. Therefore, the purpose of this literature review is to introduce the concept of developmental programming and maternal influence on neonatal development. The idea that milk serves as a conduit for delivery of MbFs and evidence in support of the lactocrine hypothesis are also reviewed. The importance of maturation of the postnatal gastrointestinal (GI) tract for absorption of MbFs, as well as porcine neonatal cervical and uterine development will be presented. Further, rationale for using next generation/high throughput RNA sequencing (RNAseq) technology to identify novel age-sensitive uterine gene expression events will be discussed. Literature in support of insulin-like growth factor 1 (IGF1) in milk and local effects on neonatal GI tract development will be reviewed. Finally, whether oral IGF1 is lactocrine-active targeting distant neonatal somatic tissues will be discussed. Given that all mammals evolved to nurse, understanding the role of lactocrine signaling for development of neonatal reproductive tissues is important with potential for long-term reproductive performance and fertility.

B. Developmental Programming

The influences of both genetic programming and environmental factors on tissue development affect resulting phenotypes. Developmental programming involves a sequence of events for establishing a developmental trajectory that ultimately dictates a particular tissue phenotype (Burggren 1999). Programming is centered upon the idea that environmental factors can influence developmental pathways during time periods where tissues are still differentiating and exhibiting plasticity (E.G. De Moura 2008). Disruptions, environmental or chemical, during normal tissue programming in early life may lead to an abnormal developmental trajectory with consequences on adult phenotype (Bartol *et al.* 2008). Environmental cues during early life may be sufficient to severely disrupt development which may lead to physiological abnormalities or more subtle changes that alter tissue function. Additionally, hormones that are important for developmental programming can induce alterations in the neonatal reproductive tract to permanently alter structure, physiology, and function (Plagemann *et al.* 2005).

In 1986 David Barker suggested a relationship between early life environmental cues and risk of disease in adulthood (Barker & Osmond 1986). Specifically, they correlated maternal under nutrition and low birth weight with ischaemic heart disease in adulthood (Barker 1989, Barker 1993) The term 'Developmental Origins of Health and Disease (DOHaD)' was coined based on Barker's hypothesis (Barker *et al.* 1989), stating that the incidence of specific adult diseases, including type II diabetes, may be linked to development *in utero* and postnatally (Silveira *et al.* 2007). Early descriptions and follow-up studies from the Dutch hunger winter famine (1944-1945), during the World War II German blockade of Holland, served as a foundation for DOHaD. During that winter, individuals in the Netherlands were restricted to 500-1000 kCal/day resulting in

thousands of deaths from starvation, particularly in women and children (Lumey *et al.* 2007). Researchers that followed-up on children born during the famine found evidence that maternal under nutrition was associated with schizophrenia (Susser & Lin 1992), elevated cholesterol (Lumey & Stein 2009)and body mass index (Roseboom *et al.* 2000), as well as impaired glucose tolerance (Ravelli *et al.* 1998).

Subsequently, in addition to epidemiological studies in humans, animal models have been employed to study DOHaD (Ganu *et al.* 2012). Rodent models have proven ideal for generational studies due to fast generation time, large litters, and easy handling (Ganu *et al.* 2012). However, physiological differences between humans and rodents have led to more studies in non-human primates (NHP) that share similar genomic relationships with humans (Raaum *et al.* 2005). For example, NHP model studies show a relationship between maternal nutrient restriction and increased fasting glucose, insulin, and β cell responsiveness in juvenile offspring (Choi *et al*., 2011). However, maternal influence on the developing neonate does not conclude at parturition, rather, it is extended through lactation by ingestion of colostrum and milk by the neonate (Bagnell *et al.* 2009). For example, in humans, Duazo and colleagues found a correlation between increased breastfeeding duration and increased adolescent psychosocial development (Duazo *et al.* 2010). Furthermore, breastfed newborns gain weight at a slower rate compared to formula-fed newborns (Boullo-Ciocca 2005, Kramer MS 2008) and have decreased risk of obesity and metabolic syndrome in adulthood (Arenz *et al.* 2004, Boullo-Ciocca 2005).

Paralleling the time period of the emergence of DOHaD, another group of researchers studying the influence of milk-based diets fed to preterm newborns on immunity, growth,

and neurophyschomotor development (Lucas 1991) proposed the term ‘programming’ (Lucas 1991) that had been originally outlined in 1975 by Dörner and colleagues. Programming refers to a stimulus applied during a critical period of development that can have long-lasting effects on tissue structure or function (Lucas 1991). Specific to the female reproductive tract (FRT), targeted disruption of FRT development during periods of neonatal life can have lasting effects on form and function of adult reproductive tissues (Crain *et al.* 2008, McLachlan *et al.* 2012). In the pig (*Sus scrofa domesticus*), uterine responses to estradiol valerate administration for 14 days from birth altered endometrial function and compromised uterine capacity for conceptus support in adults (Tarleton *et al.* 2003). In sheep (Bartol *et al.* 1999, Gray *et al.* 2001) and mice (Cooke *et al.* 2012, Filant *et al.* 2012), strategic neonatal exposure to progestins inhibited uterine gland development and suppressed fertility in adults. These studies support the idea that sensitive time periods for uterine development in the postnatal period are susceptible to perturbations that can influence the developmental trajectory into adulthood.

C. Colostrum Composition in Relation to Passive Immunity

Lactation is a defining characteristic of mammals. Through lactation maternal influence on developing offspring is extended into the neonatal period. Aside from supplying nursing young with appropriate nutrients, another benefit of lactation is protection in the form of antibodies and antimicrobials (Peaker 2002). Newborn mammals encounter a harsh environment after birth and rely on passive immunization either by prenatal transfer of immunoglobulins by the placenta or through postnatal transport of immunoglobulins via colostrum (Bainter 2007). Prenatal transfer of maternal antibodies is regulated by the placental barrier between the mother and fetus. In humans,

immunoglobulins can pass the hemochorial placenta, specifically the syncytiotrophoblast barrier (Langer 2009a). However, in pigs and other ungulate species, including sheep and cows, transfer of immunoglobulins via the epitheliochorial placenta does not take place (Bainter 2007). After birth, ungulates and humans are able to absorb immunoglobulins through their open gut after colostrum ingestion (Lascelles 1979). Colostrum from ungulates is particularly rich in protein, most likely due to antibody content (Park 2007). In general, colostrum from species with increased postnatal transfer of immunoglobulins has greater protein content than mature milk from that species (Langer 2009b). In humans, the major colostral immunoglobulin present is IgA, whereas IgG is dominant in pigs, cows, goats, and horses (Butler 1973, Widdowson 1985). Taken together, these data suggest colostrum composition of immune factors depends largely on the route of passive immunity.

Colostrum and milk contain additional immunomodulatory factors that include cytokines and chemokines (Goldman 1993, Garofalo 2010). Cyotkines are small protein hormones (5-20 kDa) that operate in extensive networks to orchestrate the development of the immune system in neonates and regulate inflammatory responses (Garofalo 2010). Cytokines are produced from a variety of cells that include B and T lymphocytes, macrophages, endothelial cells, and stromal cells (Cannon 2000) and include chemokines, interleukins, and tumor necrosis factor (TNF; (Cannon 2000). Chemokines, small chemotactic cytokines, function through activation of leukocytes and are mediatiors of inflammation (Oppenheim 1991). These milk-borne immunomodulatory factors are reported to promote growth and differentiation of B cells (Juto 1985), support proliferation of thymocytes (Soder 1987) and play a role in immune defense against

bacterial infections (Garofalo 2010). Results suggest that colostrum contains important factors important for neonatal immune development.

D. Milk as a Conduit for Bioactive Factors and the Lactocrine Hypothesis

Milk is a dynamic fluid whose composition changes throughout lactation. Colostrum, defined as first milk, is produced following parturition and gradually matures into milk by 24-48 h of lactation in pigs (Klobasa *et al.* 1987). Concentrations of total solids and protein are high in porcine colostrum compared to milk, while fat and lactose levels are comparatively low (Klobasa *et al.* 1987, Ogawa *et al.* 2014). In pigs, the high concentration of protein in colostrum reflects the increased amount of immunoglobulins, especially IgG, responsible for providing passive immunity to the nursing neonate. As colostrum transitions into mature milk in pigs there is a decline in total protein while fat and lactose increase at around 24 h of lactation. Similarly, this transition is marked by high concentrations of IgG that decline 24-48 h after birth and IgA becomes the dominant immunoglobulin (Klobasa *et al.* 1987).

Not only does milk provide energy and passive immunity, but it also supports neonatal porcine development by providing a conduit for delivery of milk borne bioactive factors (MbFs) from mother to offspring as proposed in the lactocrine hypothesis (Yan *et al.* 2006, Bartol *et al.* 2008). Relaxin (RLX), a proto-typical milk-borne hormone, is present in porcine colostrum and milk (Yan *et al.* 2006). Studies by Yan et al., 2006 showed that 1) the highest concentration of milk-borne RLX was detected during the first 24 h of lactation followed by a steady decline through lactation by PND 14; 2) nursed gilts on PND 0 and 1 had elevated serum RLX levels that then declined to levels

undetectable by PND 2-14; 3) after birth and prior to nursing, RLX was undetectable in the circulation of gilts or in those fed a RLX-free milk replacer for 12 hours on PND 0. These data support the lactocrine hypothesis; however the complete array of lactocrine-active molecules present in colostrum/milk is unknown.

MbFs present in colostrum include immunoglobulins, antimicrobial proteins, growth factors, and essential hormones that can influence neonatal tissue growth and development. Data in support of the idea that colostrum ingestion can affect postnatal development involves a study of neonatal rodents that were colostrum-deprived (Nusser & Frawley 1997). The rats were allowed to nurse from mothers that had given birth 7 days prior; thus, they were deprived of early colostrum and ingested lactation day 7 milk. Nusser and Frawley observed delayed mammotrope development throughout the prepubertal period, decreased percentages of mammotropes, and modified secretory capacity of mammotropes in adulthood. They suggested the effects were due to altered actions of the anterior pituitary; specifically they demonstrated that depriving neonatal rats of early milk delayed the development of prolactin-releasing cells (Nusser & Frawley 1997). Another example of the importance of nursing for postnatal development comes from studies in marsupials that experience a long lactational phase during which the composition of milk changes to adapt to the needs of the growing neonate (Trott *et al.* 2003). Kwek and colleagues (2009) showed that cross fostering tammar wallabies at PND 120 to host mothers at lactation day 170 supported fore-stomach maturation. Results suggest milk from a later lactational phase can trigger events important for fore-stomach development. Other findings from this study showed that neonatal wallabies in the control group were smaller sized, less developed, and weighed less when compared to

the young in the cross-fostered group (Kwek *et al.* 2009). In a study by Liu and colleagues, murine offspring of heterozygous or homozygous TNFα -knockout mice had augmented memory in adulthood when compared with offspring of wild-type mice (Liu 2013). Surprisingly, when wild-type pups were cross fostered to TNFα-deficient dams, their memory was enhanced (Liu 2013). Additionally, milk analysis showed that several chemokines were decreased in mammary secretions of TNFα-deficient dams compared to controls. When neonates born to TNFα-deficient dams were supplemented with those chemokines, adulthood memory was impaired (Liu 2013). Together, these studies involving rodents and marsupials support the concept that lactocrine signaling can influence neonatal development.

E. Use of bioassays to determine biological activity

A bioassay, by definition, involves use of a live animal or plant (*in vivo*), or tissue, or cell (*in vitro*) to determine relative strength or biological activity of a substance including a drug, pollutant, or toxicant (van Noordwijk 1989). Whole animal bioassays were introduced and practiced by Paul Ehrlich as early as 1894 (Morris 1912, van Noordwijk 1989). Ehrlich, the founder of chemotherapy, proposed the idea that chemical substances could interact with receptors expressed in pathogens. In the early 20th century, Ehrlich used rabbits infected with the syphilis-producing microorganism *Treponema pallidum* as a bioassay to evaluate arsenic-based treatments for the disease (Morris 1912, Rubin 2007). Another well-known animal bioassay, employed from the 1930s to the early 1960s, was the rabbit or 'Friedman' pregnancy test (Wide & Gemzell 1960). In 1927, Friedman discovered that when urine from a pregnant woman, containing the pregnancy hormone human chorionic gonadotropin, was injected into a

female rabbit its ovaries developed corpora lutea and corpora hemorrhagica (Friedman 1929). The rabbit model was adapted from a pregnancy test developed by Aschheim and Zondek (Aschheim S. 1928, Evans & Simpson 1930) involving female mice. They showed that a hormone from one species, human chorionic gonadotropin, could generate a biological response in another species (mice) that was a marker for human pregnancy. Later, in the 1950s and 1960s, estrogen-primed mice were used in relaxin bioassays, whereby the length of the interpubic ligament was associated with dose-proportional increases of a single relaxin injection (Steinetz *et al.* 1960). In research presented here, the neonatal porcine cervix was used as a bioassay platform for identification of lactocrine activity in colostrum as reflected by patterns of cervical cell proliferation and related biomarker expression patterns.

F. Gut Closure Dynamics and Mechanisms

The porcine digestive tract is underdeveloped in the neonate (Laskowski 1957) such that large molecules can be absorbed by the small intestine (Hardy 1969). Neonatal intestinal cells have the ability to absorb macromolecules by endocytosis and transport them intact into circulation (Sangild 2003). Increased neonatal gut permeability is observed in a variety of mammals, including humans (Catassi 1995), rodents, and ungulates (Pacha 2000, Sangild 2003). The term 'gut closure' refers to the termination of the neonatal gut's ability to absorb macromolecules and the timing of gut closure is species-dependent (Pacha 2000). In ungulates, including the pig, gut closure occurs within the first few days after birth (Sangild 2003). A study in neonatal nursing pigs

showed that absorption of the non-protein macromolecule, polyvinylpyrrolidone (mean molecular weight 160000) ceases 24-36 h after birth (Leece 1962). Similarly, in humans, gut closure corresponded with increased infant age (Eastham *et al.* 1978, Catassi 1995) . Using several different intestinal permeability assays, researchers showed that infant gut permeability decreased within the first month (Catassi 1995) to three months of age (Eastham *et al.* 1978). However, GI maturation and timing of gut closure can also depend on nursing or formula feeding. For example, in pigs, formula feeding increased the rate of gut closure to as early as 12 h postnatal when compared to nursed pigs (Jensen 2001). Jensen assessed pig gut closure in response to the differing diets through measurements of intestinal growth, IgG absorptive capacity, and intestinal enzyme activity (Jensen 2001). In contrast, in human infants formula feeding delayed gut closure compared to breast-fed infants within the first week of life (Weaver 1987, Catassi 1995). Specifically, using a lactulose/mannitol intestinal permeability test, breast-fed infants had lower lactulose/mannitol intestinal permeability than formula-fed neonates at PND 7 (Catassi 1995) Taken together, results indicate gut closure testing methods are not universal and the dynamics of gut closure are species-dependent and largely unclear.

Transfer of macromolecules across the intestinal wall facilitates the uptake of a number of protein molecules such as immunoglobulins, growth factors, and many antigens including microorganisms. Transport of macromolecules is localized in enterocytes and M cells of the intestine and follows two different pathways 1) specific receptor-mediated transcytosis and 2) nonspecific transcytosis. In specific receptor-mediated transcytosis molecules bind to its receptor in the apical membrane; the complex is transported by endocytosis through the cell within transport vesicles, and secreted into

the contraluminal compartment. Nonselective endocytotic pathway allows massive absorption when intraluminal macromolecules are endocytosed and transported across enterocytes through vacuoles (Pacha 2000). The effective transport of ingested proteins is facilitated by decreased proteolytic degradation of proteins due to the presence of colostral protease inhibitors (Westrom 1985).

Regulation of intestinal transport function is largely dependent on tight junctional complexes connecting enterocytes together. Tight junctions selectively control the passive diffusion of ions and other small solutes and act as a barrier between the luminal and basolateral compartments. Tight junctions are dynamic structures and change readily to varieties of physiological conditions and thus permeability changes as well.

G. Neonatal Development of the Porcine Cervix

Adult porcine cervical development through pregnancy has been described in detail (Eldridge-White *et al.* 1989). The general organization of the adult pig cervix includes connective tissue stroma, collagen fiber bundles, fibroblasts, and blood vessels. Unlike the sheep, rat, and human cervix, smooth muscle is predominant throughout the length of the adult pig cervix (Winn *et al.* 1993). However, a description of organizational and developmental changes in the neonatal porcine cervix has not been reported. Only a few studies have focused on neonatal cervical development in any species (hamster; (Hendry *et al.* 2004); human (Danforth 1983). Hendry et al., 2004 showed that neonatal exposure of hamsters to estradiol-17β (E2), and to a greater extent to diethylstilbestrol (DES), an estrogenic endocrine disruptor, enhanced cervical growth as early as PND 3 and continued through PND 5 and PND 21. Histologically, the luminal

epithelium of the cervix was pseudostratified at two months of age in both the control and E2-treated animals. In the neonatally DES-treated group luminal epithelial height was increased while the luminal epithelium and stroma were disorganized and contained cavities with degenerating cells. These results show that E2 and DES exposure during the perinatal period disrupts cervical morphogenesis in the neonatal hamster cervix. Whether nursing influences the cervical histology of any species is unknown. Also, studies from our laboratory demonstrate that nursing from birth to PND 2 supports porcine cervical (Frankshun *et al.* 2012) and uterine (Chen *et al.* 2011) expression patterns of proteins including the RLX receptor RXFP1. Whether porcine cervical morphology is influenced by age and nursing in a manner similar to the changes in histoarchitecture reported for the uterus (Miller *et al.* 2013) is unknown.

The protein hormone RLX not only stimulates growth and remodeling of the porcine uterus, but also plays a role in development and connective tissue remodeling of the porcine cervix (Vasilenko 1987, Min 1997). Cervical and uterine growth is a result of cellular proliferation, as well as remodeling of the connective tissue of these organs, to facilitate expansion. Highly coordinated breakdown and rebuilding of the extracellular matrix is required for remodeling and involves matrix metalloproteinases (MMPs). MMPs are a large family of proteases that include the gelatinases, MMP2 and MMP9. The production of MMPs is under the influence of a variety of stimuli including cytokines, growth factors and hormones (Woessner 1991). MMP activity is regulated by tissue inhibitors of metalloproteinases (TIMPs) secreted from adjacent cells that produce MMPs. TIMPs form complexes with MMPs and inhibit activities of MMPs (Gomez 1997). Specifically, TIMPs have an N-terminal domain that folds like a wedge and

thereby is capable of interacting with the active-site cleft of an MMP (Fernandez-Catalan *et al.* 1998) Data from our laboratory focusing on MMP2 and MMP9 proteins in the cervix showed that MMP9, but not MMP2, is lactocrine-regulated in neonatal cervical tissues at PND 2 and PND 14 (Frankshun *et al.* 2012). Specifically, gilts that nursed from birth through PND 2 or PND 14 show cervical expression of MMP2 and MMP9. However, in gilts fed milk replacer over the same period only cervical MMP2 is detected (Frankshun *et al.* 2012). These data show that some markers of reproductive tract development are lactocrine-sensitive while others are not.

H. Neonatal Development of the Porcine Uterus

The porcine uterus begins developing prenatally and completes development postnatally (Bartol *et al.* 1993). Events characteristic of uterine development in the pig from birth to PND 60 include appearance and proliferation of uterine glands, development of endometrial folds, and differentiation and growth of myometrial smooth muscle layers (Bartol *et al.* 1993). Endometrial glands are absent at birth in pigs, but begin to develop thereafter. By PND 7 the porcine endometrium contains simple, coiled, tubular glands confined to shallow stroma which continue to extend into the uterine lumen by PND 14 (Spencer *et al.* 1993). The glands begin to branch and extend into the uterine stroma by PND 28-42 with the appearance of uterine folds by PND 56 (Spencer *et al.* 1993). The period of uterine development from birth to PND 60 is ovary independent since ovariectomy at birth does not inhibit uterine gland genesis (Tarleton *et al.* 1998). The development of endometrial glands in the neonatal uterus is accompanied by an increase in DNA synthesis, located specifically in the glandular epithelium (Spencer *et al.* 1993). Additionally, there is the development of an estrogen receptor (ER)-α system

within the neonatal porcine glandular epithelium (Tarleton *et al.* 1998). A negative ER phenotype is demonstrated in PND 0 porcine uterine tissue, however tissues collected at a later time (PND 15) demonstrated positive nuclear ER staining in the glandular epithelium. Comparatively, luminal epithelial ER staining was undetectable in the uterus at PND 15 (Tarleton *et al.* 1998). Together these results suggest there is a temporal relationship between neonatal uterine gland genesis, DNA synthesis localized to the glandular epithelium, and the establishment of an ER system in glandular cells. These observations imply that ER is a marker for uterine development in pigs.

To determine whether ER activation is required for normal endometrial development in the neonatal porcine uterus, estradiol valerate (EV) and the antiestrogen, ICI 182,780 (ICI), were administered to gilts from birth to PND 14 (Tarleton *et al.* 1999). EV treatment resulted in advanced uterine development associated with increased uterine wet weight, endometrial thickness, and gland genesis (Tarleton *et al.* 1999). When ICI and EV were administered together, the precocious uterine development response to EV was attenuated (Tarleton *et al.* 1999). Taken together, these results suggest that EV is uterotrophic, ICI is antiuterotrophic, and the ER system is required for normal neonatal porcine uterine development.

There are other factors that affect the expression and activation of ER and also have the potential to impact postnatal uterine development. The 6 kDa protein hormone RLX, present in porcine colostrum (Frankshun *et al.* 2009), was reported to have uterotrophic effects that mimic estrogens and depend on the presence of ERα (ESR1) (Yan *et al.* 2006). Furthermore, nursing for 48 h from birth advanced endometrial development and adenogenesis at PND 2 and PND 14 (Miller *et al.* 2013). Effects of

implementing a lactocrine-null state, through milk replacer feeding for 48 h, were evident at PND 2, and distinct at PND 14. Endometrial glandularity, gland depth, and endometrial thickness were reduced in replacer-fed gilts at PND 14 compared to those allowed to nurse *ad libitum*. Additionally, proliferating cell nuclear antigen (PCNA) and estrogen receptor-α (ESR1) labeling indices (LI) were reduced in glandular epithelium (GE) of lactocrine-null gilts compared to nursed gilts at PND 14 (Miller *et al.* 2013).

I. Global gene expression profiling

Initial sequencing technologies that resulted in sequencing the human genome in 2001 (Lander *et al.* 2001) were based on the method of primer extension created by Frederick Sanger (Sanger *et al.* 1977). Sanger, or 'first-generation', sequencing allowed for 'reading' of DNA sequences by measuring fragment sizes on a separated gel (Metzker 2010). Along with Maxam-Gilbert sequencing that used nucleotide cleavage, these sequencing technologies enabled genetic reading for small scale, well defined targets on gene fragments (Goldman & Domschke 2014). Newer methods, termed 'next-generation sequencing' (NGS), also called deep sequencing, high throughput sequencing or massively parallel sequencing, have automated the sequencing process with higher throughput, increased efficiency and accuracy, and decreased cost (Tucker *et al.* 2009). These sophisticated DNA sequencing technologies permit: detection of differences in global gene expression, whole genome sequencing, mutation detection in Mendelian disease and cancers (Tucker *et al.* 2009). For example, NGS research has revealed isolated gene mutations found in families who pass rare Mendelian diseases (Ng *et al.* 2010) and been used to identify tumor-specific changes in gene expression that can be used to direct specific cancer therapies (Ley *et al.* 2008).

For the RNA-seq studies presented here the sequencing system used was the Illumina HiSeq 2000 (Illumina, Inc.) and details of steps included in preparation and sequencing are described here. First, total RNA (500 ng/tissue sample) is converted to cDNA for library preparation. Then, DNA is fragmented into standardized sizes and ligated to adaptors on both ends. Adaptors join to allow fragments to conform to a circular shape that is then broken into smaller fragments. However, only fragments containing adaptors are isolated for 'paired-end' reads. These isolated fragments contain an adaptor flanked on both or 'paired-ends' with DNA that previously layed on opposite ends of the original larger DNA fragment (Tucker *et al.* 2009). Paired-ended reads allow for greater accuracy in sequencing, with reduced deletions, duplications, rearrangements, and inversions of reads compared to the single-end method (Tucker *et al.* 2009). Next, fragments with associated adaptors are added to the HiSeq flow cell chamber that is coated with oligonucleotides complementary to the adaptors. Fragments undergo hybridization and amplification to form discrete clusters, all with the same sequence. Sequencing primers, DNA polymerase, and fluorescently labeled nucleotides (each containing a 3' OH group chemically inactivated) are added to the denatured gene clusters. For the sequencing steps, the HiSeq platform uses reversible terminator-based sequencing in a cyclic method using nucleotide incorporation, fluorescent imaging, and cleavage. Specifically, the fluorescently labeled terminator is imaged as each deoxynucleotide triphosphate (dNTP) is added and then cleaved to allow incorporation of the next base. The imaging step determines the identity of the ligated probe through color coding, then the blocked group (3'OH-inactivating residue) is chemically removed

to prepare each strand for the next base incorporation by DNA polymerase (Illumina HiSeq 2000 Specification Sheet, Illumina, Inc .; (Tucker *et al.* 2009, Metzker 2010).

As new emerging high throughput technologies advance, so must bioinformatic enrichment tools that allow researchers to analyze large genomic datasets. Biological interpretation of sizeable gene lists can be challenging; however, new public databases, including Gene Ontology (Ashburner *et al.* 2000), allow dissection and comprehension of datasets. Enrichment tools, each with differing enrichment and statistical algorithms, can categorize gene lists into biological processes (Huang 2009). The number of enrichment tools is actively growing and improving, however, there is not one central gold standard. Therefore, many researchers use multiple tools with differing algorithms and features most suitable to their analytic needs (Huang 2009). One such enrichment tool is Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov) that provides functional and biological interpretation of genomic studies to allow researchers to identify gene functional groups, view inter-relationships between gene groups, and search for related or new gene-terms of interest (Huang et al., 2007). DAVID statistically highlights over-represented, or enriched, gene-terms based on p-values or EASE score (Hosack *et al.* 2003, Huang da *et al.* 2007). Subsequently, a geometric mean of the EASE score for a functional group is calculated to establish rankings based on the overall participation of group members in the enriched biological process. This is defined as the 'enrichment score' (Huang da *et al.* 2007); Nature Protocols 2009). Similarly, the Kyoto Encyclopedia of Genes and Genomes (Kanehisa & Goto 2000, Kanehisa *et al.* 2014) integrated database is a resource for analyzing and categorizing biological functions from molecular-level genome sequencing

using p-values http://www.genome.jp/kegg/). The Protein Analysis Through Evolutionary Relationships (Panther; (Thomas *et al.* 2003) database of protein families, functions, and pathways is another tool for analysis of genomic data relative to known and inferred gene functions (Mi *et al.* 2005). Reactome (Matthews *et al.* 2009) provides annotated biological pathways based on molecular and orthologic processes (Croft *et al.* 2011). Finally, Ingenuity Pathway Analysis (IPA; www.qiagen.com/ingenuity; Red Wood City, CA, USA) bioinformatics software offers analysis of data and allows searches for targeted information on genes and biological pathways.

Our knowledge of the global uterine transcriptome and mechanisms controlling neonatal porcine development are limited. However, an RNASeq study focusing on the porcine uterus revealed over 1900 differentially expressed genes at pregnancy d 14 when compared to cyclic pigs (Samborski *et al.* 2013). Additional RNASeq studies assessing the uterus during pregnancy revealed novel endometrial pregnancy-associated genes in cattle (Forde *et al.* 2012) and differential myometrial transcriptomes of women in labor compared to a quiescent state (Chan *et al.* 2014). While NGS technologies have not been used to study porcine uterine development in the neonate, there is evidence for histological changes in the porcine uterine wall as early as PND 3 in that uterine GE, absent at birth, differentiates from LE to form nascent uterine glands (Masters *et al.* 2007). Also, within the first two days after birth there is differential expression of several porcine uterine genes including the relaxin receptor (*RXFP1*) and estrogen receptor-alpha (*ESR1*) in nursed gilts compared to animals fed milk-replacer over the same period (Chen *et al.* 2010, Miller *et al.* 2013). *In situ* localization of *ESR1* revealed strong expression in uterine stroma, GE, and LE at PND 2, and detection primarily in GE at PND 14 (Miller *et*

al. 2013). Additionally, proliferating cell nuclear antigen (PCNA) labeling indices, a measure of cell proliferation, were reduced for LE and nascent GE in lactocrine-null gilts, fed porcine milk replacer in lieu of nursing, by PND 2. Thus, morphologically critical events supportive of porcine uterine wall development occur within 48 h of birth. Therefore, it is important to define the uterine developmental transcriptome during this period of early neonatal life. Thus, using RNAseq, one aim of this study is to identify the developmental changes in the neonatal porcine uterine transcriptome of gilts from birth to PND 2.

J. Insulin-like Growth Factor (IGF)-1 and Binding Proteins (IGFBPs) in Mammary Secretions

IGF1, a 7.6 kDa mitogenic peptide, is a member of the insulin family of hormones primarily produced by the liver. This growth factor is important for all phases in the cell cycle including stimulating cellular proliferation, growth and differentiation, and inhibiting apoptosis (Baserga & Rubin 1993). IGF1 is present in mammary secretions of pigs (Simmen *et al.* 1988), humans (Nagashima *et al.* 1990, Donovan *et al.* 1991a), cows (Francis *et al.* 1988, Campbell & Baumrucker 1989), goats (Prosser *et al.* 1990), sheep (Simmen *et al.* 1988) and rats (Donovan *et al.* 1991b). Similar to RLX and other MbFs, milk-borne IGF1 levels are greatest during the first few days after parturition and decline as colostrum transitions to milk (Klobasa *et al.* 1987, Simmen *et al.* 1990, Donovan *et al.* 1994, Frankshun *et al.* 2009). In pig, cow, and sheep colostrum, IGF1 was found at high levels (50-500 ng/ml) while in mature porcine milk IGF1 levels were 10- to 100-fold lower than in colostrum (Simmen *et al.* 1988).

Data show that mammary tissue synthesizes IGF1 in low concentrations compared to the liver (Yee *et al.* 1989). It is suggested that the means by which IGF1 is concentrated into mammary secretions is via receptor-mediated endocytosis from maternal circulation, followed by transfer of IGF1 from the basal to apical surface of mammary epithelial cells (Wileman *et al.* 1985, Baumrucker & Blum 1993) . Prior to parturition, serum IGF1 levels in cows decline and concurrently, IGF1 levels in mammary secretions rise (Vega *et al.* 1991). Remarkably, this pattern is similar to that described for bovine IgG (Pierce & Feinstein 1965, Butler 1973).

IGFs in colostrum and milk are present in free and bound states. The amount of free or bound IGF in mammary fluid varies throughout the lactation period (Donovan *et al.* 1994, Xu & Wang 1996). The bound form refers to IGFs associated with their binding proteins. Six IGF binding proteins (IGFBPs) have been identified and cloned (Rechler & Brown 1992); the existence of different IGFBPs suggest distinctive biological functions (Rechler 1993). IGFBPs are present in biological fluids including porcine mammary secretions and exist as 150 kDa IGF-IGFBP complexes (Gelato *et al.* 1988, Donovan *et al.* 1994). IGFBPs can be either transported into milk from maternal circulation or they can be produced within the lactating mammary gland (Lee *et al.* 1993). IGFBPs function as transport vehicles (Rechler 1993), increase the half-life of circulating IGF (Walton *et al.* 1989), and augment or impede IGF activity (Baxter 1988, McCusker *et al.* 1991).

The majority of IGF in biological fluids is found in the bound state (Donovan *et al.* 1994). IGFBPs were found in porcine colostrum (Simmen *et al.* 1988, Donovan *et al.* 1994) and milk (Donovan *et al.* 1994) with similar molecular weights to those found in

maternal porcine serum (Donovan *et al.* 1994). IGFBP-1,-2,-3, and -4 have been reported in pig (Simmen *et al.* 1988, Donovan *et al.* 1994), human (Donovan *et al.* 1991a), rat (Donovan *et al.* 1991b), and goat (Prosser *et al.* 1990) milk. In pigs, IGFBPs can be transported into milk from maternal circulation or synthesized within the mammary gland (Lee *et al.* 1993). Additionally, IGFBPs are not species selective. In a study where neonatal pigs were fed labeled recombinant human IGF-1, results showed that a large proportion of labeled recombinant human IGF-1 was bound to pig IGFBPs in plasma (Xu & Wang 1996).

K. The effects of oral IGF1 supplementation on IGF1 in plasma and tissues

Whether IGF1 (7.6 kDa), a member of the insulin family of hormones, present in colostrum and milk can enter the neonatal porcine circulation is unclear. However, an early study by Mosinger and colleagues (1959) showed that orally administered insulin in PND 2 and PND 8 neonatal rats, but not PND 21 or PND 30 rats, induced hypoglycemia (Mosinger *et al.* 1959). Results indicate that in rats, insulin permeates the intestinal wall during the same postnatal time period when maternal immunoglobulins are derived after birth from colostrum and milk (Mosinger *et al.* 1959).

GI permeability and maternal glucocorticoid absorption is increased in earlier aged compared to later aged neonatal rats (Henning & Sims 1979). In oral supplementation studies in rodents, lactating dams whose drinking water was supplemented with corticosterone throughout the lactation period, had offspring with improved learning and memory (Casolini *et al.* 1997, Catalani *et al.* 2002) and reduced anxiogenic and fear responses that persisted into adulthood (Catalani *et al.* 2000, Catalani

et al. 2002), as well as resistance to ischemic brain damage (Casolini *et al.* 2007) compared to control rats. A similar response was shown in rhesus macaques, where 'confident' temperament characteristics were increased in infants that nursed from mothers with high milk cortisol levels (Hinde 2007). Data indicate that lactational programing can influence neonatal behavioral phenotypes, whereby less fearful responses in offspring were attributed to increased maternal glucocorticoid production in milk (Hinde & German 2012).

Data from our lab show that RLX (6 kDa), another member of the insulin family of protein hormones that is found in porcine colostrum, was undetectable at birth but measurable in the circulation within 24 h in nursing pigs (Yan *et al.* 2006). Whether IGF1 is able to enter circulation of neonatal pigs is debatable. Oral administration of IGF1 to pigs in milk replacer did not increase serum IGF1 concentrations compared to pigs fed milk replacer alone for four days from birth when evaluated at 24 h, 48 h, 72 h, and 96 h postnatal (Burrin *et al.* 1996). Similarly, porcine serum IGF1 concentrations assessed on PND 14 were unaffected by oral supplementation of IGF1 delivered in milk replacer (Houle *et al.* 2000). Nevertheless, both studies reported proliferative effects in the GI tract for IGF1-supplemented pigs (Burrin *et al.* 1996, Houle *et al.* 2000). In these two studies, it may be that serum IGF1 concentrations were evaluated at time points when exogenous IGF1 had already cleared the circulation and was retained in tissues. Pharmacokinetic data for IGF1 plasma clearance in rats indicated at 2 h post-administration less than half of labeled IGF1 was detectable compared to 15 min post-administration (Ballard et al., 1990). Meanwhile, reduced blood IGF1 at 2 h post-administration was associated with increased kidney, adrenal, brain, and gut IGF1 levels

(Ballard et al., 1990). Additionally, when fed orally to pigs at birth or on PND 3, ^{125} I-IGF1, administered in colostrum, was detected in peripheral plasma within 1 h after administration, and was found in the stomach, small intestine, kidney, brain, and other somatic tissues by 4 h post-administration (Xu & Wang 1996). Further, in a comparison of pigs that either nursed or were replacer-fed, nursed pigs displayed higher plasma IGF1 concentrations and increased growth rate (Dauncey *et al.* 1994). Taken together, the preponderance of data supports the idea that IGF1 family molecules acquired orally can enter the neonatal circulation

Oral IGF1 can stimulate growth of neonatal porcine GI tissues. In replacer-fed pigs, oral IGF1 promoted cell proliferation in intestinal crypts (Xu *et al.* 1994), increased jejunal and ileal villus height by PND 4 (Burrin *et al.* 1996), and both jejunal villus height and mucosal DNA content and synthesis by PND 14 (Houle *et al.* 2000). As mentioned above, Xu and Wang detected labeled IGF1 in somatic tissues beyond the GI tract (Xu & Wang 1996), however, whether orally administered IGF1 has direct actions at the level of the cervix is unknown.

L. IGF1 Cascade in Reproductive Tissues

IGF1 receptors (IGF1R) have been localized on mucosal and serosal surfaces of the intestine of the pig (Laburthe *et al.* 1988, Schober *et al.* 1990). Taken together with the presence of IGF1 in porcine colostrum and milk (Simmen *et al.* 1988), IGF1 may play a role in neonatal intestinal development. Furthermore, IGF1Rs have been localized to neonatal ovine uterine LE, GE, and stroma (Hayashi *et al.* 2005), endometrium of primates (Hild-Petito *et al.* 1994) and outer myometrium of rat uteri (Ghahary & Murphy

1989). Mammary IGF1R was most abundant during lactogenesis, and then declined during the lactation period; similar to the decline in IGF1 concentration as colostrum transitions to milk (Dehoff *et al.* 1988, Hadsell *et al.* 1990, Simmen *et al.* 1990).

IGF1 is thought to exert its mitogenic effects predominantly through the IGF1 receptor (IGF1R), supported by the observation that newborn mice that have altered IGF1 or IGF1R gene expression have significant growth retardation (Baker *et al.* 1996). Additionally, adult mice that are homozygous for an IGF1 gene mutation are infertile dwarfs; females specifically have underdeveloped uteri, do not ovulate, and display hypoplastic myometrium (Baker *et al.* 1996). The IGF1 signaling pathway within the porcine cervix has not been studied. However, it is known that IGF1 stimulates cellular proliferation, differentiation, and survival through interactions with its receptor (Ullrich *et al.* 1986). Upon ligand binding, a conformational change occurs within the receptor, thereby activating tyrosine kinase activity resulting in autophosphorylation and phosphorylation of cellular substrates at tyrosine residues (Li & Miller 2006). When phosphorylated, the tyrosine residues provide docking domains for the Src homology 2 domain of adaptor proteins including insulin receptor substrate (IRS)-1 and -2 (Dews *et al.* 1997, Valentinis *et al.* 1997). The downstream phosphatidylinositol-3 kinase (PIK3)/AKT and MAPK signaling pathways can then be activated. Within the PIK3/AKT pathway, PIK3 promotes synthesis of phosphatydylinolistol 3'-phosphate (PIP3) at the plasma membrane. AKT, a serine/threonine kinase protein, is then translocated to the plasma membrane where 3-phosphoinositide-dependent kinase (PDK) 1 and PDK2 activate AKT by phosphorylation of the Thr 308 and Ser 473 residues

(Woodgett 2005). AKT can then be translocated into the cellular cytoplasm and nucleus to play a role in cell survival and maintenance (Kulik *et al.* 1997).

Although the role of IGF1 signaling molecules in neonatal porcine endometrium and cervix is not clear, it has been shown that *AKT* increases in uterine LE and GE of rats during embryo implantation (Toyofuku *et al.* 2006). *In vitro* studies have shown that IGF1 treated porcine trophectoderm cells had increased pAKT protein in the cytoplasm and nucleus (Jeong *et al.* 2014). Together, these studies suggest that IGF1 signaling is important during the peri-implantation period in pigs (Stroband & Van der Lende 1990, Jeong *et al.* 2014); which is one of the most critical stages of pregnancy and marks the period when conceptus mortality increases and litter size decreases in pigs (Stroband & Van der Lende 1990). Whether oral IGF1, a candidate MbF, is lactocrine-active in the neonatal pig and targets the cervix to activate the IGF1 signaling cascade is unknown

M. Significance

There is a general understanding that nursing promotes a variety of beneficial effects on postnatal development with potential long-term consequences. However, how lactocrine signaling affects developmental programming of postnatal tissues is not completely understood. Colostrum is a potent stimulator of growth (Donovan & Odle 1994) and contains over 60 milk-borne agents including hormones and growth factors (Grosvenor *et al.* 1993, Donovan & Odle 1994, Koldovsky *et al.* 1995). Although the list of known MbFs is growing, their role in neonatal reproductive tract development remains largely unknown. The goal of this research is to better understand lactocrine signaling and its role in the development of the porcine reproductive tract. Together, these studies

are important in examining and extending the lactocrine hypothesis; specifically by determining lactocrine signaling effects on the developmental program and trajectory of the neonatal porcine cervix and uterus. Knowledge of the relevance of MbFs in colostrum using a neonatal pig bioassay could be used to improve commercial and human milk formulas. This research may be important in promoting breastfeeding awareness and its impact on neonatal health.

N. Objectives

The focus of the proposed work is to extend the lactocrine hypothesis by determining whether lactocrine signaling affects the porcine cervical developmental program and trajectory, using a neonatal pig bioassay as a tool to study lactocrine signaling. The overall hypothesis to be tested in this proposed research is that factors in colostrum support the developmental program of the female reproductive tract in the neonate. Thus, the objectives of this dissertation research include the following:

1) To identify age-dependent changes in the neonatal porcine uterine transcriptome from birth (PND 0) to PND 2 using RNA sequencing.

2) To evaluate the effects of age and nursing from birth on porcine cervical (a) histoarchitecture and (b) cell proliferation at PND 2 and PND 14.

3) To determine the effects of a single feeding of colostrum or milk-replacer at birth, with or without oral IGF1 supplementation, on porcine cervical cell proliferation and IGF1 signaling molecules at 12 h postnatal.

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CHAPTER II

Defining the Age-Sensitive Neonatal Porcine Uterine Transcriptome

ABSTRACT

Uterine development in the pig begins prenatally and is completed postnatally. Maternal support of neonatal development in mammals continues after birth through the lactation period. At birth (postnatal day = PND 0), the porcine endometrium consists of a simple, corrugated luminal epithelium (LE) supported by stroma that extends to the myometrial interface. Glandular epithelium (GE), absent at birth, differentiates from LE shortly thereafter. Onset of uterine gland genesis is marked by estrogen receptor-alpha (ESR1) expression and marked immunostaining for proliferating cell nuclear antigen (PCNA) in nascent GE, evident by PND 2. The fact that PCNA labeling indices were reduced for LE and nascent GE in lactocrine-null gilts, fed porcine milk replacer in lieu of nursing, by PND 2 indicates that morphogenetically critical events supportive of porcine uterine wall development occur within 48 h of birth. Therefore, it is important to define the uterine developmental transcriptome during this period of early neonatal life. Consequently, the objective of this study was to determine effects of age on the porcine uterine transcriptome between birth and PND 2 using RNA Sequencing (RNAseq). Newborn gilts (n = 4/group) were assigned to be: 1) euthanized at birth; or 2) nursed *ad libitum* for 48 h. Uteri were collected either at PND 0 or at 50 h postnatal. Total RNA was extracted from each uterus and analyzed qualitatively, for integrity, and quantitatively. RNA (500 ng/per uterus) was used to create cDNA libraries for each uterus. These were bar-coded individually and sequenced at > 90 million reads per sample. Raw reads were mapped to the most recent Sscrofa 10.2 build. Results of RNAseq analyses were validated using qPCR. Gene enrichment and functional analyses were conducted with software analysis programs. Results of RNAseq revealed 3283 genes for which expression changed from birth to PND 2 ($P \leq 0.05$). Of these,

expression of 737 genes were up-regulated while 2546 transcripts were down-regulated at least 2-fold or more in uteri from PND 2 as compared to PND 0 gilts. Multiple up- and down- regulated biological processes were identified to be affected by age using gene enrichment and functional analysis tools including chemokine, Wnt β-catenin, transforming growth factor-β (TGFβ), matrix metalloproteinase (MMP), and estrogen receptor-α (ESR1) signaling pathways. RNAseq results extend previous findings that identify age-sensitive events associated with porcine neonatal uterine development.

INTRODUCTION

In the pig, as in other mammals, reproductive tract development begins prenatally and is completed postnatally. Prenatally, the mammalian uterus develops from paired Müllerian ducts which are tubular structures consisting of simple epithelium surrounded by undifferentiated mesenchyme (Cooke *et al.* 2013). Postnatally, events associated with uterine wall development include: (i) organization and stratification of endometrial stroma; (ii) differentiation and growth of myometrium; and (iii) development of endometrial glands (Bartol *et al.* 1993, Bartol *et al.* 1999, Tarleton *et al.* 1999). In the pig, transformation of the immature neonatal uterus to functional maturity occurs within 120 days of birth when, with supplemented progestin, the uterus is able to support pregnancy (Dziuk & Cook 1966).

Members of the Wnt gene family are hypothesized to play a role in radial patterning and gland formation in the postnatal uterus (Mericskay *et al.* 2004). Specifically, Wnt-β-Catenin signaling is necessary for epithelial organization, radial patterning of mesenchyme, and smooth muscle organization (Mericskay *et al.* 2004). Additionally, Wnt signaling is involved in cell behaviors including adhesion, differentiation, and proliferation that are involved in uterine gland genesis (Cunha 1976, Sharpe & Ferguson 1988, Cooke *et al.* 2013).

At birth, [postnatal day (PND) 0], simple columnar luminal epithelium (LE) lines the porcine uterine lumen and is supported by undifferentiated mesenchyme and an undeveloped myometrium (Bartol *et al.* 1993, Spencer *et al.* 1993, Bartol *et al.* 1999). Shortly after birth, the porcine endometrium undergoes organizational and morphogenetic changes as nascent uterine glands begin to form. This is marked by differentiation of

glandular epithelium (GE) from LE (Bartol *et al.* 1993) and onset of estrogen receptor-alpha (ESR1) expression in nascent GE (Tarleton *et al.* 1998, Nielsen *et al.* 2001, Bartol *et al.* 2013). Uterine ESR1 protein expression, absent at birth, is detectable in nascent GE as early as 24 h postnatal (Bartol *et al.* 2013) and is consistently evident by PND 15 (Tarleton *et al.* 1998, Cooke *et al.* 2013). Similarly, uterine epithelial proliferation begins shortly after birth when a developmental transition from a morphogenetically inactive to a proliferative state occurs by PND 3 (Masters *et al.* 2007). Proliferating cell nuclear antigen (PCNA), a marker of proliferation, was observed inconsistently in porcine uterine LE at PND 0, and regularly in LE and developing GE on PND 3 through PND 6 (Masters *et al.* 2007). Recent data for the pig (Miller *et al.* 2013) showed that: (1) lactocrine-sensitive uterine cell behaviors associated with the onset of endometrial adenogenesis, including cell compartment-specific changes in PCNA labeling index and ESR1 immunostaining patterns, were evident by PND 2; and (2) lactocrine disruption for two days from birth by milk replacer feeding altered endometrial histoarchitecture by PND 14, as reflected by inhibition of adenogenesis. Collectively, observations indicate that the period from birth to PND 2 is important with respect to establishment of an optimal uterine developmental trajectory (Bartol *et al.* 2008).

Gene expression events associated with and supportive of porcine uterine development during the first two days of neonatal life have yet to be defined globally. This can now be accomplished efficiently and effectively using high throughput, RNA sequencing (RNAseq) technologies (Tucker *et al.* 2009). Consequently, the objective of this study was to define changes in the porcine uterine transcriptome associated with the period from birth to PND 2 using RNAseq.

MATERIALS AND METHODS

Animals and Experimental Design

Gilts (*Sus scrofa domesticus*) were born and raised in an established herd of crossbred (Duroc, Hampshire, Yorkshire and Landrace genetics) pigs at the Swine Unit of the New Jersey Agricultural Experiment Station, Rutgers University. All procedures involving animal work were reviewed and approved by the Rutgers Institutional Animal Care and Use Committee (IACUC) and conducted in accordance with the Guide for the Care and Use of Agricultural Animals in Agriculture Research and Teaching (Gentry *et al.* 2004). Attention was given to ensure that sows nursed litters of similar size.

The experimental design is illustrated in Figure 1. At birth gilts (N = 8) were assigned randomly to be either: 1) sacrificed on PND 0, prior to nursing (n = 4); or 2) nursed *ad libitum* from birth through 48 h of age (PND 2, n = 4). Gilts were euthanized and uterine tissues were collected on either PND 0 or 50 h postnatal. Uteri were trimmed of associated connective tissues and uterine wet weights (mg) were recorded. Uterine tissue samples were stored in RNALater at -80°C until used for RNA extraction.

Uterine RNA Isolation and Analysis

Total RNA was isolated from 50-60 mg of wet tissue from each uterine sample using the miRNA-easy kit (Qiagen Inc., Valencia, CA, USA). RNA quantity and integrity were determined 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 an RNA integrity number (RIN) $\geq$ 8.0 were used for RNASeq.

Preparation of cDNA libraries

All RNAseq procedures were performed at the HudsonAlpha Institute for Biotechnology (HAIB; Huntsville, AL, USA) in the Levy laboratory (www.hudsonalpha.org/levy-lab). The workflow for RNAseq analyses (Figure 2) began with conversion of total RNA (500 ng/sample) from each uterus into cDNA for library preparation using the NEBNext® mRNA Library Prep Reagent Set for Illumina (New England Biolabs Inc., Ipswich, MA, USA). Bar codes with uniquely indexed primers were attached to each cDNA library and amplified through six PCR cycles using KAPA HiFi HotStart Ready Mix (Kapa Biosystems Inc., Woburn, MA, USA). cDNA library quality was assessed using a Qubit® 2.0 Fluorometer and library concentration was determined using a DNA 1000 chip and an Agilent 2100 Bioanalyzer. Further library quantification was performed using the qPCR-based KAPA Biosystem Library Quantification kit (Kapa Biosystems Inc., Woburn, MA, USA). Individual sample libraries were diluted to a final concentration of 12.5 nM and equimolar amounts of each sample were pooled (n=4/pool/group) and amplified prior to RNA Sequencing.

RNA Sequencing, alignment, and data analysis

RNA sequencing at over 90 million reads per sample was performed at the HAIB using a 200 cycle TruSeq SBS HSv3 kit on a HiSeq2000 (Illumina Inc., San Diego, CA, USA). Reads were demultiplexed using default settings on bcl2fastq conversion software v1.8.3 (Illumina Inc., San Diego, CA, USA). Quality control checks on raw sequencing data for each sample were performed using FastQC (Babraham Bioinformatics, London, UK). Raw reads were mapped to the Sscrofa 10.2 build of the porcine genome [National

Center for Biotechnology Information (NCBI); Bethesda, MD, USA] using TopHat v1.4.0 (Trapnell *et al.* 2009) with default settings that allowed only two mismatches per read. Alignment of mapped reads was estimated using SAMtools (Li *et al.* 2009) and imported onto Avadis NGS (Strand Scientifics, CA, USA), a commercial data analysis platform. Next, aligned reads were filtered based on read quality metrics such that reads with a base quality score of < 30, alignment score of < 95, and mapping quality of < 40 were removed. Remaining reads were filtered based on read statistics, where missing mates, translocated reads, unaligned reads, and flipped reads were removed. The remaining read list was filtered to remove duplicates. Reads were grouped according to their respective identifiers (barcodes) followed by quantification of transcript abundance using Trimmed Means of M-values (TMM; (Robinson & Oshlack 2010)) for normalization. Differentially expressed genes (DEGs), based on fold change ($\geq \pm 2.0$), were identified between groups (PND 0 vs PND 2). P-values for each DEG were estimated by z-score calculations using a false discovery rate (FDR) correction of 0.05 (Reiner *et al.* 2003). Human orthologs of unannotated DEGs were identified by Dr. Anthony McNeel [United States Department of Agriculture, Agriculture Research Service (USDA-ARS), Clay Center, NE, USA] using NCBI BLAST (Bethesda, MD) and a custom computer program written in Python (www.python.org; Haddock and Dunn 2011) using NCBI modules within BioPython (Cock *et al.* 2009). Data were subjected to principle component analysis (PCA) to determine sample and group (PND 0 and PND 2) clustering patterns. A volcano plot, used to illustrate significant up- and down-regulated gene expression events, was generated using R Programming (GNU General Public License; www.r-project.org).

Gene enrichment analyses were performed using: a) Database for Annotation, Visualization, and Integrated Discovery (DAVID; (Huang da *et al.* 2007a, Huang da *et al.* 2007b); b) Kyoto Encyclopedia of Genes and Genomes (KEGG; (Kanehisa & Goto 2000, Kanehisa *et al.* 2014)); c) Protein Analysis Through Evolutionary Relationships (Panther; (Thomas *et al.* 2003)); and d) Reactome (Matthews *et al.* 2009, Croft *et al.* 2011). Finally, Ingenuity Pathway Analysis (IPA) software (www.qiagen.com/ingenuity; Red Wood City, CA, USA) was used for analysis, integration, and understanding of targeted information on genes and biological pathways (www.ingenuity.com).

Quantitative (q)PCR

RNA used to generate cDNA libraries for RNAseq was also used to validate RNAseq results using qPCR. Uterine RNA from individual animals was pooled to create PND 0 and PND 2 samples. Reverse transcription was performed using 100 ng of total RNA per sample, a Peltier Thermal Cycler-200 (Bio-Rad Laboratories Inc, Hercules, CA, USA) and the SuperScript III First-Strand Synthesis System (Life Technologies; Grand Island, NY, USA) for RT-PCR. qPCR was performed using SYBR Green and universal thermal cycling parameters (40 cycles) indicated by the manufacturer on a StepOne Plus System (Applied Biosystems/Life Technologies). Primers were designed using Primer Quest software (Integrated DNA Technologies, Inc; Coralville, IA, USA) and synthesized by Sigma Aldrich (City, State). Primer sequences (Table 1), directed to the porcine genome, were evaluated for quality by amplifying serial dilutions of the cDNA template. Control qPCR reactions included substitution of water only in place of primers, and template to ensure specific amplification in all assays. Dissociation curves for primer

sets were evaluated to ensure that no amplicon-dependent amplification occurred (Real-time PCR Handbook 2012; Life Technologies)

Data generated by qPCR were analyzed using the $\Delta\Delta C_T$ method described by Applied Biosystems (ABI User Bulletin 2, 2001). In this protocol, C_T values of both sample and calibrator are normalized to an endogenous reference gene, in this case *cyclophilin*, for which expression was unaffected by neonatal age. Amplification efficiencies for target genes were determined to be similar to that calculated for the reference gene, *cyclophilin* (ABI User Bulletin 2, 2001).

Nine genes were targeted for qPCR validation of RNAseq results. Included were genes for which uterine expression was either: (1) up-regulated more than 2-fold [*glutamate receptor, ionotropic, N-methyl-D-aspartate* (*GRIN2C*), and *matrix metalloproteinase (MMP)-7*; (2) down-regulated more than 2.0-fold [*progesterone receptor* (*PGR*) and *transmembrane receptor 126b* (*TMEM126b*)]; or (3) unchanged [< ± 2.0 fold change; *fibrinogen gamma chain* (*FGG*), *insulin-like growth factor binding protein 5* (*IGFBP5*), *follistatin* (*Foll*), *interleukin 5* (*IL5*), and *kalirin* (*KALRN*)] on PND 2 as compared to PND 0.

RESULTS

Effects of age on group clustering and differential gene expression

Principal component analysis showed that uterine RNASeq data for PND 0 and PND 2 clustered independently based on transcript expression profiles (Figure 3). Results indicated unique uterine gene expression signatures for each day. Volcano plot analysis revealed 3283 genes that were differentially expressed on PND 2 when compared with PND 0 ($P \leq 0.05$; Figure 4A). Gene expression events that differed by at least 2-fold were identified. Expression was up-regulated for 737 genes and down-regulated for 2546 genes on PND 2 as compared to PND 0.

qPCR validation of RNAseq results

Results of qPCR analyses for nine targeted transcripts employed to validate RNAseq data are shown in Figure 4B. A positive correlation ($r=0.77$, $P < 0.05$) between qPCR and RNAseq results was identified, supporting the validity of RNAseq data. Gene selections for qPCR validation were made such that at least two transcripts in up-regulated, down-regulated, or unchanged categories were included.

Gene Enrichment Analysis

Presented in Table 2 are selected results for DAVID functional annotation clustering, indicated terms for which associated genes were up- or down-regulated in uteri of gilts at PND 2 versus birth. Enriched canonical pathway results identified using KEGG, Panther, and Reactome analyses are shown in Table 3. Functional terms for the top up-regulated processes identified by DAVID analysis were immune-related and

included: ‘immune response’, ‘cell-cell signaling’ and ‘response to wounding’. Results of KEGG analyses identified ‘cytokine-cytokine receptor interaction’ and ‘chemokine signaling pathway’ as up-regulated processes. Further, DAVID analysis indicated ‘protein amino acid phosphorylation’ and ’cell adhesion’ as functional terms down-regulated in uteri from PND 2 as compared to PND 0 gilts (Table 1). Results of KEGG, Panther, and Reactome analyses (Table 3) indicated that elements of uterine gene expression pathways associated with ‘Wnt’, ‘transforming growth factor-β (TGFβ)’ signaling, and ‘angiogenesis’ were also down-regulated in uteri from PND 2 as compared to PND 0 gilts.

Network IPA analysis of neonatal age-sensitive biological pathways

Network IPA of the chemokine signaling pathway revealed higher expression ($P < 0.05$) of *chemokine receptor-4* (*CXCR4*), *calmodulin* (*CaM*), *phosphatidylinositide-3 kinase* (*PI3K*), and *monocyte chemoattractant protein-1* (*MCP-1*) in uteri of PND 2 gilts compared to gilts at PND 0. Genes that exhibited lower expression ($P<0.05$) included *calcium/calmodulin-dependent protein kinase* (*CAMK*), *Rho-associated, coiled-coil containing protein kinase 2* (*ROCK2*), *protein kinase C* (*PKC*), *myosin light chain phosphatase* (*MCLP*), *mitogen activated protein kinase 1/2* (*MEK1/2*), *jun proto oncogene* (*JUN*), and *fos gene family* (*FOS*).

IPA of the Wnt-β-Catenin pathway revealed lower expression ($P < 0.05$) of genes including *secreted frizzled-related protein 1* (*SFRP1*), *Dickkopf Wnt Signaling Pathway Inhibitor* (*DKK*), *LEF/TCF* transcription factors, *transforming growth factor-β* (*TGFβ*), and its receptor (*TGFBR*), *adenomatous polyposis coli* (*APC*), *axin*, *AKT*, *transcription*

factor 4 (*TCF*)*4, JUN, sex determining region Y-box* (*SOX*), *Groucho,* and *low density lipoprotein receptor-related protein* (*LRP*)*-1/5/6* in PND 2 nursed gilts compared to at birth gilts. PND 2 uterine up-regulated (P<0.05) genes included the *cadherin* gene family, *MMP-7, ubiquitin* (*Ub*), and *orphan nuclear receptor* (*LRH*)*-1*. Genes that were both up- and down-regulated in uteri of PND 2 gilts compared to those at birth included *frizzled receptors* (*FZDs*), *protein phosphatase* (*PP*)*2A,* and *Wnt* gene family members. Review of fold change data indicated that *Wnt2B* and *Wnt 4* were up-regulated, while *Wnt10a* was downregulated (P<0.05) in uteri of PND 2 gilts.

Results of IPA for the MMP signaling pathway indicated that uterine *MMP-9* was up-regulated (P<0.05) on PND 2 compared to tissues obtained at birth. Down-regulated (P<0.05) expression of genes associated with the MMP signaling pathway on PND 2 included *tissue inhibitor of metalloproteinases* (*TIMP*)*-4, LRP1, thrombospondin* (*TSP*)*-2, Disintegrin And Metalloproteinase Domain* (*ADAM*)*-10/12/17,* and *heparan sulfate proteoglycan* (*HSPG*).

Results of IPA for the ESR1 revealed increased uterine expression (P<0.05) of *ESR1, TATA box binding protein-associated factor* (*TAF*), and *Dosage-Sensitive Sex Reversal-Adrenal Hypoplasia Congenital Critical Region on the X Chromosome, Gene 1* (*DAX1*) on PND 2. Genes for which expression was down-regulated (P<0.05) on PND 2 included: *progesterone receptor* (*PGR*), *MEK1/2, triiodothyronine receptor auxiliary protein* (*TRAP*), *Transcription activator* (*BRG1*; also known as *ATP-dependent helicase SMARCA4*), *ACTR* (also called *AIB1* and *SRC-3*) was identified as a coactivator for nuclear receptors, *glutamate receptor interacting protein 1* (*GRIP1*), *nuclear receptor corepressor 1* (*NCOR1*), and *nuclear receptor corepressor* (*SMRT*).

DISCUSSION

RNAseq was used to identify novel elements of the neonatal porcine uterine transcriptome for which expression was affected between birth and PND 2. Overall, 3283 differentially expressed genes were identified. Results indicate that major, global changes in patterns of uterine gene expression occur within two days of birth. Deep sequencing approaches have transformed transcriptomics research (McGettigan 2013, Mutz *et al.* 2013) and enabled generation of large, unique datasets. The power of sequencing lies in the ability to identify biologically significant information including gene expression patterns, differentially expressed and novel genes (McGettigan 2013), epigenetic events, and predictions of genetic disease (Goldman & Domschke 2014). This RNAseq study was conducted in collaboration with researchers at the HudsonAlpha Institute for Biotechnology and the USDA-ARS. These collaborations provided essential expertise and guidance pertaining to experimental design, workflow, gene annotation, and bioinformatics needed to generate and interpret this complex dataset.

Bioinformatic software programs, including DAVID, KEGG, Panther, and Reactome, allow researchers to interpret large lists of genes derived from genomic studies (Huang da *et al.* 2007b). These resources were used to identify biological processes, pathways and associated genes that were up- or down-regulated during neonatal porcine uterine development in the period between birth and PND 2. Supplemental to these programs, IPA was used to provide insight into specific elements of signaling pathways that are differentially expressed over this period. Here, in-depth review of pathways previously identified to be associated with uterine development, including Wnt-β-catenin, TGFβ, MMP, and ESR1 signaling were studied. In addition,

elements of pathways not previously associated with porcine uterine development were identified, including chemokine signaling. Some of the biological processes and pathways identified here were expected, while others were novel.

Based on DAVID and KEGG analyses, biological processes, associated genes and pathways enriched in the PND 2 uterus included those related to 'immune response' and similar immune-related functional terms including 'chemokine signaling' and 'cytokine-cytokine receptor interaction'. Originally described as immune-related intercellular messengers, chemokines and cytokines are not only mediators of inflammation (Oppenheim 1991), but are also involved in organogenesis and morphogenesis by: (i) altering expression patterns of adhesion molecules (Borish & Rosenwasser 1996, Cale 1999); (ii) extracellular matrix (ECM) remodeling (Vaday *et al.* 2001, Nieto & Lutolf 2011); (iii) influencing transcription factor-cytokine interactions (Richer *et al.* 1998, Dimitriadis *et al.* 2005); and (iv) modulating cellular processes like growth, differentiation, apoptosis, cell-cell interactions, and directed cell migration (Borish & Rosenwasser 1996, Cale 1999). These processes are also important for neonatal uterine development and adenogenesis. For example, epithelial-mesenchymal, cell-cell, and cell-ECM interactions support uterine morphogenesis and differentiation of uterine cells and tissues (Bartol *et al.* 1993). In the present study, DAVID analysis also revealed processes affected by age involving 'cell-cell signaling', 'cell adhesion' and 'regulation of transportation' that can be associated broadly with processes known to be influenced by chemokines and cytokines (Cale 1999, Park & Yang 2011).

Although effects of chemokines and cytokines on neonatal porcine uterine development have not been explored, other studies implicate these molecules in tissue

development and morphogenesis. For example, in the developing kidney, cytokines such as tumor necrosis factor-α (TNFα) and TGFβ inhibited murine nephrogenesis *in vitro* (Rogers & Berman 1993, Cale *et al.* 1998). Like the neonatal uterus (Cunha *et al.* 1983, Sanders 1988), the developing kidney undergoes morphogenesis and mesenchymal to epithelial conversion (Cale 1999). These processes are important for generation of new tissue architecture from undifferentiated epithelium (Cunha *et al.* 1983, Sanders 1988, Sharpe & Ferguson 1988). In addition to DAVID and KEGG, IPA of the chemokine signaling pathway revealed uterine genes differentially expressed at PND 2, including up-regulation of the chemokine receptor, *CXCR4*. A functional role for *CXCR4* in uterine development has yet to be established. However, *CXCR4* supports blood vessel formation in other developing tissues, including the heart (Tachibana et al., 1998). Also of interest, a positive feedback loop between *CXCR4*, its ligand *CXCL12*, and *ESR1* has been documented in *vitro*; whereby *CXCL12/CXCR4* signaling promotes transcriptional activation of *ESR1* (Sauve *et al.* 2009). Results support the present study where uterine *ESR1* is up-regulated at PND 2.

The Wnt gene family plays an important role in female reproductive tract (FRT) development in several species, including pigs (Bartol *et al.* 2006, Cooke *et al.* 2013). Prenatally, *Wnt-4a* supports Müllerian duct formation while *Wnt-7a* regulates Müllerian duct differentiation (Miller & Sassoon 1998, Parr & McMahon 1998). Postnatally, uterine differentiation and maturation involving mesenchymal-epithelial interactions affect expression patterns of *Wnt-4*, *Wnt-5a*, and *Wnt-7a* (Cunha 1976, Brody & Cunha 1989, Miller *et al.* 1998, Miller & Sassoon 1998). Both KEGG and Panther analyses indicated that elements of the uterine "Wnt signaling pathway" were down-regulated by

PND 2 when compared to uteri of gilts at birth. These observations support other studies indicating age-associated changes in Wnt gene expression in the postnatal period. For example, uterine expression of several Wnts, including *Wnt-7a* and *Wnt-11*, are temporo-spatially regulated in mice (Hayashi *et al.* 2011). Expression levels change as epithelium differentiates and uterine glands form (Miller *et al.* 1998, Miller & Sassoon 1998, Hayashi *et al.* 2011). Specifically, *Wnt-7a*, localized to LE on PND 1 and PND 5, was also apparent in superficial ductal GE on PND 10 and PND 15. Similarly, *Wnt-11*, low in uterine LE at birth, increased in LE and superficial ductal GE at PND 10 and PND 15 (Hayashi *et al.* 2011). IPA review of the Wnt-β-catenin pathway revealed both up- and down- regulation of *Wnt* and *frizzled receptor* (*FZD)* genes as an effect of age (PND 2 vs. PND 0). For example, fold change analysis showed that uterine *Wnt-2b* and *Wnt-4* were up-regulated, while *Wnt-10a* was down-regulated on PND 2. This was not surprising, as there are 19 different mammalian Wnt ligands and 10 different FZDs (Dijksterhuis *et al.* 2014) for which expression could be differentially regulated depending on timing or phase of development (Miller *et al.* 1998).

Wnts activate the canonical Wnt-β-catenin pathway via FZD receptors which, in turn, leads to β-catenin accumulation in target cells. β-catenin acts as a transcription factor in the nucleus and can bind epithelial (E)-cadherin. It is through this pathway that Wnts, cooperating with β-catenin and E-cadherin, affect events regulating FRT development, including cytodifferentiation, morphogenesis, and adenogenesis (Miller & Sassoon 1998, Mericskay *et al.* 2004, Hayashi *et al.* 2011). Results of IPA for the Wnt-β-catenin pathway revealed that cadherin gene family members, including epithelial (E), neural (N), placental (P), and vascular endothelial (VE) cadherins, important for cell

adhesion (Takeichi 1991, Gumbiner 1996), were up-regulated in uteri of nursed gilts on PND 2. In prepubertal pigs, E-cadherin was localized in pig endometrial epithelial cells and increased with uterine growth (Ryan *et al.* 2001). Epithelial proliferation in the developing neonatal porcine uterus (Miller *et al.* 2013) may be supported by increased cadherin expression as reported here.

Consistent with results of KEGG and Reactome analyses, IPA of the Wnt-β-catenin pathway also indicated that transcripts for *TGFβ* and its receptor,*TGFBR*, were down-regulated in PND 2 uteri when compared to uteri at birth. Although the TGFβ pathway and its relation to postnatal uterine development have not been studied in neonatal pigs, *TGFBR* is required for formation of the murine myometrium (Gao *et al.* 2014). *TGFBR*-null female mice have disorganized uterine smooth muscle, oviductal diverticula, or fluid filled cysts within the oviduct, and are infertile (Li *et al.* 2011). TGF-β signaling activates Wnt-β-catenin signaling in various cell types (Cheon *et al.* 2004), supports ECM production (Zode *et al.* 2011, Busnadiego *et al.* 2013) and regulates the expression of genes, including *MMP-7*, associated with ECM breakdown (Bruner *et al.* 1995). Together, these studies suggest factors in the Wnt-β-catenin and TGFβ signaling pathways play a role in postnatal uterine developmental events that determine tissue function.

Uterine growth involves both cell proliferation and ECM synthesis and remodeling. Similarly, these processes are also involved in wound repair (Belacortu & Paricio 2011), or 'response to wounding', identified here by DAVID analysis as a biological process affected by age. ECM remodeling is regulated locally by MMPs and tissue inhibitors of metalloproteinases (TIMPs; (Nagase *et al.* 1996, Gillard *et al.* 2004)).

For example, MMP-9 is involved in basement membrane degradation, a process important for reproductive tissue growth and remodeling (Nagase *et al.* 1996, Gillard *et al.* 2004). The increase in uterine *MMP-9* expression 2 d after birth, identified here by IPA, supports studies by Chen et al (2011) who found uterine MMP-9 protein increased with age from birth to PND 2 (Chen *et al.* 2011). Another important MMP for ECM remodeling is epithelial-specific *MMP-7*, localized to GE and LE in human endometrium (Rodgers 1993). Evidence that elements of the Wnt-β-catenin pathway, including TGFβ, regulate MMP-7 expression (Bruner *et al.* 1995, Brabletz *et al.* 1999) was supported by IPA data indicating increased uterine *MMP-7* and decreased *TGFβ* expression by PND 2. This increase in *MMP-7* gene expression may be due to LE and GE proliferation associated with uterine development at PND 2 (Miller *et al.* 2013).

Results of IPA analyses support earlier observations (Tarleton *et al.* 1998, Miller *et al.* 2013), indicating an increase in uterine *ESR1* expression, particularly in GE, by PND 2 in the pig. However, evidence reported here for neonatal porcine uterine *PGR* expression, which decreases from birth to PND 2, is novel. In sheep, PGR was detected by immunohistochemistry in uterine epithelium and stroma at PND 1 (Taylor *et al.* 2000) and neonatal progestin exposure inhibited uterine adenogenesis (Tarleton *et al.* 1998), similar to that observed in mice (Cooke *et al.* 2012, Filant *et al.* 2012). Further, progestin-induced inhibition of ovine adenogenesis was reported to involve down-regulation of epithelial ESR1 expression (Gray *et al.* 2000a). Studies *in vitro* (Savouret *et al.* 1991) and *in vivo* (Spencer & Bazer 1995), showed down-regulation of epithelial *PGR* was associated with up-regulation of *ESR1*, consistent with the idea that neonatal

PGR/ESR1 expression changes can support GE differentiation (Tarleton *et al.* 1999, Gray *et al.* 2000a, Gray *et al.* 2000b).

Results of this RNAseq analysis revealed elements of the neonatal porcine uterine transcriptome associated with development between birth and PND 2. Evidence for differential uterine expression of over 3000 genes during this period provides substantial fuel for future investigations aimed at understanding mechanisms regulating uterine development. In this study, RNAseq analyses of the whole, neonatal porcine uterine transcriptome were performed, including epithelium, stroma, myometrium and perimetrium. Thus, a limitation of this study was that no compartment-specific information was obtained. Studies by Miller and colleagues (2013), using laser microdissection, indicated differential gene expression in porcine endometrial stroma and epithelium (LE and GE) as early as PND 2. Such studies, considered in light of present results, encourage investigation of developmentally related, global, cell compartment-specific gene expression, as reported for the mouse (Niklaus & Pollard 2006, Filant & Spencer 2013). This type of information will provide insight into gene expression events regulating postnatal FRT development.

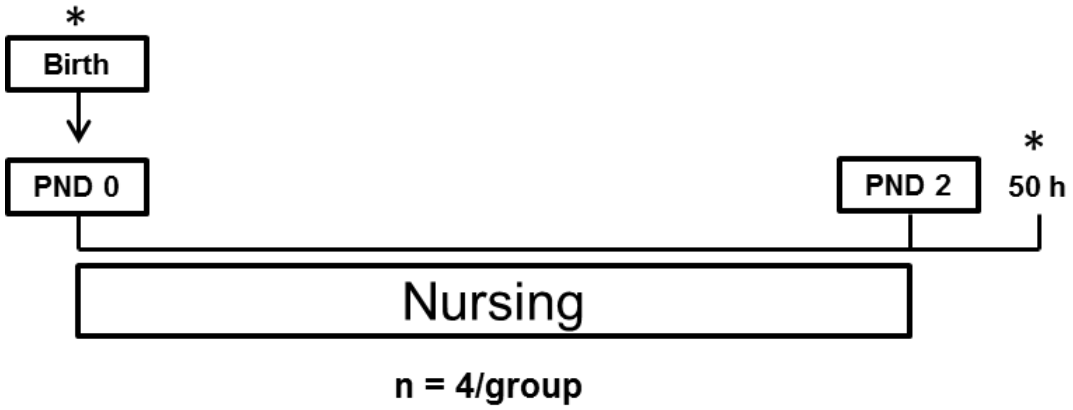


Figure 1: Experimental Design. Gilts were assigned to either be sacrificed at birth (PND 0) prior to feeding, or nurse normally *ad libitum* until PND 2. Uteri were collected on both PND 0 and PND 2, as denoted by asterisks.

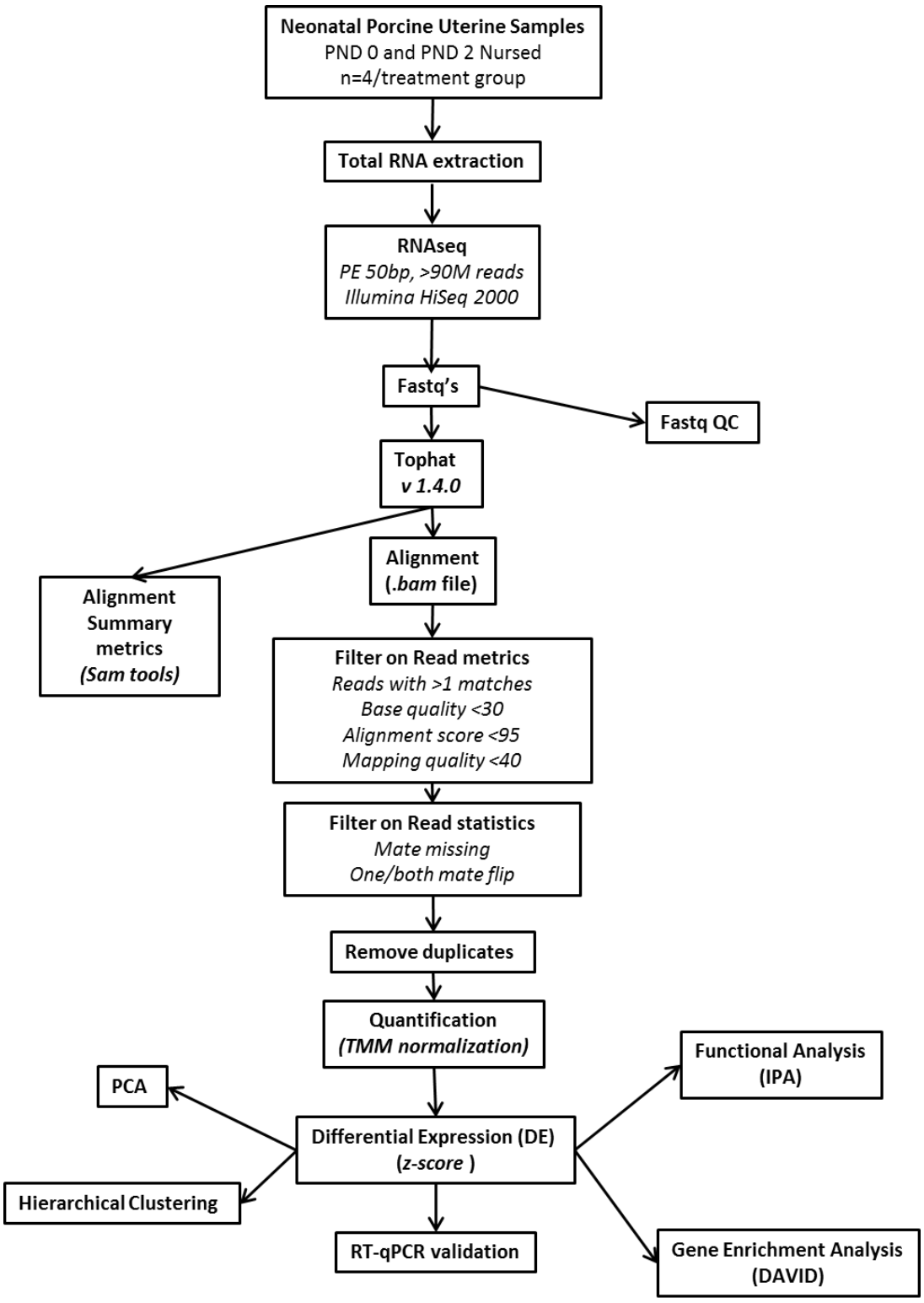
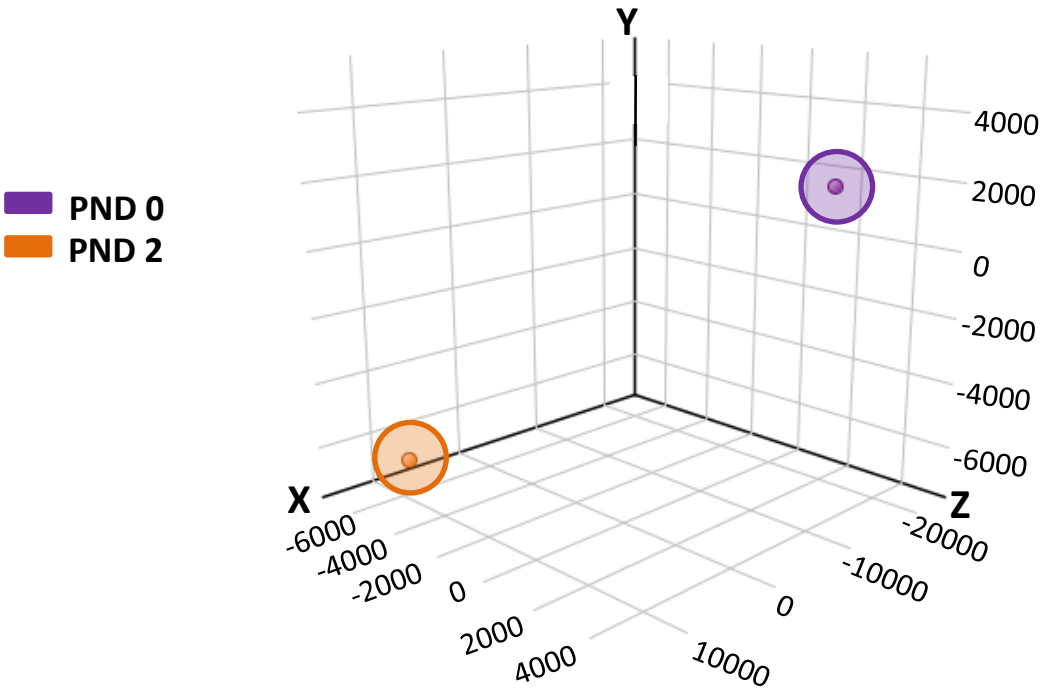


Figure 2: RNAseq flow chart listing step-wise processes conducted during preparation, running, alignment, analysis, and validation of the data sets.

Figure 3: Principal component analysis (PCA) plot obtained from RNAseq analysis of pooled neonatal porcine uterine samples showing the clustering of PND 0 (purple) and PND 2 (orange) based on the transcriptomic expression profiles.



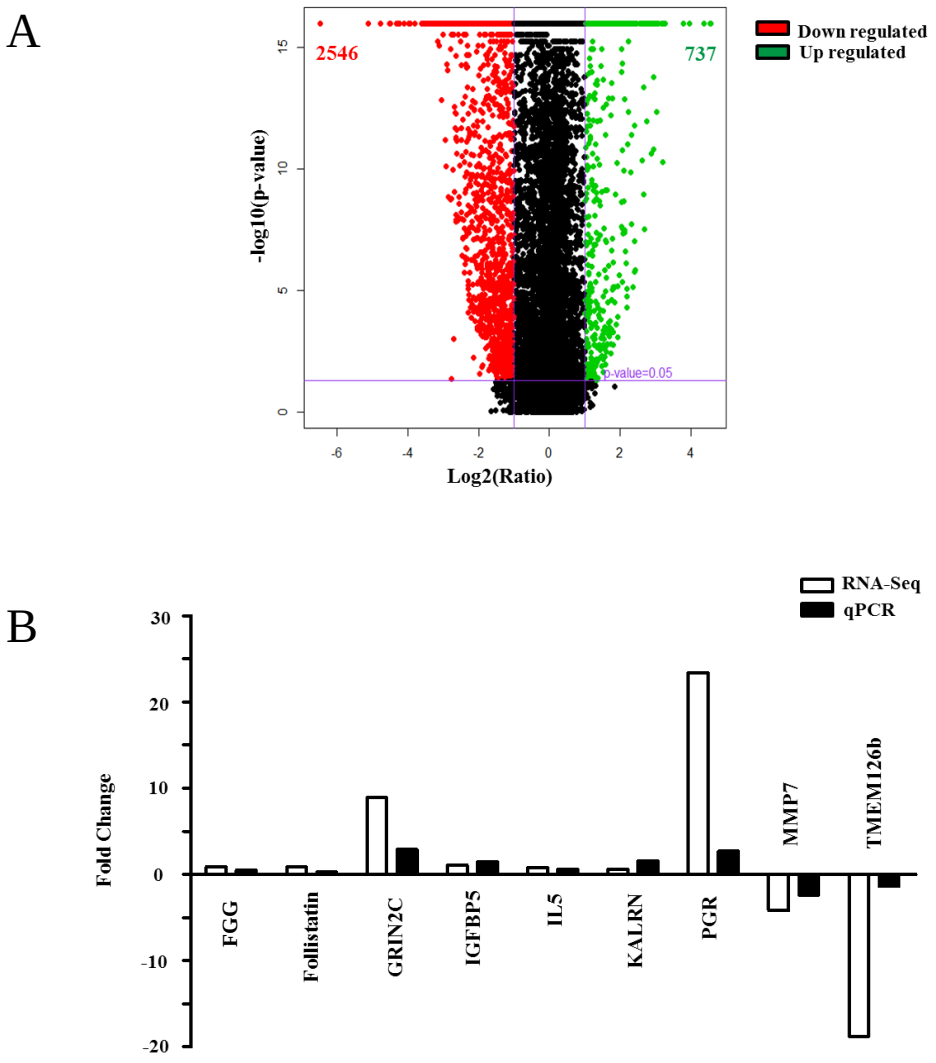


Figure 4: A) Volcano plot for differences in gene expression between PND 2 vs. PND 0. The purple horizontal line denotes P < 0.05; red color denotes genes that were down regulated (fold change > 2); green color denotes genes that were up regulated (fold change > 2); and black denotes insignificant gene changes (fold change < 2). Colored values indicate the number of differentially expressed genes that were down- (red) or up-regulated (green). B) Results of qPCR validation for nine genes that were differentially regulated in the RNAseq data set for PND 2 vs. PND 0; white bars = RNAseq fold change; black bars = qPCR fold change.

Table 1: Gene and primer information for targeted porcine uterine transcripts used for qPCR validation.

Gene Symbol	Gene Name	Accession #	Forward and Reverse Primer Sequences
FGG	Fibrinogen gamma chain	NM_001244524	**F:** TCTCCTACTGGAACACAGA **R:** CCTCCAGCTGCACTCTTAAT
Foll	Follistatin	AJ_715530	**F:** GGCCTATGAGGGAAAGTGTATC **R:** ACAGGCTCCTCAGACTTACT
GRIN2C	Glutamate receptor, ionotropic, N-methyl D-aspartate	XM_003131237.3	**F:** TGTGTACTTGCTGTTCTGTCT **R:** CACCTCTCTGGGTCTTCATTTC
IGFBP5	Insulin-like growth factor binding protein 5	NM_214099.1	**F:** TGAAGAAGGACCGCAGAAAG **R:** CTGCTCAGATTCCTGTCTCATC
IL5	Interleukin 5	KC_660157	**F:** CGTTAGTGCCATTGCTGTAGAA **R:** CATCAAGTTCCCATCGCCTATC
KALRN	Kalirin	EF_443102	**F:** GAGGATCCAGAACACTGAAGAC **R:** TCACGTCTCCTCACAGATACT
PGR	Progesterone receptor	GQ_903679	**F:** CCAGCTTGTCGCCTTAGAAA **R:** CGGCATCTAGTGCTCTCATAAC
MMP7	Matrix metalloproteinase 7	NM_214207	**F:** CGCCTGCCTATAACTGGAAT **R:** TTTGGCTGGCTTGGGAATAG
TMEM126b	Transmembrane receptor 126b	NM_001243600	**F:** CAGGTAACATAAGCCGGGAA **R:** GTGGCAGTGGAACAGTATGA
Cyclophillin	Cyclophilin	AU_058466	**F:** TTATAAAGGTTCCTGCTTTCACAGA **R:** TGCCATTATGGCGTGTGAAG

Table 2: Selected results for DAVID functional annotation clustering indicating terms for which associated genes were up- or down-regulated in uteri of gilts from birth (PND 0) to PND 2.

Functional Terms of Overrepresented Biological processes[a]	Enrichment Score[b]
Up-Regulated	
Immune response (50, 2.1)	5.7
Inflammatory response (29, 2.5)	5.0
Defense response (44, 2.0)	4.9
Cell-cell signaling (41, 2.0)	4.1
Response to wounding (37, 2.0)	4.0
Behavior (32, 1.9)	3.3
Response to calcium ion (9, 4.7)	3.2
Regulation of secretion (18, 2.5)	3.1
Positive regulation of transportation (19.2.4)	2.8
Regulation of interleukin-8 production (5, 9.5)	2.8
Down-Regulated	
Protein amino acid phosphorylation (120, 1.6)	6.3
Regulation of small GTPase mediated signal transduction (56, 1.9)	5.7
Enzyme linked receptor protein signaling pathway (69, 1.7)	5.4
Transmission of nerve impulse (70, 1.7)	5.3
Macromolecular catabolic process (128, 1.4)	4.5
Ion transport (126, 1.4)	4.5
Synaptic transmission (59, 1.7)	4.4
Cell adhesion (116, 1.4)	4.4
Biological adhesion (116, 1.4)	4.3
Phosphate metabolic process (152, 1.4)	4.2

[a]Values within parentheses indicate 1) the number of genes involved in, and 2) 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 gene list. Enrichment scores are calculated by taking the geometric mean of the P values associated with the differentially expressed genes involved in the corresponding annotation cluster (in-$\log_{10}$ 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 at PND 2 versus PND 0.

Bioinformatics Program	Pathways	P-value
	Up-Regulated	
KEGG	Cytokine-cytokine receptor interaction	0.0024
	Histidine-metabolism	0.031
	Chemokine signaling pathway	0.047
Reactome	Muscle contraction	0.008
	Down-Regulated	
KEGG	Hedgehog signaling pathway	4.86E-04
	Endometrial cancer	6.49E-04
	MAPK signaling pathway	7.70E-04
	Vascular smooth muscle contraction	0.002
	ECM-receptor interaction	0.008
	Apoptosis	0.011
	ER-β signaling pathway	0.013
	Calcium signaling pathway	0.013
	Wnt signaling pathway	0.016
	TGFβ signaling pathway	0.022
Panther	PDGF signaling pathway	0.007
	Angiogenesis	0.009
	Endothelin signaling pathway	0.009
	VEGF signaling pathway	0.011
	Wnt signaling pathway	0.040
	EGF receptor signaling pathway	0.047
Reactome	Signaling by PDGF	0.001
	Integrin cell surface interactions	0.002
	Signaling by NGF	0.009
	Hemostasis	0.010
	Signaling by TGFβ	0.013
	Signaling by insulin receptor	0.038

Figure 5: IPA results for the chemokine signaling pathway. Effects of age on elements of this signaling pathway are shown below. Red coloration denotes down-regulation while green denotes up-regulation ($P < 0.05$) in PND 2 compared to PND 0 uteri of gilts. Elements of the pathway that are white were not affected by age. CXCR4 = chemokine receptor-4 ; CaM = calmodulin; CAMK = calcium/calmodulin-dependent protein kinase; ROCK2 = Rho-associated, coiled-coil containing protein kinase-2; MLCP = myosin light chain phosphatase; MCP-1 = monocyte chemoattractant protein-1; MEK1/2 = mitogen activated protein kinase kinase 1/2; PKC = protein kinase C; PI3K = phosphatidylinositide 3- kinase; JUN = jun proto oncogene; FOS = Fos gene family

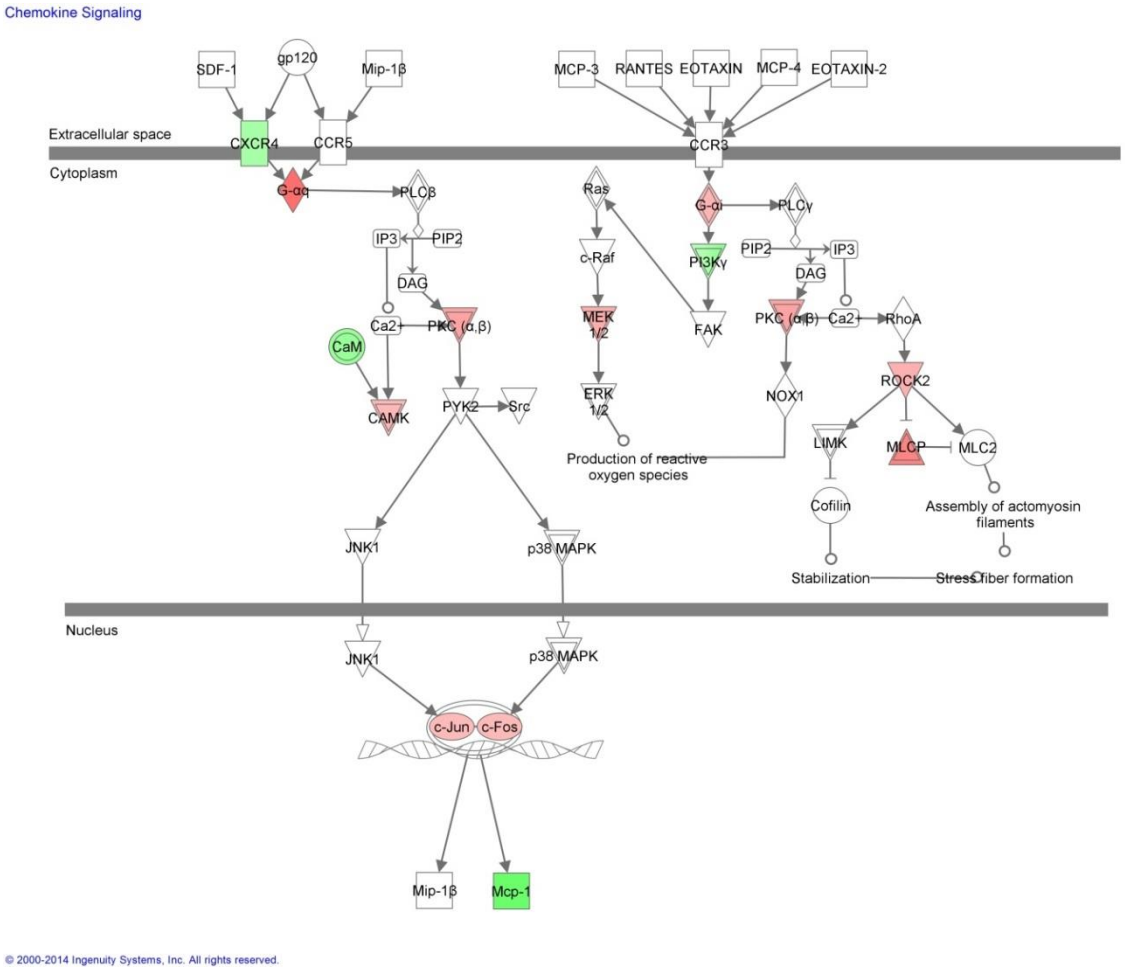


Figure 6: IPA results for Wnt β-catenin signaling pathway. Effects of age on elements of this signaling cascade are shown below. Red color denotes down-regulation, green color denotes up-regulation ($P < 0.05$), and red/green denotes both up- and down-regulation in PND 2 compared to PND 0 uteri of gilts. Elements in white color are not affected by age. Wnts = Wingless type gene family; TGFβ = transforming growth factor beta; TGFBR = transforming growth factor, beta receptor; AKT = protein kinase B; SOX = SRY; sex determining region Y box gene family of transcription factors; MMP-7 = matrix metallopeptidase 7; SFRP = secreted frizzled-related protein; TCF4 = transcription factor 4; LRH-1 = liver receptor homolog 1; LEF/TCF = LEF/TCF family of transcription factors; PP2A = protein phosphatase 2; DKK = Dickkopf Wnt signaling pathway inhibitor; LRP 1/5/6 = low density lipoprotein receptor-related protein 1/5/6

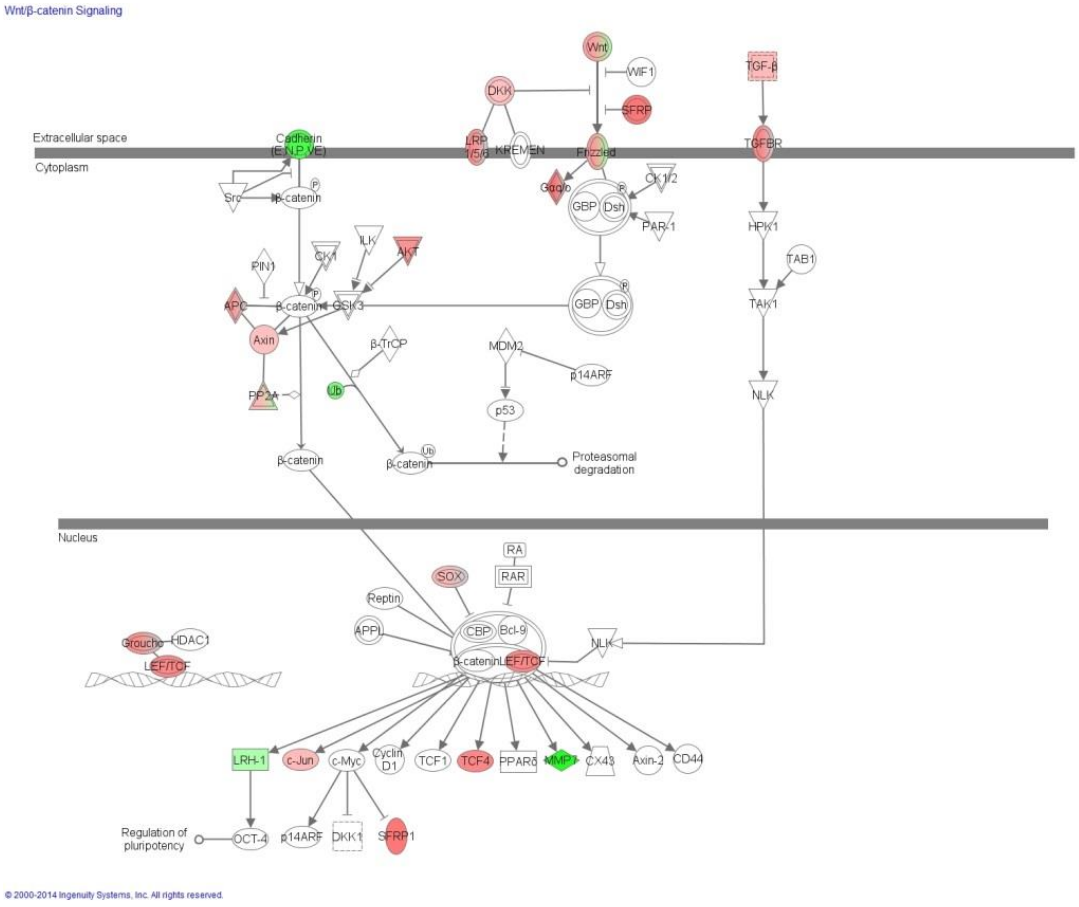


Figure 7: IPA results for the matrix metalloproteinase (MMP) signaling pathway. Effects of age on elements of this signaling cascade are shown below. Red color denotes down-regulation, green color denotes up-regulation ($P < 0.05$), and red/green denotes both up- and down-regulation in PND 2 compared to PND 0 uteri of gilts. Elements in white color are not affected by age. Genes affected by age include: MMP-9, tissue inhibitor of metalloproteinases = TIMP-4, low density lipoprotein receptor-related protein = LRP1, thrombospondin = TSP-2, Disintegrin And Metalloproteinase Domain = ADAM-10/12/17, and heparan sulfate proteoglycan =HSPG.

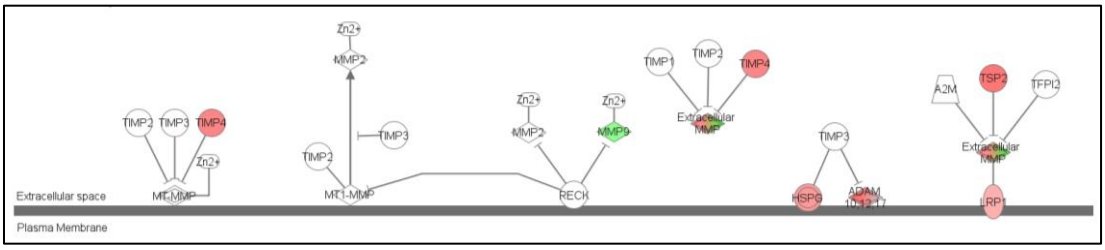
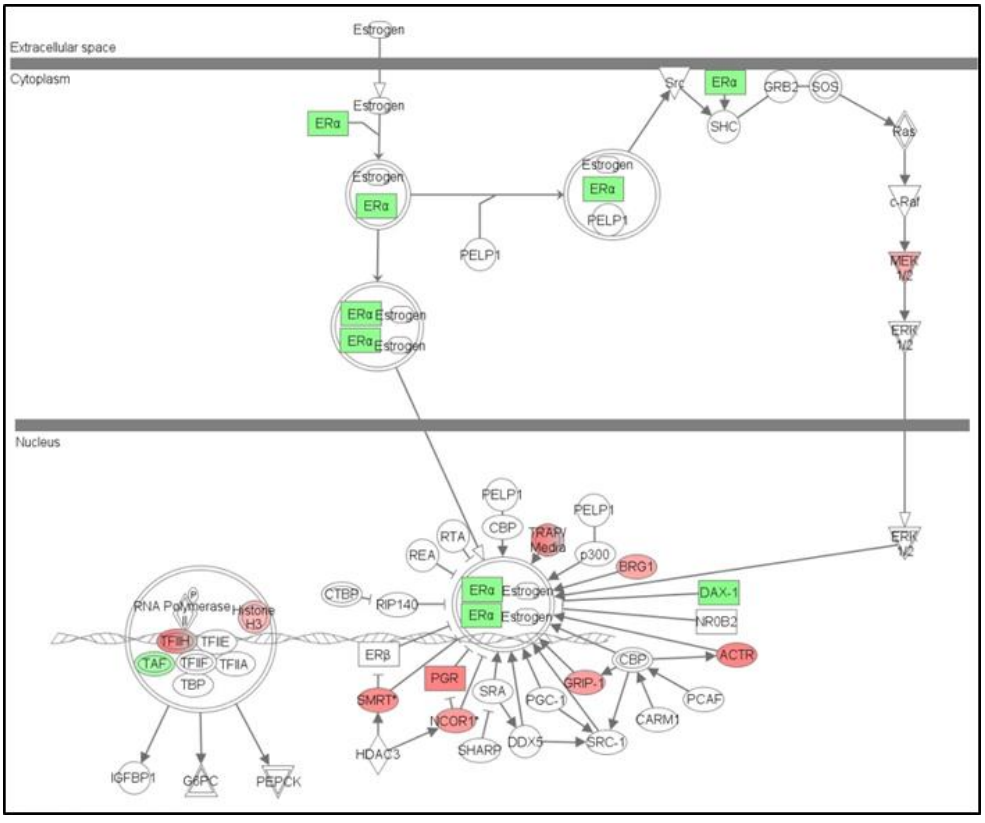


Figure 8: IPA results for the estrogen receptor α (or ESR1) signaling pathway. Effects of age on elements of this signaling cascade are shown below. Red color denotes down-regulation, green color denotes up-regulation ($P < 0.05$), and red/green denotes both up- and down-regulation in PND 2 compared to PND 0 uteri of gilts. Elements in white color are not affected by age. Genes affected by age include: ERα , progesterone receptor = PGR, mitogen activated protein kinase kinase ½ = MEK1/2, triiodothyronine receptor auxiliary protein = TRAP, Transcription activator = BRG1 (also known as ATP-dependent helicase SMARCA4), ACTR (also called AIB1 and SRC-3) was identified as a coactivator for nuclear receptors, glutamate receptor interacting protein 1 = GRIP1, nuclear receptor corepressor 1 = NCOR1, nuclear receptor corepressor =SMART, TATA Box Binding Protein-Associated Factor = TAF, and Dosage-Sensitive Sex Reversal-Adrenal Hypoplasia Congenita Critical Region on the X Chromosome, Gene 1 = DAX1.



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CHAPTER III

The Effects of Age and Nursing on Porcine Cervical Histoarchitecture and Cell Proliferation

ABSTRACT

Nursing supports neonatal porcine uterine and testicular development, however, lactocrine effects on cervical development are undefined. Studies were conducted to determine effects of: (1) age and imposition of the lactocrine-null state from birth [postnatal day (PND) 0] by milk replacer feeding on cervical histology; and (2) imposition of the lactocrine-null state for two days from birth on cervical cell proliferation, as reflected by proliferating cell nuclear antigen (PCNA) immunostaining. Study 1 and 2 gilts were randomly assigned (n = 5-10/group) to nurse *ad libitum* or to be gavage-fed porcine milk replacer (30 ml/kg BW/2 h) for 2 days or 14 days when cervices were collected. Tissues were also obtained from gilts at birth (PND 0) prior to nursing. Histologically, cervical crypt depth and height of luminal epithelium (LE) increased with age by PND 14 ($P < 0.001$) when both responses were reduced ($P < 0.001$) in replacer-fed gilts. Cell proliferation was reduced in LE at PND 2, and in cryptal epithelium and stroma by PND 14 in replacer-fed gilts compared to those that nursed. Returning replacer-fed gilts to nursing on PND 2 did not rescue the cervical phenotype by PND 14. Data indicate that age and nursing support neonatal porcine cervical development and cell proliferation and reinforce the importance of lactocrine signaling in porcine reproductive tissues.

INTRODUCTION

Maternal influence on neonatal development in mammals continues after birth through lactation and nursing (Peaker 2002). Nursing supports neonatal porcine reproductive tract development by delivery of milk-borne bioactive factors (MbFs) from mother to offspring as proposed in the lactocrine hypothesis (Yan *et al.* 2006, Bartol *et al.* 2008). In the neonatal pig, lactocrine signaling is important for uterine (Miller *et al.* 2013) and testicular (Rahman 2014) development. However, the extent to which age and nursing affect cervical histogenesis during the early neonatal period is unknown. Histologically, the adult porcine cervix is lined by columnar luminal epithelium (LE), and contains epithelial crypts that penetrate the adluminal stroma (Eldridge-White *et al.* 1989, Winn *et al.* 1993). Features of neonatal porcine uterine development are defined more completely with respect to both patterns of endometrial histogenesis (Bartol *et al.* 1993), and periods of postnatal life during which disruption of the uterine developmental program can affect the organizational trajectory and, ultimately, phenotype of the endometrium (Tarleton *et al.* 2001). For the porcine uterus, imposition of the lactocrine-null state for 48 h from birth [postnatal day (PND) 0], by feeding milk replacer in lieu of nursing, alters patterns of endometrial development (Miller *et al.* 2013). Effects of imposition of the lactocrine-null condition, detected as early as PND 2, were pronounced by PND 14 (Miller *et al.* 2013).

Colostrum (first milk) contains MbFs found in high concentrations that decline within the first 24-36 h of lactation during the transition from colostrum to milk (Klobasa *et al.* 1987, Neville *et al.* 2001, Nguyen 2001). In the porcine neonate a window for transmission of macromolecules, including MbFs, is open prior to gut closure, which is

estimated to occur within 48 h of birth (Leece 1973). When lactocrine-null neonatal gilts, deprived of colostrum for the first two days after birth, with substitution milk replacer feeding, were allowed to nurse in synchrony with littermates at PND 2, the uterine phenotype was not rescued. Whether the neonatal porcine cervix responds to the lactocrine-null challenge similar to the uterus is unknown.

Given that the impact of nursing on cervical histogenesis has not been described, the objective of study 1 was to determine the effects of age and imposition of the lactocrine-null state from birth through milk replacer feeding on cervical histology at PND 2 and PND 14. Study 2 objective was to determine the effects of imposition of the lactocrine null-state (i.e., absence of nursing) for two days from birth on patterns of cervical cell proliferation at PND 2 and PND 14. The working hypothesis for these objectives is that nursing supports neonatal porcine cervical development at PND 2 and PND 14.

MATERIALS AND METHODS

Animals

Crossbred gilts (*Sus scrofa domesticus;* Duron, Hampshire, Yorkshire, and Landrace genetics) were obtained from the swine unit of the New Jersey Agricultural Experiment Station, Rutgers University or at the Auburn University Swine Research and Education Center. At birth, gilts, born from sows at their first to fourth parity, were randomly assigned to nurse normally on their dams or to be fed porcine milk replacer (Advance Liqui-Wean ®; Carpentersville, IL, USA). Replacer-fed gilts were kept in a separate pen in close proximity to their dams. All pens were equipped with heat lamps. Gilts admitted to these studies had a minimum birth weight of 1.3 kg, based on evidence that postnatal development is retarded in lower body weight pigs (Rehfeldt & Kuhn 2006). Two studies were conducted, as outlined in Figure 1. In all studies, care was taken to insure that treatments were balanced for potential effects of litter (n = 22 litters for study 1; n = 12 litters for study 2.) Nursing groups were normalized by number of offspring and male siblings were not removed. All procedures involving animals were reviewed and approved by Institutional Animal Care and Use Committees and were conducted in accordance with the Guide for the Care and Use of Agriculture Animals in Research and Teaching (2010; Federation of Animal Science Societies, IL, USA).

Study 1 was conducted to determine the effects of age and nursing from birth on patterns of cervical histogenesis at PND 2 and PND 14 (Figure 1A). Gilts in this study were either euthanized at birth (PND 0; n = 10), prior to nursing, nursed *ad libitum* from birth to PND 2 (n =7) or PND 14 (n =6), or gavage-fed milk replacer (30 ml/kg BW/2 h;

(Houle *et al.* 2000) from birth to PND 2 (n =5) or PND 14 (n = 5). Cervices were collected on PND 0, PND 2, or PND 14.

Study 2 was conducted to determine the effects of replacer-feeding for two days from birth on cervical cell proliferation and histology (Figure 1B) at PND 2 and PND 14. Here, gilts were either 1) nursed *ad libitum* from birth (n =7); 2) gavage-fed milk replacer from birth (n=7) until PND 2; 3) nursed *ad libitum* from birth to PND 14 (n = 5); or 4) gavage-fed milk replacer for 48 h from birth and then switched to nursing until PND 14 (n = 6). Cervices were collected on PND 2 or PND 14.

Histology and Histomorphometry

At the time of tissue collection, cervices were trimmed of connective tissue, fixed in Xpress Molecular Fixative (Sakura Finetek, Torrance, CA, USA) or 4% paraformaldehyde and embedded in Paraplast Plus (Sigma-Aldrich, St. Louis, MO, USA). Preliminary experiments testing fixatives used on tissues at the same timepoint showed no difference on either hematoxylin and eosin (H&E) or proliferating cell nuclear antigen (PCNA) staining. Cross-sections (6 µm) of cervical tissues were affixed to Superfrost Plus slides (VWR International, LLC, Radnor, PA, USA). Tissues were deparaffinized, rehydrated, stained with Immunomaster hematoxylin (American Master Tech Scientific, Lodi, CA, USA) and counterstained with eosin (Fisher Scientific, Fair Lawn, NJ, USA). General histological observations and measurements were made in four to five nonsequential sections per gilt using an Olympus FSX100® microscope at 4.2x and 20x magnifications. As illustrated in Figure 2, cervical crypt depth for all crypts (both deep and shallow) in each section was measured from the basal aspect of the

luminal epithelium (LE) in the cervical crypt to the mouth of the crypt at the central lumen. Porcine cervical crypts were defined as columnar epithelia that form distinct folds that protrude from the lumen. Assessment of cervical luminal epithelial height was determined by measuring from the basal aspect to the apical surface of LE. A minimum of ten sites were chosen at random for each cervical tissue section (Ryan *et al.* 2001). Stromal thickness was measured from the basal border of the LE to the interface of stroma and the inner circular smooth muscle layer. Four quadrants were identified for each cervical cross section and one measurement was made at random in each quadrant of each section.

Immunohistochemistry (IHC)

Immunostaining to detect PCNA *in situ* was performed using a VectaStain ABC Elite kit (Vector Laboratories, Burlingame, CA, USA). Cervical sections were subjected to antigen retrieval in boiling sodium citrate buffer (pH = 6), blocked with normal horse serum at room temperature and incubated with mouse anti-rat PCNA IgG (1:100; 0.91 µg/ml; Invitrogen Life Technologies, Grand Island, NY, USA) overnight at 4°C. After incubation with biotinylated secondary antibody, endogenous peroxidase activity was blocked (3% hydrogen peroxide for 5 min. at room temperature) and sections were incubated with ABC reagent and developed using 0.1% diaminobenzidine substrate (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Negative control sections were incubated with mouse isotype control IgG (1.06 µg/ml; Invitrogen). Images were obtained using a QImaging camera and analyzed using ImageJ software (National Institute of Health, Bethesda, MD, USA).

PCNA Labeling Index (LI)

Digital images (10x magnification) of PCNA-stained cervical sections were converted to 8-bit grayscale using Adobe Photoshop (CS5 Version12.1; Adobe Systems Inc., San Jose, CA, USA). Nuclei were identified as positive when staining intensity values were at or above 30% of the highest values generated by ImageJ (Masters *et al.* 2007, Miller *et al.* 2013). For LE, cryptal epithelium (CE), and stroma, a minimum of 1000 cells (labeled and unlabeled) were counted for each gilt. Grayscale images were converted to pseudocolor using ImageJ in order to better illustrate staining intensity. Here, positively stained cells appear yellow-green on a black background. For each cell compartment (LE, CE, stroma), PCNA labeling index (LI), expressed as a percentage, was determined by dividing the number of PCNA-positive cells by the total number of cells counted and multiplying that value by 100 (Masters *et al.* 2007, Miller *et al.* 2013).

Statistics

Quantitative data were subjected to analyses of variance using GLM procedures (SAS 2013, Cary, NC, USA) and are presented as least squares means (LSM) ± SEM. Analyses considered variation due to main effects of treatment (nursing vs. replacer), age, and cell compartment (epithelium vs. stroma), as well as interactions as appropriate.

RESULTS

Study 1: Effects of age and nursing on cervical histoarchitecture

Effects of neonatal age on cervical luminal epithelial height, crypt depth, and stromal thickness are shown in Figure 3. Both luminal epithelial height (Figure 3A) and crypt depth (Figure 3B) were similar on PND 0 and PND 2, and increased ($P < 0.001$) by PND 14. Stromal thickness was not affected by age.

Figure 4 illustrates effects of neonatal age and milk replacer-feeding on cervical histoarchitecture. Overall, both luminal epithelial height and crypt depth increased ($P < 0.001$) from PND 2 to PND 14. Imposition of the lactocrine-null state from birth by replacer-feeding reduced ($P < 0.001$) luminal epithelial height and crypt depth by PND 14, when treatment effects were most pronounced (treatment x day; $P < 0.001$). In contrast, stromal thickness was unaffected by age or treatment.

Cervical histology on PND 0 and PND 14 in nursed and replacer-fed gilts is shown in Figure 5. Consistent with histological observations, cervical crypt development advanced from PND 0 to PND 14 and was most pronounced in nursed gilts in which, compared to replacer-fed gilts, more extensive branching of cervical crypts was observed by PND 14. Cervices from replacer-fed gilts obtained on PND 14 were similar histologically to tissues obtained on PND 0.

Study 2: Effects of age and nursing on cervical cell proliferation

Pseudocolored images illustrating effects of nursing for two days from birth on PCNA immunostaining patterns and histograms depicting PCNA LI data for cervical

tissues obtained on PND 2 and PND 14 are shown in Figure 6. Signal indicative of PCNA labeling above background was observed in all cell compartments. On PND 2 cervical histology of nursed gilts (Figure 6A) was similar to that of replacer-fed gilts (Figure 6B). However, imposition of the lactocrine-null state for two days from birth reduced ($P < 0.05$) PCNA LI compared to nursed gilts (Figure 6C) by PND 2. Overall, in nursed and replacer-fed gilts, PCNA LI was lower ($P < 0.01$) in epithelium (LE and CE) when compared to stroma (St) on PND 2. This effect was most pronounced for LE (treatment x cell compartment; $P < 0.05$).

Generally, in comparison with gilts nursed for two weeks from birth, cervical development on PND 14 was retarded in replacer-fed gilts that returned to nursing on PND 2 (Figure 6D vs. 6E). Overall, PCNA LI was lower (Figure 6F; $P < 0.001$) in epithelium in comparison to stroma, and higher ($P < 0.05$) in nursed as compared to replacer-fed gilts. Reduced PCNA LI associated with imposition of the lactocrine-null condition for two days from birth was most pronounced in CE and stroma (treatment x cell compartment; $P < 0.05$).

DISCUSSION

Results confirm and extend previous findings for the porcine uterus (Miller *et al.* 2013) and testes (Rahman 2014) by showing effects of age and nursing on cervical development that are evident on or before PND 14. New observations were presented regarding porcine cervical histogenesis that illustrate effects of imposition of the lactocrine-null state for two days from birth on cellular and molecular aspects of cervical development in the neonate. Results provide evidence that lactocrine signaling 1) contributes to the variety of factors that affect neonatal porcine cervical development, and 2) the first two days of neonatal life trigger cell compartment specific events that support cervical morphogenesis.

In nursed pigs, events associated with cervical histogenesis reported here include age-related increases in crypt depth, luminal epithelial height, and branching of crypts into the cervical stroma. Results indicate that, as observed for the uterus (Miller *et al.* 2013), cervical histology does not change appreciably between birth and PND 2, and that cervical crypt depth and luminal epithelial height increase with age by PND 14. However, as observed for endometrial gland development in the neonatal pig (Miller *et al.* 2013), cervical crypt development was retarded on PND 14 in lactocrine-null gilts. In the adult pig, cervical crypts form interdigitations important for copulation, insemination and fertility (Eldridge-White *et al.* 1989, Winn *et al.* 1993). Whether alterations in cervical development reported here for replacer-fed gilts affect reproductive performance later in life has yet to be determined. However, a relationship between the amount of colostrum consumed by female pigs on PND 0 and their lifetime fecundity was documented (Bartol *et al.* 2013). In a retrospective study involving 381 sows, low serum

immunoglobulin immunocrit values on their day of birth (Vallet *et al.* 2013), indicating minimal colostrum consumption, were associated with reduced litter size over four parities. Taken together, studies emphasize the importance of colostrum ingestion shortly after birth for support of female reproductive tract (FRT) development. Mechanisms through which lactocrine programming of FRT development may affect reproductive capacity remain to be defined.

Targeted disruption of FRT development during organizationally critical periods of postnatal life can have lasting effects on form and function of adult reproductive tissues (Crain *et al.* 2008, McLachlan *et al.* 2012). In the hamster, neonatal exposure to diesthylstilbesterol increased cervical diameter and altered LE histology, reflected by increased luminal epithelial height and cornification, in a manner that persisted into adulthood (Hendry *et al.* 2004). In sheep (Bartol *et al.* 1999, Gray *et al.* 2001) and mice (Cooke *et al.* 2012, Filant *et al.* 2012), strategic neonatal exposure to progestins inhibited development of uterine glands and suppressed fertility in adults. Such lasting effects are likely to reflect disruption of postnatal organization events that determine cell fate and, by consequence, morphogenetic processes that dictate tissue structure and function (Bartol & Bagnell 2012).

Although cervical histology at PND 2 was similar in nursed and replacer-fed gilts, reduced proliferation of LE was observed in replacer-fed gilts as reflected by PCNA LI. Treatment effects on patterns of cervical LE proliferation were similar to those described for the PND 2 porcine endometrium (Miller *et al.* 2013). Consistently, as observed for the neonatal porcine uterus (Miller *et al.* 2013), imposition of the lactocrine-null state from birth reduced PCNA LI in CE and stroma, but not in LE, on PND 14. Collectively,

results are consistent with the idea that lactocrine support is important for both cervical and endometrial development in the neonatal pig, and that mitogenic factors in colostrum support development (Donovan & Odle 1994, Blum & Baumrucker 2008).

Returning replacer-fed gilts to nursing on PND 2 did not rescue cervical phenotype by PND 14. Again, results for the cervix agreed with observations reported for the developing porcine endometrium (Miller *et al.* 2013). Factors to be considered in explanation for these observations include potential interactions of gastrointestinal (GI) tract maturation with parallel changes in the composition of colostrum that are associated with its transition to mature milk. In the pig, gut closure occurs within 24 h to 48 h after birth (Leece 1973). This event prevents further transfer of macromolecules, including potential MbFs, across the GI tract and into neonatal circulation. Milk replacer feeding can shorten the period from birth to gut closure to as early as 12 h postnatal (Jensen *et al.* 2001), thereby narrowing the window for MbF transfer. In parallel with such changes in GI tract function, changes in colostrum composition also occur within 48 h of parturition in the pig (Klobasa *et al.* 1987, Ogawa *et al.* 2014). Such changes may alter the nature and array of available lactocrine-active factors with potential to be transferred to the neonate by PND 2. This is when replacer-fed gilts were returned to nursing in study 2. The observation that mammotrope development was delayed throughout the prepubertal period when neonatal rats, deprived of lactation day 1 colostrum, were cross-fostered to dams on lactation day 7 supports this idea (Nusser & Frawley 1997). Both changes in neonatal gut closure dynamics and colostrum composition over time may define the critical window for lactocrine signaling.

Taken together, results presented here establish that, as reported for the neonatal porcine uterus (Miller *et al.* 2013) and testis (Rahman 2014), as well as for the murine hippocampus (Liu *et al.* 2014), lactocrine support is necessary for normal development of the neonatal porcine cervix. Disruption of cervical development by transient imposition of the lactocrine-null state for two days from birth altered the cervical developmental program in a manner that, as assessed on PND 14, could not be reversed by returning gilts to nursing in synchrony with their littermates. Data presented here reinforce that lactocrine regulation of postnatal events associated with cervical and, by extension, FRT development is initiated with ingestion of colostrum shortly after birth.

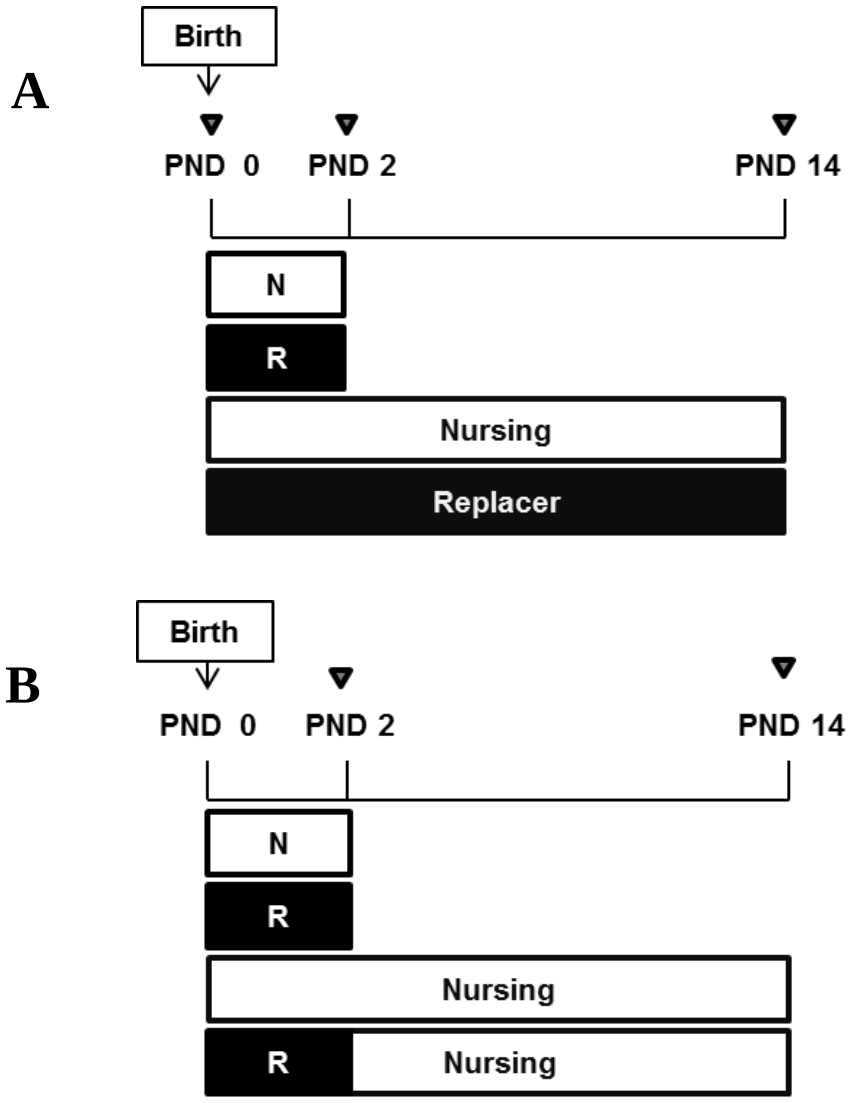
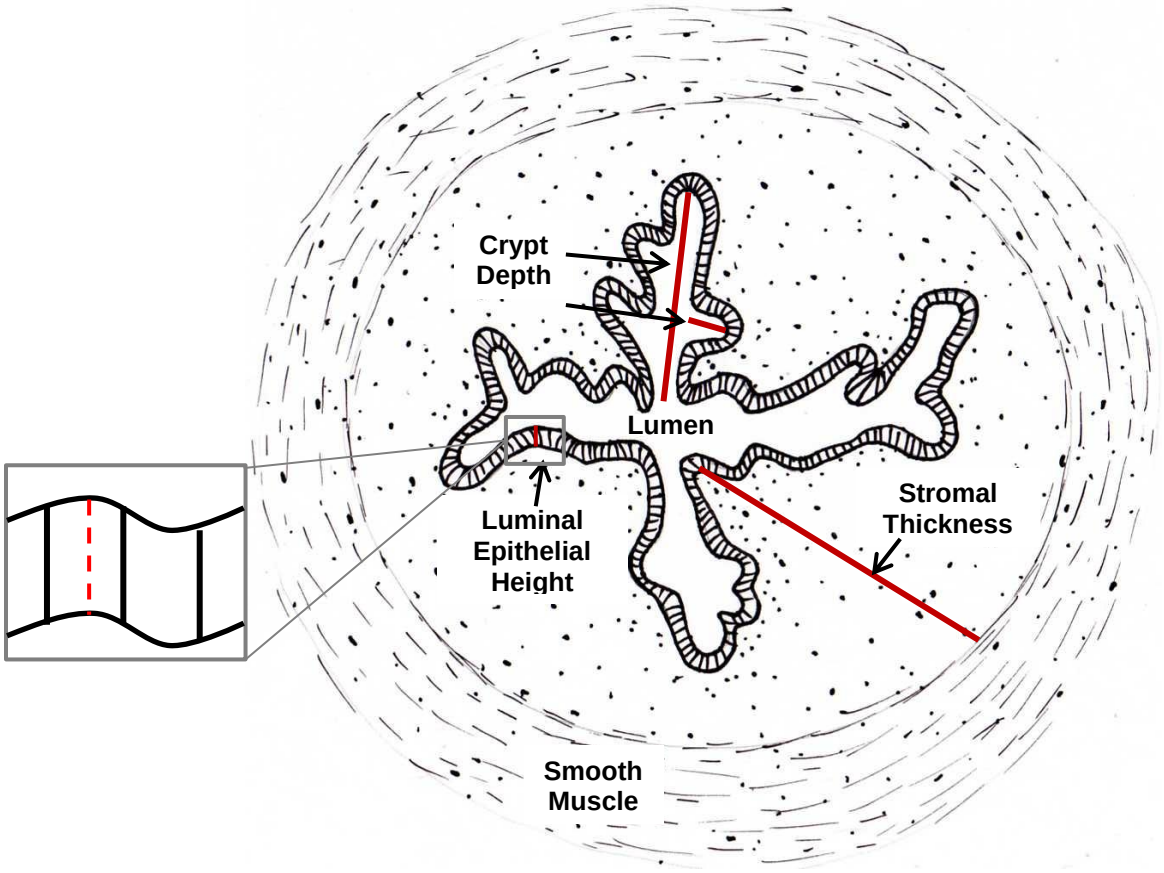


Figure 1: Experimental designs for A) study 1, and B) study 2. Tissues were obtained at birth (PND 0), prior to nursing, or gilts were allowed to either nurse (N) *ad libitum* or were gavage-fed milk replacer (R) from birth to PND 2 or PND 14. Cervices were obtained at time points as indicated by arrowheads; n = 5-10/group.

Figure 2: Schematic of the neonatal porcine cervix (cross section) illustrating the histomorphometric protocol employed to obtain measurements of luminal epithelial height, crypt depth, and stromal thickness. Red lines show how depth of 1) deep and 2) shallow cervical crypts was measured from the basal aspect of the luminal epithelium (LE) to the mouth of the crypt at the lumen. Magnified inset shows how cervical luminal epithelial height was measured from the basal aspect to the apical surface of LE. Stromal thickness was measured from the basal border of the LE to the interface of stroma and the inner smooth muscle layer.

Illustration by M.E. Camp



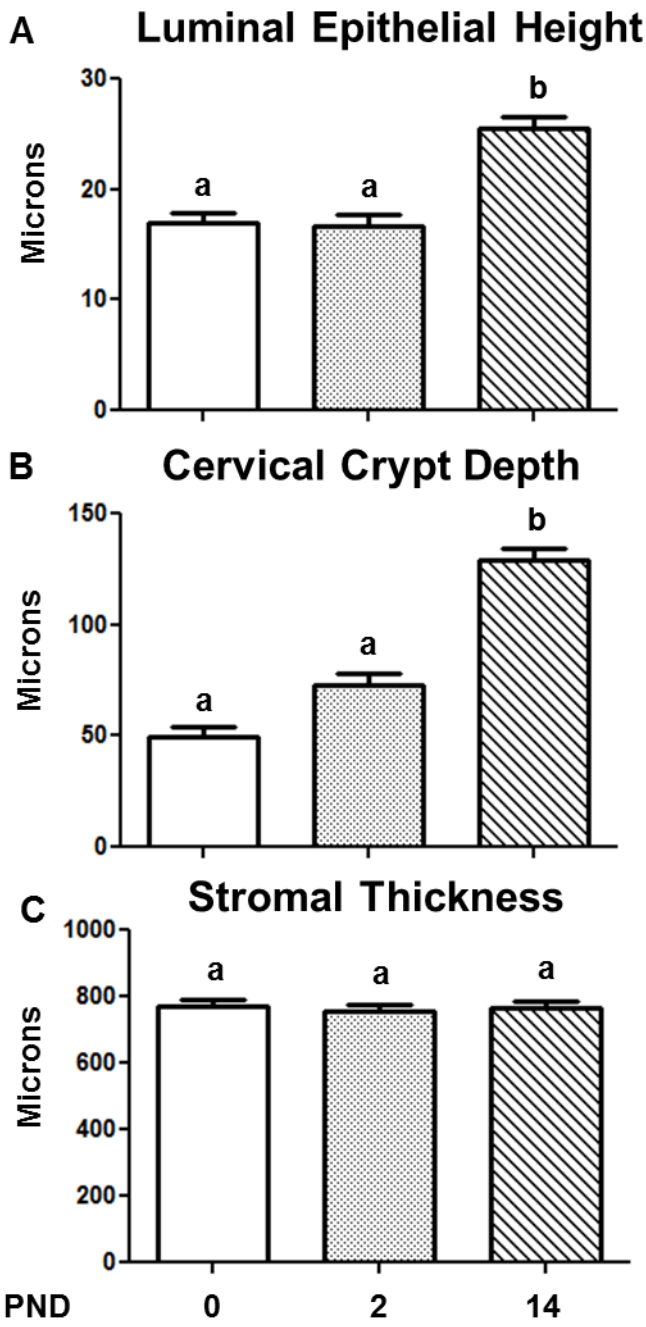


Figure 3: Effects of neonatal age on porcine cervial A) luminal epithelial height, B) crypt depth, and C) stromal thickness. Data are expressed as LSM ± SEM. Different letters dennote differences ($P < 0.001$).

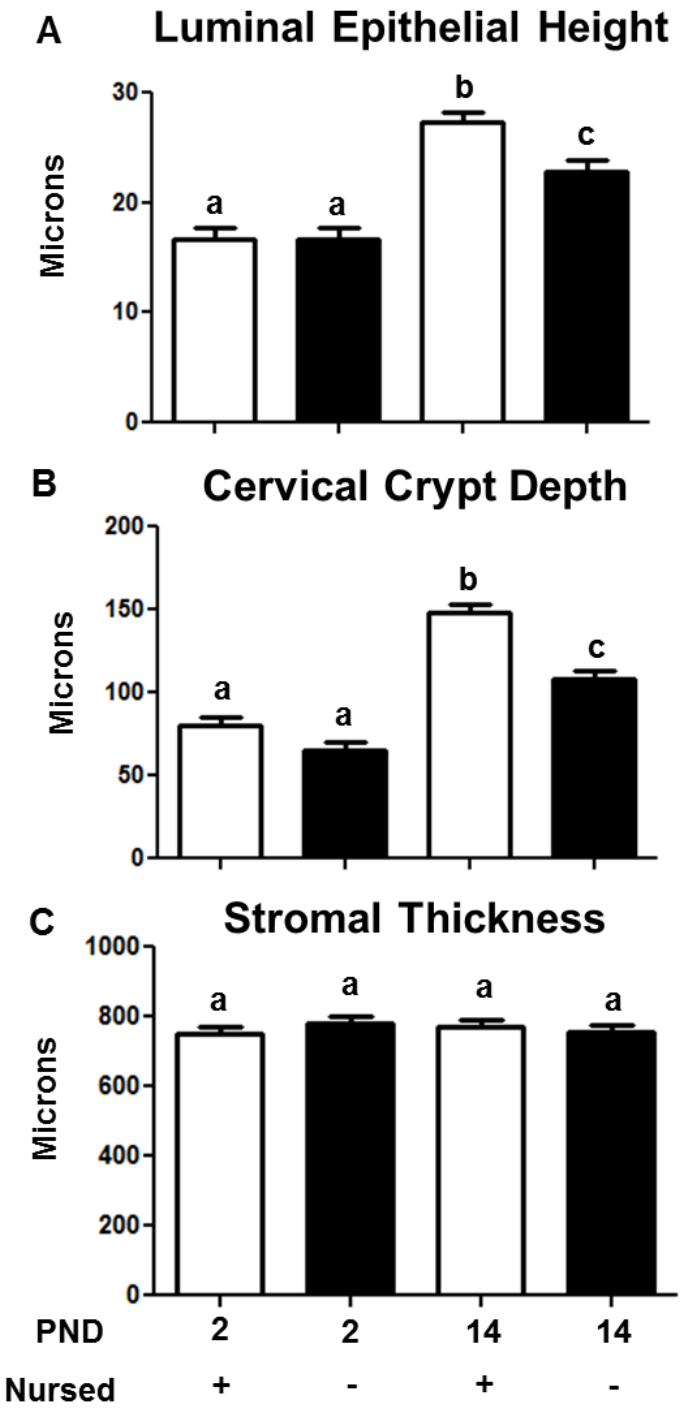


Figure 4: Effects of nursing (+) or milk replacer feeding (-) and neonatal age on A) luminal epithelial height, B) crypt depth, and C) stromal thickness. Data are expressed as LSM ± SEM. Different letters denote differences ($P < 0.01$).

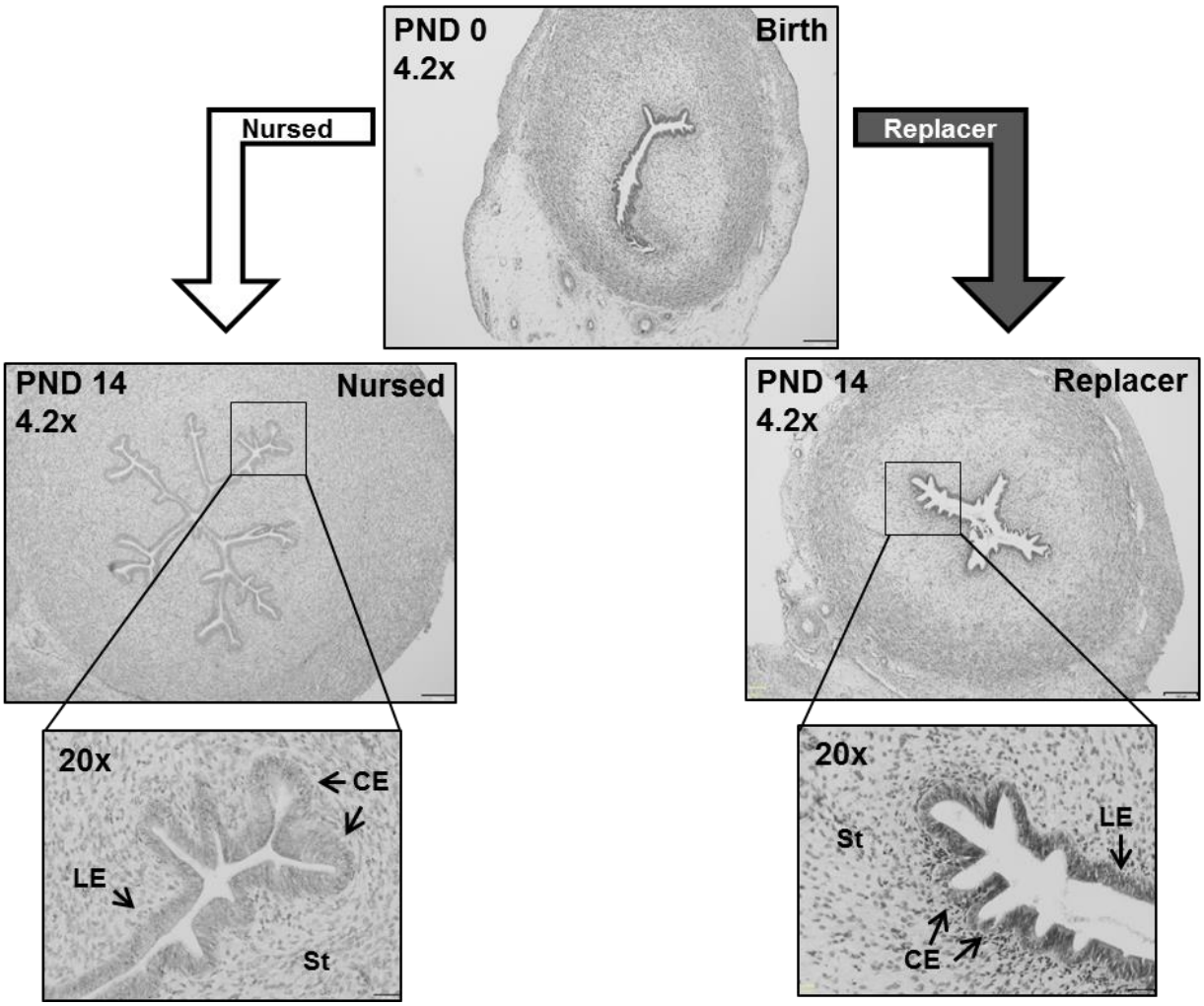


Figure 5: Cervical histoarchitecture affected by age and nursing. Photomicrographs of porcine cervical tissues obtained at birth (PND 0; top) and from nursed (left) as compared to replacer-fed (right) gilts on PND 14. Lower power images (4.2x; bar = 153 µm) and high power detailed (20x; bar = 32 µm) images illustrate effects of both age (PND 0 vs. PND 14) and imposition of the lactocrine-null condition from birth by replacer feeding on cervical histology. Crypt epithelium (CE), luminal epithelium (LE), and stroma (St) are indicated in 20x images.

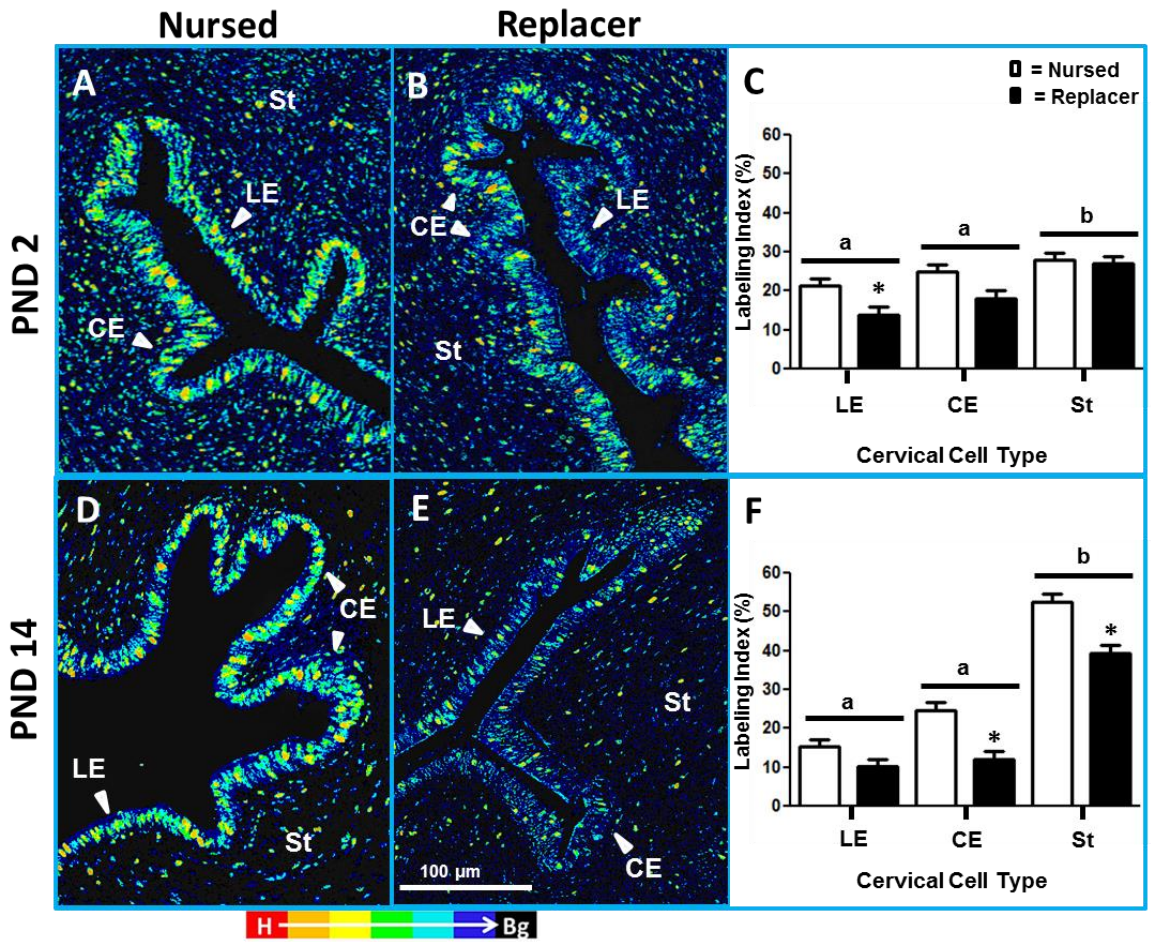


Figure 6: Effects of neonatal age and nursing on cervical PCNA immunostaining and PCNA labeling indices (LI). Pseudocolored images illustrate PCNA labeling patterns for cervical tissues obtained on PND 2 (A,B) and PND 14 (D,E) from nursed (A,D) and replacer-fed (B,E) gilts. Positively immunostained cells appear yellow-green on a black background; size bar indicates 100 µm. Associated data for PCNA LI by cervical cell compartment are shown on PND 2 (C) and PND 14 (F). White bars denote nursed and black bars denote replacer-fed groups. Data are presented as LSM ± SEM. Horizontal lines with different letters denote differences between cell compartments at PND 2 ($P < 0.01$) and PND 14 ($P < 0.001$). Asterisks denote cell compartment specific treatment effects (nursed vs. replacer) at PND 2 and PND 14 ($P < 0.05$).

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CHAPTER IV

Colostrum and Oral Insulin-like Growth Factor 1 Support Neonatal Porcine Cervical Proliferation and Development

ABSTRACT

Nursing supports neonatal porcine cervical development by providing a conduit for delivery of milk-borne bioactive factors (MbFs) from mother to offspring as proposed in the lactocrine hypothesis. Lactocrine effects on the porcine cervix are detectable as early as postnatal day (PND) 2, suggesting that such effects are induced shortly after first ingestion of colostrum (first milk). Insulin-like growth factor 1 (IGF1), a candidate MbF found in porcine colostrum, is recognized to stimulate growth of intestinal villi by PND 1. However, it is not known if oral IGF1 affects development of other neonatal somatic tissues. Here, the objective was to determine effects of a single feeding of colostrum compared to milk replacer feeding from birth, with or without orally supplemented IGF1, on cellular and molecular events associated with porcine cervical development at 12 h postnatal. Gilts were gavage-fed a single feeding (15 ml/kg BW) of either colostrum collected at birth or milk replacer, with or without supplemental IGF1 (1 µg/ml). After the initial feeding, all gilts were gavage-fed milk replacer until 12 h postnatal when cervical tissues were collected. Cervical cell proliferation patterns, reflected by proliferating cell nuclear antigen (PCNA) immunostaining and labeling indices (LI), and expression of proteins associated with the IGF1 signaling pathway [phosphorylated (p)AKT and antiapoptotic B-cell lymphoma 2 (BCL2)] were evaluated. PCNA LI were determined for crypt epithelium (CE), luminal epithelium (LE) and stromal cells. Cervical tissue lysate proteins were subjected to immunoblot analysis for pAKT and BCL2 using total AKT as a loading control. Cervical PCNA immunostaining was more consistent and intense at 12 h postnatal in gilts fed colostrum, colostrum with IGF1, or replacer with IGF1 as compared to gilts fed replacer alone. Immunostaining patterns were

similar in gilts fed colostrum with or without IGF1. Oral supplementation of IGF1 in replacer-fed gilts resulted in PCNA staining comparable to that seen in gilts fed colostrum. Compared to gilts given a single feeding of colostrum at birth, replacer-fed gilts had reduced cervical PCNA LI for CE ($P<0.01$), LE ($P<0.05$) and stroma ($P<0.05$) at 12 h postnatal. Cervical PCNA LI for all cell compartments were similar for gilts fed colostrum with or without IGF1. However, IGF1 supplementation of replacer-fed gilts produced cervical cell PCNA LI similar to those determined for gilts fed colostrum. Both BCL2 and pAKT were detectable on immunoblots at 12 h postnatal in gilts fed colostrum or replacer alone. However, signal intensity increased for BCL2 ($P<0.05$) and pAKT ($P<0.001$) in gilts given IGF1 in either colostrum or milk replacer. Results indicate that: (1) lactocrine effects of a single feeding of colostrum at birth can be identified in developing porcine cervical tissues at 12 h postnatal; (2) cervical cell proliferation is reduced in gilts fed milk replacer from birth; and (3) IGF1 is orally active in the neonatal pig as reflected by increases in cervical cell proliferation and expression of proteins in the IGF1 signaling pathway. Results also establish a shorter, more efficient bioassay system for identification of colostral lactocrine activity at 12 h postnatal, and implicate IGF1 as a potential lactocrine-active factor affecting female reproductive tract development.

INTRODUCTION

Maternal support of neonatal development in mammals continues after birth through lactation to insure an optimal developmental program (Peaker 2002). Nursing supports neonatal porcine reproductive tract development by delivery of milk-borne bioactive factors (MbFs) from mother to offspring as proposed in the lactocrine hypothesis (Yan *et al.* 2006, Bartol *et al.* 2008). In the neonatal pig, nursing supports cervical histogenesis by PND 14, and cell compartment specific proliferation at PND 2 and PND 14, as compared to lactocrine-null gilts fed milk replacer from birth (Camp *et al.* 2014).

Colostrum (first milk) contains bioactive factors including relaxin, a prototypical MbF (Frankshun *et al.* 2011), detected in circulation of nursed pigs by 12 h postnatal (Yan *et al.* 2006). Insulin-like growth factor 1(IGF1; 7.6 kDa), another member of the insulin family of hormones, is found in porcine colostrum at high concentrations (Simmen *et al.* 1988, Simmen *et al.* 1990, Donovan *et al.* 1994, Donovan & Odle 1994) that decline within the first 24 h to 36 h of lactation during the transition from colostrum to mature milk (Klobasa *et al.* 1987, Neville *et al.* 2001, Nguyen 2001). In the porcine neonate, a window for transmission of macromolecules, including MbFs, is open prior to gut closure in pigs, which is estimated to occur 24 h to 48 h after birth (Leece 1973). Orally administered IGF1 is locally bioactive in neonatal pigs promoting intestinal crypt cell proliferation and increasing villus height (Xu *et al.* 1994, Burrin *et al.* 1996, Houle *et al.* 2000). Whether oral IGF1 affects neonatal somatic tissue development beyond the gastrointestinal (GI) tract is unknown. IGF1 signaling can act via the phosphatidylinositol-3 kinase (PI3K)/AKT pathway (Tseng *et al.* 2002, Shelton *et al.*

2004) to promote cell proliferation, differentiation, and survival (Baserga & Rubin 1993). A serine/threonine kinase, AKT (Shelton *et al.* 2004), activated when phosphorylated (pAKT), increases levels of the anti-apoptotic protein B-cell lymphoma 2 (BCL2; (Mora *et al.* 2005). Thus, broadly, pAKT and BCL2 can be used as indicators of IGF1-mediated signaling.

Since lactocrine effects on cervical (Frankshun *et al.* 2012, Camp *et al.* 2014), uterine (Miller *et al.* 2013), and testicular (Rahman 2014) development are reported as early as 48 h after birth, it is likely that such effects could be detected earlier in development. This study was designed to determine if a single feeding of colostrum, as compared to milk replacer, administered at birth, with or without supplemental IGF1, affect cell behaviors associated with cervical development when assessed at 12 h postnatal. Evidence of such comparatively short-term effects would support establishment of a more efficient bioassay.

MATERIALS AND METHODS

Animals

Crossbred gilts (*Sus scrofa domesticus;* Duroc, Hampshire, Yorkshire, and Landrace genetics) were obtained from the swine unit of the New Jersey Agriculture Experiment Station, Rutgers University. At birth, gilts (n = 4-5/group), born from sows at their first to fourth parity, were gavage-fed a single dose (15 ml) of either 1) colostrum collected at the onset of parturition (0 hour lactation); 2) colostrum supplemented with recombinant human IGF1 (1 µg/ml; Pepro Tech, Rocky Hill, NJ, USA); 3) milk-replacer alone or 4) milk-replacer supplemented with IGF1 (1 µg/ml). Experimental design is illustrated in Figure 1. The dose of IGF1 used in these studies was based on the concentration of IGF1 found in porcine colostrum as reported by Simmen (Simmen *et al.* 1990). All pens were equipped with heat lamps. Gilts admitted to these studies had a minimum birth weight of 1.3 kg, based on evidence that postnatal development is retarded in lower body weight piglets (Rehfeldt & Kuhn 2006). After the initial feeding, all gilts were gavage-fed milk replacer (30 ml/kg BW/2 h) until 12 h when animals were euthanized and tissues were collected.

All procedures involving animals were reviewed and approved by relevant Institutional Animal Care and Use Committees and were conducted in accordance with the Guide for the Care and Use of Agriculture Animals in Research and Teaching (2010; Federation of Animal Science Societies, IL, USA).

Immunohistochemistry (IHC)

Immunostaining to detect PCNA *in situ* was performed using a VectaStain ABC Elite kit (Vector Laboratories, Burlingame, CA, USA). Cervical sections were subjected to antigen retrieval in boiling sodium citrate buffer (pH = 6), blocked with normal horse serum at room temperature and incubated with mouse anti-rat PCNA IgG (1:100; 0.91 µg/ml; Invitrogen Life Technologies, Grand Island, NY, USA) overnight at 4°C. After incubation with biotinylated secondary antibody, endogenous peroxidase activity was blocked (3% hydrogen peroxide for 5 min. at room temperature) and sections were incubated with ABC reagent and developed using 0.1% diaminobenzidine substrate (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Negative control sections were incubated with mouse isotype control IgG (1.06 µg/ml; Invitrogen). Images were obtained using a QImaging camera and analyzed using ImageJ software (National Institute of Health, Bethesda, MD, USA).

PCNA Labeling Index (LI)

Digital images (10x magnification) of PCNA-stained cervical sections were converted to 8-bit grayscale using Adobe Photoshop (CS5 Version12.1; Adobe Systems Inc., San Jose, CA, USA). Nuclei were identified as positive when staining intensity values were at or above 30% of the highest values generated by ImageJ (Masters *et al.* 2007, Miller *et al.* 2013). For LE, cryptal epithelium (CE), and stroma, a minimum of 1000 cells (labeled and unlabeled) were counted for each gilt. Grayscale images were converted to pseudocolor using ImageJ in order to better illustrate staining intensity. Here, positively stained cells appear yellow-green on a black background. For each cell compartment (LE, CE, stroma), PCNA labeling index (LI), expressed as a percentage,

was determined by dividing the number of PCNA-positive cells by the total number of cells counted and multiplying that value by 100 (Masters *et al.* 2007, Miller *et al.* 2013).

Protein extraction and Immunoblot analysis

Total cervical protein was extracted by homogenizing tissues (20 mg) in lysis buffer (1% Triton X-100, 10% glycerol, 150 mM Tris-HCl, 200 mM NaCl, and 1 mM $MgCl_2$, pH = 7.5)). Samples were centrifuged (12,000 x g/4°C/10 min), protein supernatant removed and stored (-80°C). Protein concentrations were measured using the DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Cervical lysates (20 µg) were resolved on 12% Bis-Tris-HCl-buffered polyacrylamide gels (Invitrogen, CA, USA) under reducing conditions followed by transfer onto nitrocellulose membranes (Bio-Rad Laboratories). Membranes were blocked with 10% nonfat milk powder (NFMP) in Tris-buffered saline containing Tween (TBST; 25 mM Tris (pH = 7.5), 0.14 mM NaCl, 3 mM KCl, and 0.05% Tween 20) for 1 h at room temperature. Membrane was incubated with rabbit anti-mouse phospho-AKT (pAKT; 1:1000; 60 kDa; Cell Signaling, Beverly, MA, USA) in TBST-5% NFMP overnight at 4°C. Membranes were washed with TBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000; Invitrogen) for 1 h at room temperature. After washing in TBST, membrane bound antibodies were detected by enhanced chemiluminescence (ECL; Perkin Elmer Life Sciences, Waltham, MA, USA) per manufacturer's recommendations on film. Membranes were washed with stripping buffer (Restore Western Blot Stripping Buffer, Thermo Scientific, Waltham, MA, USA) to remove antibodies. To ensure all antibodies were removed after stripping, ECL was added and membrane developed using film to confirm no signal present. Membranes

were then incubated with rabbit anti-mouse AKT (1:1000; 60 kDA; EMD Millipore, Burlington, MA, USA) overnight at 4°C, washed with TBST, then incubated with goat anti-mouse IgG (1:10,000; Invitrogen) for 1 h at room temperature. Signal was detected using ECL and film. Lastly, membranes were probed with rabbit anti-human B-cell lymphoma 2 (BCL2; 1:1000; 26 kDA; sc-492, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C, washed with TBST, then incubated with goat anti-rabbit IgG (1:10,000; Invitrogen) for 1 h at room temperature. The pAKT controls were lysates (10 µg) from bovine mammary epithelial cells (MAC-T) incubated with (+ control) or without (- control) IGF1 (Fleming *et al.* 2006). The BCL2 positive control lysate was human BCL2 transfected 293T cells (10 µg; sc-176463; Santa Cruz Biotechnology). Protein loading was monitored using total AKT. For AKT, pAKT, and BCL2, antibody specificity was evaluated by omission of primary antibodies in the immunoblot analysis. Incubation with secondary antibody alone showed no evidence for cross reactivity with cervical proteins. ECL signals were quantified densitometrically from film using Scion Image for Windows (Scion Corp., Frederick, MD, USA). All data were corrected for background signals and expressed as target protein to total AKT ratio.

Statistics

Quantitative data were subjected to analyses of variance using GLM procedures (SAS 2013, NC, USA) and are presented as least squares means (LSM) ± SEM. Analyses considered variation due to main effects of a single feeding of colostrum or milk replacer with or without oral IGF1 (colostrum vs. replacer feeding; colostrum alone vs. colostrum + IGF1; replacer alone vs. replacer + IGF1).

RESULTS

Effects of a single feeding of colostrum or milk replacer with or without oral IGF1 on cervical cell proliferation at 12 h postnatal

Images illustrating the effects of a single feeding of colostrum or milk replacer at birth, with or without oral IGF1, on cervical PCNA immunostaining patterns and PCNA LI at 12 h postnatal are shown in Figure 2. In comparison to gilts fed a single dose of colostrum at birth, replacer feeding alone was associated with a marked reduction in PCNA immunostaining at 12 h (Figure 2A vs. B). Colostrum-fed gilts showed similar PCNA immunostaining with or without oral IGF1 (Figure 2A vs. C). In contrast, oral IGF1 supplementation of replacer-fed gilts resulted in cervical immunostaining similar to gilts fed colostrum (Figure 2D vs. A and C). These observations were reflected by data for PCNA LI (Figure 2E-G). For cervical LE ($P < 0.01$), CE ($P < 0.001$) and stroma ($P < 0.05$), PCNA LI at 12 h postnatal was reduced in replacer-fed gilts when compared to gilts fed a single dose of colostrum. Colostrum-fed gilts supplemented with IGF1 showed similar PCNA LI compared to colostrum only controls for all cervical cell types. However, when replacer-fed gilts were supplemented with IGF1, PCNA LI for LE, CE, and stroma increased compared to replacer-alone gilts ($P < 0.01$); and were comparable to colostrum-fed gilts with or without oral IGF1.

Effects of a single feeding of colostrum or milk replacer with or without oral IGF1 on cervical BCL2 and pAKT proteins at 12 h postnatal

A representative immunoblot image showing BCL2, pAKT, and total AKT proteins at 12 h postnatal in cervical protein lysates from colostrum- or replacer-fed gilts,

with or without oral IGF1, is shown in Figure 3A. Immunoreactive BCL2 (26 kDa), pAKT (60 kDa) and AKT (60 kDa) proteins were detected in all cervical tissues. Colostrum- and replacer-fed gilts showed comparable expression of BCL2 protein. However, oral supplementation of IGF1 in colostrum-fed gilts increased BCL2 ($P < 0.05$) levels when compared to control gilts fed colostrum alone. Likewise, replacer-fed gilts supplemented with IGF1 had higher cervical BCL2 ($P < 0.05$) protein levels when compared to gilts fed replacer alone (Figure 3B). Cervical pAKT protein levels were similar in gilts fed colostrum or replacer alone. Cervical pAKT levels increased ($P < 0.001$) in colostrum-fed gilts supplemented with IGF1 when compared to gilts fed colostrum alone. Similarly, IGF1 supplemented replacer-fed gilts had increased ($P < 0.001$) pAKT protein levels when compared to gilts fed only replacer (Figure 3C).

DISCUSSION

Results confirm and extend previous findings for the porcine cervix (Frankshun *et al.* 2012, Camp *et al.* 2014), uterus (Miller *et al.* 2013), and testes (Rahman 2014). Results indicate that a single feeding of colostrum at birth supports cervical cell proliferation and establish that such effects are detectable by 12 h postnatal. Further, results support a shorter 12 h bioassay protocol for evaluation of potential lactocrine active factors. Data shows that IGF1 is orally active in the porcine neonate as reflected by effects on cervical PCNA LI, and patterns of cervical pAKT and BCL2 expression.

A candidate MbF, IGF1 is present in colostrum and milk of multiple species including humans (Nagashima *et al.* 1990, Donovan *et al.* 1994, Donovan & Odle 1994) cows (Francis *et al.* 1988, Campbell & Baumrucker 1989) goats (Prosser *et al.* 1990) and pigs (Simmen *et al.* 1988). Similar to milk-borne relaxin in pigs (Frankshun *et al.* 2011), IGF1 concentrations in porcine milk are highest during the first few days of lactation and decline during the transition of colostrum into milk (Simmen *et al.* 1990, Donovan *et al.* 1994). Milk-borne IGFs are present in free and bound states throughout lactation (Donovan *et al.* 1994, Xu & Wang 1996). IGF binding proteins (IGFBPs) -1, -2, -3, and -4, present in pig milk (Simmen *et al.* 1988, Donovan *et al.* 1994) function as transport vehicles, increase the half-life of circulating IGF1 (Walton *et al.* 1989), and can modulate IGF activity (Baxter 1988, McCusker *et al.* 1991).

Whether orally administered IGF1 can enter the neonatal porcine circulation is unclear. Serum IGF1 concentrations did not differ between replacer-fed pigs with or without supplemental IGF1 for four days from birth when evaluated at 24 h, 48 h, 72 h,

or 96 h postnatal (Burrin *et al.* 1996). Similarly, porcine serum IGF1 cocnentrations assessed on PND 14 were unaffected by oral supplementation of IGF1 delivered in milk replacer (Houle *et al.* 2000). Nevertheless, both studies reported proliferative effects in the GI tract for IGF1-supplemented pigs (Burrin *et al.* 1996, Houle *et al.* 2000). By contrast, serum IGF1 concentrations in neonatal rats fed formula supplemented with IGF1 for three days were higher than those found in rats fed formula devoid of IGF1 (Philipps *et al.* 1997). Additionally, when fed orally to pigs at birth or on PND 3, ^{125}I-IGF1, administered in colostrum, was detected in peripheral plasma within 1 h after administration, and was found in the stomach, small intestine, kidney, and other somatic tissues by 4 h post-administration (Xu & Wang 1996). These results indicated that orally administered IGF1 can and does enter the neonatal circulation. Consistently, in a comparison of neonatal pigs that were either nursed or replacer-fed (Dauncey *et al.* 1994), nursed pigs displayed higher plasma IGF1 concentrations and increased growth rate. In that study, hepatic IGF1 levels were similar in nursed and replacer-fed pigs, suggesting that differences in plasma IGF1 concentrations were due to high levels of milk-borne IGF1 (Dauncey *et al.* 1994). Currently, the preponderance of data supports the idea that IGF1 acquired orally can enter the neonatal circulation. To the extent that this is the case, effects of orally administered IGF1 reported here could reflect direct actions of this peptide growth factor at the level of the cervix.

Oral IGF1 can stimulate growth of neonatal porcine GI tissues. In replacer-fed pigs, oral IGF1 promoted cell proliferation in intestinal crypts (Xu *et al.* 1994), increased jejunal and ileal villus height by PND 4 (Burrin *et al.* 1996), and both jejunal villus height and mucosal DNA content and synthesis by PND 14 (Houle *et al.* 2000). Present

results indicate that the porcine cervix is also a target for oral IGF1. The extent to which these effects reflect direct actions of orally administered IGF1 at the level of the cervix or an alternate, indirect mechanism (Baumrucker & Blum 1993) is not known.

Supplementation of colostrum with IGF1 did not affect cervical cell proliferation. However, in replacer-fed gilts oral IGF1 administration increased cervical cell proliferation to levels equivalent to those observed for colostrum-fed gilts at 12 h postnatal. One explanation for these effects is that agents in colostrum that are absent in milk replacer, including IGFBPs (Donovan *et al.* 1994), caseins and/or lactoferrin (Xian *et al.* 1995), may bind IGF1 and impede its uptake into the neonatal circulation, thereby reducing IGF1 bioavailability. The most abundant binding protein in peripheral circulation, IGFBP3 can bind substantial amounts of circulating IGF1, thereby increasing its half-life (Baxter 1991). Also present at high concentrations in the circulation of young pigs (Dauncey *et al.* 1993), IGFBP2, together with IGFBP3, could further affect IGF1 bioavailability. To the extent that IGFBP profiles differ between colostrum- and replacer-fed gilts, this could explain, in part, the proliferative responses of cervical cells seen in IGF1 supplemented, replacer-fed gilts, in which IGF1 bioavailability would be greater.

Cervical pAKT and BCL2 responses indicated that orally administered IGF1 was active in colostrum- and replacer-fed gilts. IGF1 stimulates cell proliferation and promotes cell differentiation and survival through interactions with the IGF1 receptor and subsequent activation of signaling cascades involving the PI3K/AKT pathway (Woodgett 2005). A serine/threonine kinase, AKT plays a role in inhibition of apoptosis by phosphorylating and inactivating several proteins involved in the apoptotic cascade

(Dudek *et al.* 1997, Mora *et al.* 2005). Activated pAKT can increase levels of anti-apoptotic proteins including BCL2 (Mora *et al.* 2005).

The fact that cervical pAKT and BCL2 expression levels were similar at 12 h postnatal in gilts given a single feeding of either colostrum or milk replacer at birth indicates that cervical expression of these molecules is not acutely lactocrine sensitive. In contrast to results obtained for cervical cell proliferation, oral IGF1 supplementation increased cervical pAKT and BCL2 expression regardless of the medium in which it was delivered. An explanation for these observations may be that pAKT and BCL2 responses are more sensitive to induction by IGF1. Reduced IGF1 bioavailability in colostrum (Simmen *et al.* 1988, Xian *et al.* 1995) could also contribute to differing results, as indicated above. Furthermore, the capacity of endogenous IGFBPs to bind additional, exogenous IGF1 can be limited by the degree of binding protein saturation (Umezawa *et al.* 1991). The extent to which agents in colostrum affect the bioavailability of IGF1 or other potentially lactocrine-active MbFs will be important to determine.

Taken together, results presented here establish evidence that lactocrine effects of a single feeding of colostrum at birth can be detected in developing porcine cervical tissues by 12 h postnatal. Results indicate that lactocrine regulation of postnatal events associated with cervical and, by extension, FRT development is initiated with colostrum ingestion shortly after birth. This observation provides the basis for development of a more efficient bioassay for identification of lactocrine-active MbFs. Present results also implicate IGF1, biologically active in the piglet, as a potential lactocrine-active MbF factor affecting FRT development.

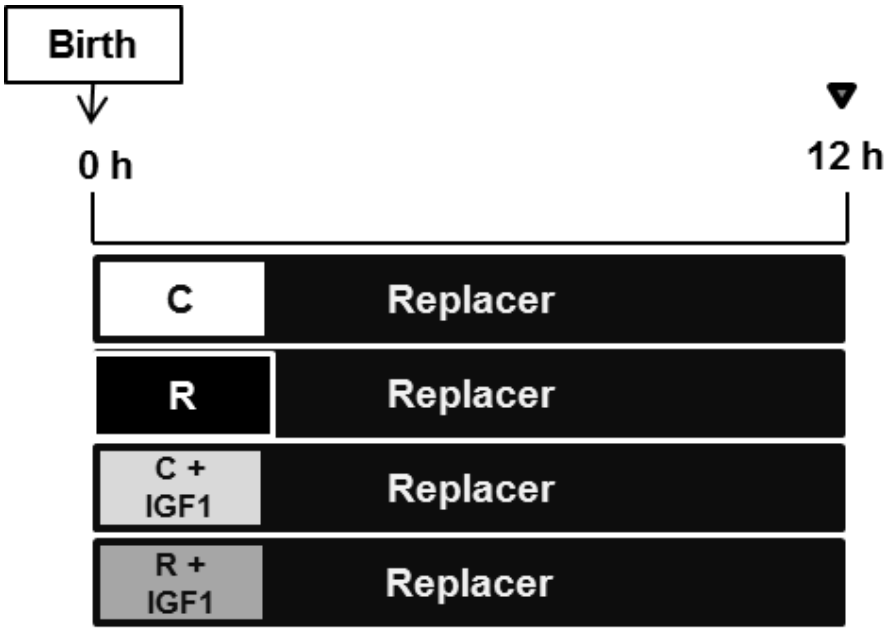

Figure 1: Experimental design of the oral IGF1 study. Gilts (n=4-5/group) were gavage-fed a single feeding of colostrum (C; collected at the onset of parturition at 0 h lactation) or milk replacer at birth, with or without IGF1, followed by milk replacer feeding (30 ml/kg BW/2 h) until 12 h when tissues were collected. Cervices were obtained at 12 h postnatal as indicated by arrowhead.

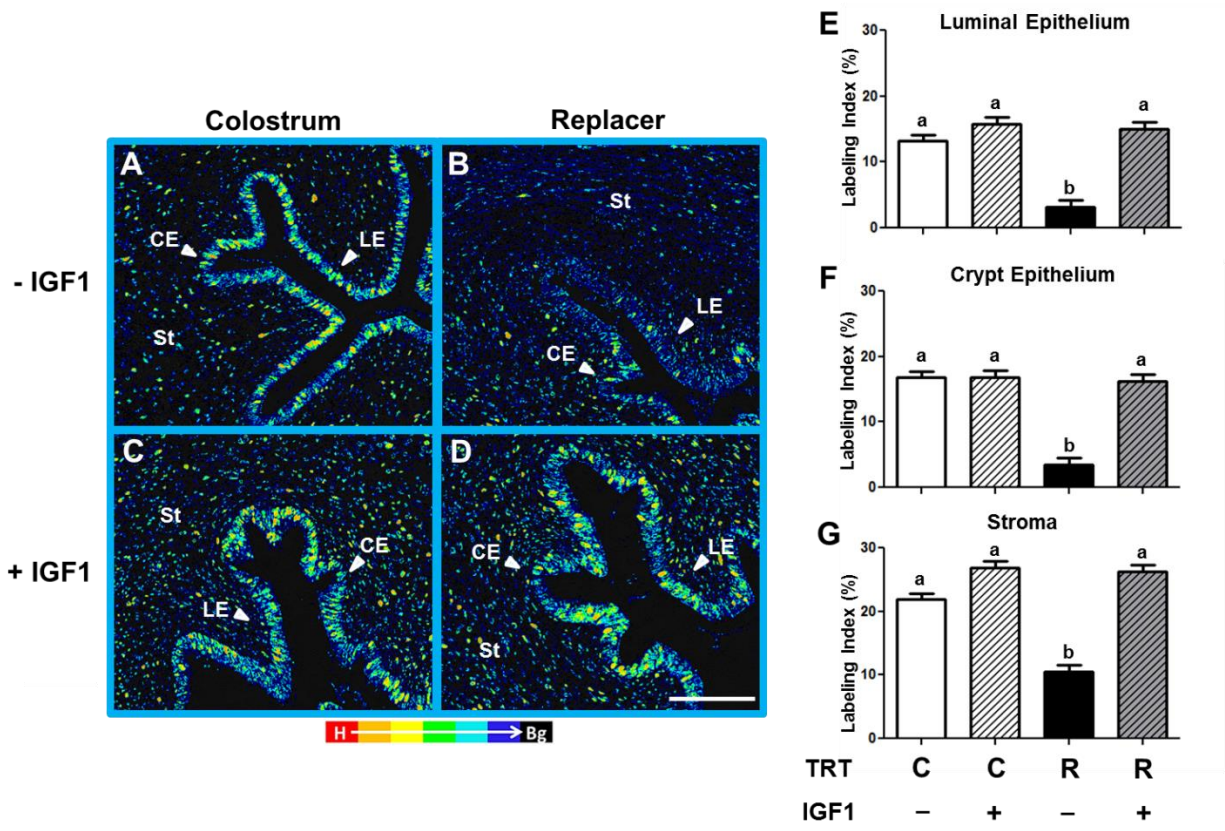

Figure 2: Effects of a single feeding of colostrum (C) or milk replacer (R), with (+) or without (-) oral IGF1, on cervical PCNA immunostaining patterns (2A,B,C,D) and PCNA LI (2E,F,G) at 12 h postnatal. Pseudocolored images illustrate PCNA labeling patterns in luminal epithelium (LE), crypt epithelium (CE), and stroma (St). Positively stained cells appear yellow-red on a black background; size bar indicates 100 µm. For histograms, data are presented as LSM ± SEM and different letters denote differences (LE, $P < 0.01$; CE, $P < 0.001$; St, $P < 0.05$).

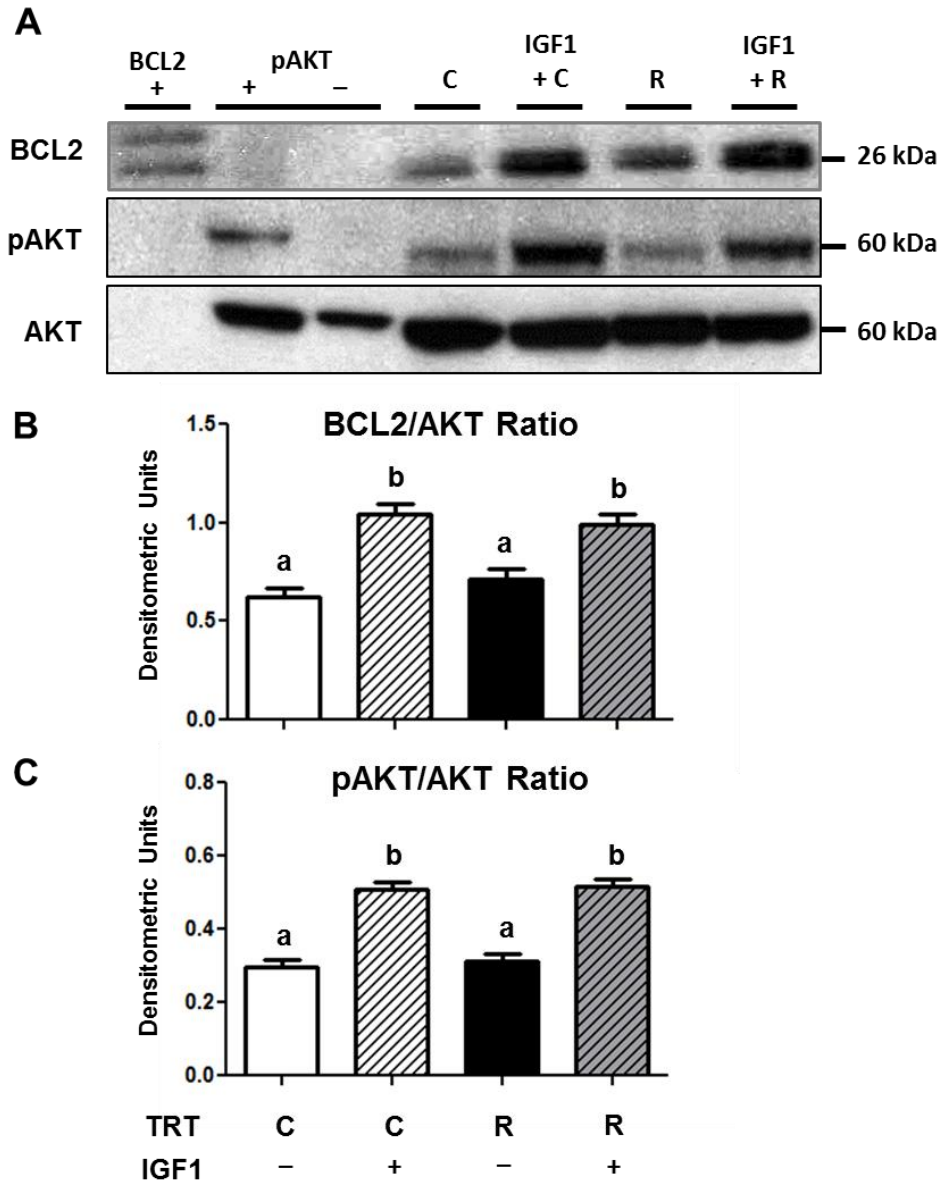

Figure 3: Effects of a single oral feeding of colostrum (C) or milk replacer (R), with (+) or without (-) IGF1, on cervical BCL2 and phosphorylated (p)AKT proteins at 12 h postnatal. Representative immunoblot (A) images show positive (+; BCL2 and pAKT) and negative (-; pAKT) control samples as described in methods. Densitometric data for the relative expression of BCL2 (B) and pAKT (C) were normalized to total AKT and expressed as LSM ± SEM. Different letters indicate differences (BCL2, $P < 0.05$; pAKT, $P < 0.001$).

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CHAPTER V

DISSERTATION CONCLUSIONS

DISSERTATION CONCLUSIONS

Ingestion of colostrum during nursing supports neonatal development by delivering milk-borne bioactive factors (MbFs) from mother to offspring as proposed in the lactocrine hypothesis. Lactocrine support of neonatal porcine uterine (Miller *et al.* 2013) and testicular (Rahman 2014) development have been reported. Results of experiments described in this dissertation extend the scope of the lactocrine hypothesis to include: 1) effects of neonatal age from birth to PND 2 on global uterine gene expression; 2) effects of age and nursing on porcine cervical histoarchitecture and cell proliferation; and 3) evidence that oral IGF1 is lactocrine-active in promoting cervical cell proliferation using neonatal pig bioassay for identifying lactocrine activity at 12 h postnatal. Observations provide the basis upon which future directions will continue testing the lactocrine hypothesis.

The porcine uterus begins developing prenatally and completes development postnatally (Bartol *et al.* 1993). Events characteristic of early uterine development in the pig include appearance and proliferation of uterine glands, development of endometrial folds, and differentiation and growth of the myometrial smooth muscle layers (Bartol *et al.* 1993). Imposition of the lactocrine-null state, through milk replacer feeding, has illustrated the importance of nursing from birth for support of endometrial adenogenesis and markers and mediators of uterine development, including ESR1 by PND 2 (Miller *et al.* 2013); thereby indicating a sensitive 48 h window for postnatal uterine development. Results of the RNA-seq study presented in Chapter II provide new information about the postnatal porcine uterine transcriptome during the first two days of life and reveal more

than 3000 differentially expressed genes and functional pathways associated with the first 48 h of neonatal porcine uterine development. Global, age-sensitive effects on the neonatal uterine transcriptome revealed pathways known to be important for uterine development, including Wnt-β-Catenin, MMP, and ESR1 signaling. In addition, RNA-seq analysis also uncovered novel processes and differentially expressed genes not previously associated with uterine development within two days postnatal, including genes in the chemokine and cytokine signaling pathway. Although a role for chemokines and cytokines on neonatal porcine uterine development had not been previously explored, other studies implicate these molecules in tissue development and morphogenesis.

Although neonatal porcine uterine development has been described (Tarleton *et al.* 1999, Bartol *et al.* 2006, Miller *et al.* 2013), Chapter III provides the first report of neonatal porcine cervical development from birth as assessed by histological measurements and cervical cell proliferation. Previous studies from our lab demonstrated the effects of age and lactocrine signaling on markers of cervical development, including the relaxin receptor (RXFP1; (Frankshun *et al.* 2012)). Here, observations for the neonatal porcine cervix show it develops in a manner similar to that of the neonatal uterus, in that cervical histology appears similar at birth and PND 2. However, from PND 2 to PND 14, changes in cervical histology include 1) extensive branching of cervical crypts and 2) age-related increases in measurements of cervical crypt depth and luminal epithelial height. As reported for endometrial gland development in the neonatal pig (Miller *et al.* 2013), cervical crypt development was retarded and cell proliferation was decreased on PND 14 in lactocrine-null gilts and their cervices appeared similar histologically to cervices of gilts at birth. Returning gilts to nursing in synchrony with

littermates after two days of milk replacer feeding did not rescue the uterine phenotype at PND 14 (Miller *et al.* 2013) nor the cervical phenotype reported here. In adult pigs, the cervix is lined with columnar LE and contains epithelial crypts or folds that penetrate the adluminal stroma important for insemination and fertility (Eldridge-White *et al.* 1989, Winn *et al.* 1993). Whether disruption of neonatal cervical development persists to influence reproductive performance in adults is unknown. However, long-term effects of colostrum consumption in neonatal pigs on fecundity in adulthood were documented in a retrospective study (Bartol et al., 2013). Decreased serum immunoglobulin immunocrit values on PND 0, an indicator of minimal colostrum ingestion in pigs (Vallet *et al.* 2013), were associated with reduced litter size in adulthood (Bartol *et al.* 2013).

Previous reports from our laboratory established relaxin (RLX) as a prototypical, lactocrine-acting MbF in the porcine neonate (Yan *et al.* 2006, Frankshun *et al.* 2009). There are many other potential lactocrine-active factors in porcine mammary secretions (Grosvenor *et al.* 1993, Donovan & Odle 1994, Playford *et al.* 2000), including another member of the insulin family of hormones, IGF1 (Simmen *et al.* 1988). Peaker and Neville (1991) proposed a set of criteria to be fulfilled in determining whether an MbF influences the suckling neonate that can be applied to determine whether IGF1 acts through a lactocrine mechanism (Peaker & Neville 1991). First, IGF1 must be present and active in porcine colostrum and milk. Second, greater IGF1 levels must be present in the neonatal circulation after nursing. Third, expression of IGF1 receptors must be present in target tissues and lastly, biological activity of IGF1 on the neonate must occur (Peaker & Neville 1991, Peaker 2002, Yan *et al.* 2006). Based on literature, IGF1 meets the first criterion in that it was identified in colostrum and milk of several species

(Nagashima *et al.* 1990, Prosser *et al.* 1990, Donovan *et al.* 1991a, Donovan *et al.* 1991b), including the pig (Simmen *et al.* 1988). Whether milk-borne IGF1 meets the second criterion is debatable however, the preponderance of data supports this idea (Dauncey *et al.* 1994, Xu & Wang 1996, Philipps *et al.* 1997). Receptors for IGF1 (IGFR) have not been detected in the cervix. However, IGF1R was detected in uterine GE in primates (Hild-Petito *et al.* 1994), LE and GE of mice (Baker *et al.* 1996), and myometrium of immature rats (Ghahary & Murphy 1989). In the adult pig, uterine IGF1R were also detected (Hofig *et al.* 1991), although it has not yet been determined whether they are present in the neonate. However results presented in Chapter IV indicate that oral IGF1 is biologically active in the neonatal porcine cervix, as reflected by positive effects on cervical cell PCNA LI, and expression of pAKT and BCL2 protein.

Observations presented in Chapter IV indicate the effects of a single feeding of colostrum or milk replacer, at birth, with or without oral IGF1 supplementation, on cervical cell proliferation at 12 h postnatal. Furthermore, to determine if the downstream PI3K/AKT signaling pathway was activated following oral IGF1 ingestion, cervical pAKT and BCL2 were examined. Colostrum delivered by orogastric gavage was effective in promoting cervical cell proliferation when compared to replacer-fed gilts. IGF1 supplementation increased cervical cell proliferation in replacer-fed gilts compared to gilts fed replacer alone, and increased cervical pAKT and BCL2 proteins in both colostrum- and replacer-fed gilts. These observations indicate IGF1 is orally active in the neonatal pig, yet IGF1 bioavailability may be regulated by unique factors in colostrum that are absent in milk replacer, including IGFBPs (Simmen *et al.* 1988, Donovan *et al.* 1994). Because the majority of IGF1 is associated with carrier proteins (Simmen *et al.*

1988) or caseins (Xian *et al.* 1995), the extent to which such proteins alter IGF1 bioavailability may explain results presented here. Furthermore, the capacity of endogenous IGFBPs to bind additional, exogenous IGF1 can be limited by the degree of binding protein saturation (Umezawa *et al.* 1991). Results implicate IGF1 as orally active in the neonatal pig and identify it as a candidate lactocrine-active factor.

This dissertation research provides support for the lactocrine hypothesis; specifically by determining lactocrine signaling effects on the developmental program and trajectory of the neonatal porcine cervix and uterus. Results establish that neonatal age has a global effect on the neonatal porcine uterine transcriptome from birth to PND 2. The RNAseq study supports the idea that high throughput technologies can reveal novel genes, pathways and processes involved in postnatal uterine development and provide the rationale for further studies analyzing age-sensitive biological processes and mechanisms, including the uterine microRNAome, from birth to PND 2. Observations have expanded the scope of the lactocrine hypothesis to include developmental changes in the cervix observed in response to age and nursing. Results from Chapter IV established a new shorter 12 h bioassay to determine lactocrine activity and oral IGF1 is bioactive in the neonatal pig. Finally, long term studies should be designed to determine whether nursing or implementation of the lactocrine-null state from birth persist into puberty and have effects on reproductive success in adulthood. Additionally, knowledge of the relevance of lactocrine-active factors in colostrum using a neonatal pig bioassay could be used to improve milk-based formulas used for agricultural animals and/or non-breast fed infants. Results support the World Health Organization's message regarding the importance of nursing in humans. Considering that all mammals nurse their young, this

research may be important in promoting breastfeeding awareness and its impact on neonatal health.

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