MATERNAL LACTOCRINE PROGRAMMING OF THE PORCINE UTERUS

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ABSTRACT OF THE DISSERTATION

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Nursing ensures lactocrine delivery of milk-borne bioactive factors to offspring and the lactocrine hypothesis for maternal programming proposes that disruption of lactocrine communication from birth (postnatal day = PND 0) affects both the program and trajectory of porcine uterine development. Establishment of a model system for study of lactocrine-sensitive uterine organizational events shortly after birth in the neonate and for identification of potentially lactocrine-active factors found in porcine colostrum, such as insulin-like growth factor 1 (IGF1), is important. Studies show that the neonatal porcine uterine transcriptome is age- and lactocrine-sensitive on PND 2. However, whether uterine microRNA (miRNA) expression or the uterine miRNA-mRNA interactome is affected similarly is unknown. Lactocrine deficiency from birth, reflected by low serum immunoglobulin immunocrit, was associated with alterations in aspects of the neonatal uterine developmental program and, long-term, was linked to reduced lifetime fecundity in adult gilts. These observations suggested lactocrine effects on programming of adult uterine function. However, whether lactocrine deficiency and

disruption of neonatal uterine development is ultimately reflected by effects on patterns of endometrial gene expression during the periattachment period of early pregnancy in adulthood is unknown. Consequently, research objectives were to: (1) determine acute effects of (a) nursing vs milk replacer feeding, and (b) method of feeding a single dose of colostrum at birth, with or without supplemental IGF1, on porcine uterine development at 12 h postnatally; (2) determine short-term effects of age and nursing on porcine uterine (a) miRNA expression between birth and PND 2 and (b) miRNA-mRNA interactions using integrated target prediction analysis; and (3) determine long-term effects of lactocrine-deficiency from birth on adult endometrial (a) mRNA and miRNA expression during the periattachment period of early pregnancy (pregnancy day 13), including identification of affected miRNA–mRNA interactions. Results showed nursing for 12 h from birth supports rapid establishment of a uterine developmental program, and that a single feeding of colostrum at birth increased endometrial cell proliferation at 12 h, regardless of method of feeding. Further, oral IGF1 was sufficient to support endometrial cell proliferation at 12 h in replacer-fed gilts, and supplementation of colostrum with IGF1 further increased endometrial cell proliferation. Between birth and PND 2, novel age- and lactocrine-sensitive uterine miRNAs and miRNA-mRNA relationships associated with porcine neonatal development were identified. On pregnancy day 13, lactocrine deficiency from birth did not affect corpora lutea number, uterine horn length, uterine wet weight, embryo recovery, or uterine luminal fluid estrogen content. However, next-generation sequencing analyses revealed lactocrine-sensitive endometrial mRNAs and miRNAs associated with aspects of solute transport, endometrial receptivity, and immune response. Collectively, results showed that adequate colostrum consumption

within 12-24 hours of birth in pigs is important for establishment of a uterine developmental program required to insure normal patterns of endometrial gene expression during the periattachment period of early pregnancy.

DEDICATION

"*For I know the plans I have for you, plans to give you a hope and a future.*" Jer 29:11

I dedicate this work to my parents, Hank and Candis George, for always supporting me and encouraging me to accomplish my dreams, even in the darkest of times. Thank you so much for your unconditional love and 'words of wisdom'. I wouldn't be the person I am today without both of you and despite life's struggles, I wouldn't change a thing. I am forever grateful I was lucky enough to have you both as my parents and my best friends. I love you both dearly.

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ASSOCIATED PUBLICATIONS

Chapter II has been published as:

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CHAPTER I

Review of Literature

A. The Purpose and Scope of the Literature Review

Research described herein was designed to test the lactocrine hypothesis for maternal programming of porcine uterine development. The lactocrine hypothesis suggests that milk-borne bioactive factors (MbFs), communicated from mother to offspring in colostrum (first milk) during nursing, determine the neonatal uterine developmental program. Further, the lactocrine hypothesis predicts that disruption of lactocrine communication will alter the developmental trajectory of uterine tissues with lasting consequences. Goals of this research were to identify lactocrine effects on aspects of porcine uterine development in the neonate, and to determine if such effects program uterine endometrial response to pregnancy during the periattachment period in adults.

The purpose of this literature review is to provide the rationale for research described here. Included is an overview of literature pertaining to the concept of maternal programming of neonatal development; a description of studies indicating that colostrum acts as a conduit for the delivery of MbFs, including review of data supporting insulin-like growth factor I (IGF1) as a porcine MbF; discussion of the concept of critical developmental periods and the rationale for existence of a lactocrine programming window; review of evidence in support of the lactocrine hypothesis; review of current understanding of neonatal uterine development; and review of the biology of the periattachment period of early pregnancy in the pig. Finally, the significance of this work with regard to maternal lactocrine programming of porcine uterine function, and long-term consequences of lactocrine deficiency on reproductive performance will be provided.

B. Maternal Programming of Development

Throughout development, there are periods of organizational plasticity, when the genome can react to the environment and generate different phenotypes (West-Eberhard 2003). The timing and duration of such windows of susceptibility to developmental disruption are both species- and tissue-specific (Zambrano *et al.* 2014). Developmental programming can be defined as the sequence of events that establish the developmental trajectory resulting in a specific phenotype (Burggren 1999). Both environmental and chemical insults to developing offspring can alter developmental trajectory with lasting consequences for form, function, and health (Bartol *et al.* 2008, Rabadan-Diehl & Nathanielsz 2013, Hanson & Gluckman 2014).

Evidence in support of developmental programming stems from epidemiological studies that identified a relationship between early environment and later risk of metabolic and other diseases (Barker & Osmond 1986). For example, fetal undernutrition during pregnancy, indicated by low birth weight, was correlated with development of ischemic heart disease in adulthood (Barker *et al.* 1989, Barker *et al.* 1993, Barker 1995). The epidemiological studies that first indicated a relationship between intrauterine environment and later disease served as the basis for what would become ‘Barker’s Hypothesis’, and what is now described as ‘Developmental Origins of Health and Disease (DOHaD)’(Barker *et al.* 1989). Further evidence of DOHaD comes from retrospective studies of the Dutch hunger winter famine during World War II (1944-1945), when a German blockade resulted in severe undernutrition in Holland. Undernourished mothers gave birth to offspring that had a greater risk of cardiovascular disease (Roseboom *et al.* 2000), obesity (Ravelli *et al.* 1999), and impaired glucose

tolerance (Ravelli *et al.* 1998) in adulthood, emphasizing the importance of the maternal environment on developing offspring.

Maternal influence on development of offspring begins during gestation and, in mammals, extends into postnatal life through lactation and factors delivered to nursing offspring in colostrum (first milk). Maternal programming is defined as the ability of maternal factors to affect development with lasting consequences on offspring phenotype (Bartol *et al.* 2013). Where postnatal maternal programming is at work, perturbations of the maternal environment have the potential to affect both the developmental program and trajectory neonatal tissues (Bartol *et al.* 2008). For example, maternal consumption of a high-fat diet during lactation resulted in impaired hypothalamic neurocircuit formation and glucose homeostasis in adult murine offspring (Vogt *et al.* 2014). Further, rat offspring that nursed from dams fed a high-fat diet displayed features of metabolic syndrome in adulthood (Khan *et al.* 2005). Data indicate that alterations in nutrition of maternal origin during postnatal life can impact developmental trajectory and adult metabolism.

Studies in a variety of species indicate that exposure to an altered hormonal milieu during critical periods of neonatal development can have long-term effects on adult uterine morphology and function (Crain et al. 2008). While timing of events associated with uterine histogenesis varies among species, uterine endometrial gland development (adenogenesis) is completed postnatally in most, if not all mammals (Cooke *et al.* 2013). In mice and sheep, neonatal progestin exposure throughout that period associated with endometrial adenogenesis resulted in a uterine gland knockout phenotype in adults, characterized by lack of endometrial glands (Bartol *et al.* 1999, Gray *et al.*

2001a, Cooke *et al.* 2012, Filant *et al.* 2012). In rats, neonatal progesterone exposure disrupted endometrial gland genesis, and resulted in progesterone resistance and uterine dysfunction at maturity (Dhakal *et al.* 2015). Importantly, effects of exogenous progestins were dependent upon the developmental period when neonatal exposure occurred, with attenuated or negligible effects on endometrial adenogenesis in beef heifers (Bartol et al. 1995) and mice (Cooke et al. 2012) when exposure was delayed. Data for the pig (*Sus scrofa domesticus*) showed that estradiol valerate exposure from birth (postnatal day = PND 0) to PND 13 altered aspects of neonatal uterine wall uterine development and, ultimately, function in adults (Tarleton *et al.* 2003, Chen *et al.* 2010). Together, these data suggest that neonatal perturbations during critical periods of uterine development can alter developmental trajectory and affect adult uterine/endometrial phenotype.

C. Milk as a Conduit for Bioactive Factors

Lactogenesis, involving the transition of mammary epithelial cells to a secretory state, occurs in two stages, starting with differentiation of alveolar epithelial cells, and followed by milk secretion (Kensinger *et al.* 1986). In pigs, the first stage of lactogenesis occurs between days 90 and 105 of pregnancy and the second between days 112 of pregnancy until early lactation in the postpartum period (Kensinger *et al.* 1986). At parturition (~day 114 of pregnancy), secretions within the alveolar lumina begin to accumulate and resemble colostrum (Kensinger *et al.* 1986). In the pig, colostrum, or first milk, can be defined as the initial pigmented and viscous secretion from the mammary gland produced for 24-48 h from the onset of parturition (Klobasa *et al.* 1987). Colostrum contains bioactive, nutritive, and immune factors including proteins, peptides, growth factors, steroid hormones, fatty acid-derived molecules, and oligosaccharides (Grosvenor

et al. 1993, Donovan & Odle 1994, Schanbacher *et al.* 1998, Blum & Baumrucker 2008, Lefevre *et al.* 2010, Frankshun *et al.* 2011, Tao *et al.* 2011, Sharp *et al.* 2014). Many hormones and growth factors are synthesized *de novo* within the mammary gland from precursors delivered from the maternal circulation (Grosvenor *et al.* 1993). Other components of colostrum are transferred from maternal blood into mammary secretions (Grosvenor *et al.* 1993). Many hormones present in porcine colostrum are detectable in maternal plasma before parturition and during lactation (Devillers *et al.* 2004).

Colostrum and milk also contain microRNAs (miRNAs), as indicated by data for several species including the pig (Gu *et al.* 2012, Chen *et al.* 2014), human (Kosaka *et al.* 2010, Liao *et al.* 2017), cow (Hata *et al.* 2010), goat (Ji *et al.* 2012), tammar wallaby (Modepalli *et al.* 2014), and giant panda (Ma *et al.* 2017). MicroRNAs are short non-coding RNAs ranging from about 18-25 nucleotides in length that regulate post-transcriptional gene expression (Bartel 2004). In milk and other body fluids, miRNAs are relatively stable and exist in exosomes (Zhang *et al.* 2015), which can then be communicated intercellularly. In the pig, exosomal miRNA profiles for colostrum (lactation days 0-3) grouped separately from those defined for mature milk (lactation days 7-28) (Gu *et al.* 2012). Further, neonatal pigs fed only colostrum had increased serum concentrations of immune-related miRNAs compared to those fed only milk (Gu *et al.* 2012), suggesting that these exosomes are transferred from mother to offspring by nursing and may play a role in immune system development.

At parturition, porcine colostrum consists of high concentrations of solids and protein with lower amounts of fat and lactose, which decrease linearly within the first 12 h of lactation (Klobasa *et al.* 1987, Langer 2009). By 24-48 h the composition of

colostrum changes substantially during the transition to mature milk which, relative to colostrum, contains decreased amounts of protein and increased amounts of fat and carbohydrates (Klobasa *et al.* 1987, Langer 2009). In pigs, the predominant proteins observed in colostrum are immunoglobulins (Klobasa *et al.* 1987). This is important since the porcine epitheliochorial placenta does not support transplacental transport of immunoglobulins (Baintner 2007). Therefore, transient passive immunity is established in nursing piglets by consequence of nursing, providing immune protection before active immune defenses are developed (Baintner 2007). Immunoglobulins (Ig), particularly IgG, are present in high concentrations in porcine colostrum, and levels of IgG in plasma of piglets one day after birth are positively correlated with survival (Vallet *et al.* 2013). In addition to IgGs, cytokines, including interleukin (IL)-6, interferon (IFN)-γ, IL-12, IL-10, and IL-4, are also transferred from mother to neonate via lactation for additional immune support (Nguyen *et al.* 2007).

D. Lactocrine Hypothesis for Maternal Programming of Postnatal Development

The lactocrine hypothesis suggests that bioactive factors in colostrum are delivered from mother to offspring as a specific consequence of nursing and enter the neonatal circulation to affect development (Bartol *et al.* 2008). Evidence in support of the lactocrine hypothesis comes from several species. For example, a study in rhesus macaques showed that glucocorticoids in milk, independent of available milk energy, were associated with more nervous and less confident behavior in neonates (Hinde *et al.* 2015). Additionally, mothers of lower parity (< 7) had greater cortisol concentrations in their milk, and their offspring exhibited greater neonatal weight gain (Hinde *et al.* 2015). This suggests that mothers under stress may be programming their neonates through

lactocrine-active cortisol to grow more quickly and be more cautious. If so, then offspring nursing mothers with fewer resources that are, therefore, more stressed, may have a greater chance of surviving.

Studies in rodents provide additional support for lactocrine-active MbFs in regulation of development. Neonatal mice that ingested TNFα-deficient milk by nursing TNFα-null mothers displayed increased hippocampal cell proliferation as neonates, and increased spatial memory as adults (Liu *et al.* 2014). Importantly, these results were recapitulated through administration of an anti-TNF drug to wild-type lactating mothers, suggesting that observed effects were due to postnatal influences (Liu *et al.* 2014). Milk from TNFα-deficient mothers had reduced chemokine levels compared to controls, and supplementation of those chemokines to pups restored both postnatal hippocampal cell proliferation and adult memory to normal levels (Liu *et al.* 2014). Further, timing of mammotrope development, as well as mammotrope function in adulthood were altered in rats deprived of early colostrum through cross-fostering of nursing young to mothers in later lactation (Nusser & Frawley 1997).

Additional evidence for lactocrine programming stems from macropodid marsupials that display asynchronous concurrent lactation. In these mammals, multiple-aged offspring (newborn to juvenile) nurse from specific mammary glands, ingesting milk specific to their stage of development (Nicholas *et al.* 1997). A study involving two species of wallabies, parma and tammar, showed that cross-fostering nursing parma wallabies to tammar mothers carrying young of equivalent developmental stages altered postnatal grow patterns and delayed developmental events markedly in parma young, suggesting that maternal milk regulates development in a species-specific manner

(Menzies *et al.* 2007). All of these young wallabies received maternal care, but consumed milk of differing composition. Results reinforce that importance of lactocrine conditions for normal development.

With regard to pigs, colostrum intake varies substantially among nursing piglets (Devillers *et al.* 2011), and can be influenced by factors including birth weight, litter size, and birth rank (Devillers *et al.* 2007, Devillers *et al.* 2011, Declerck *et al.* 2015), as well as teat position and competition for the teat (Wu *et al.* 2010). Gilts that ingest small amounts of colostrum during the first day of life, as reflected by low serum immunoglobulin immunocrit (iCrit) values, display poor survival outcomes (Vallet *et al.* 2013). Low iCrit gilts that do survive showed reduced growth rates, increased age at puberty and, as adults, gave birth to smaller litters compared to their higher iCrit littermates (Bartol *et al.* 2013, Vallet *et al.* 2015). Results, involving data generated from 799 gilts over four parities, were interpreted to indicate permanent impairment of reproductive performance in female pigs that did not receive sufficient colostrum from birth (Vallet et al., 2105). The fact that all gilts in that study consumed colostrum via nursing but, otherwise, were raised together under standard husbandry conditions, reinforced the idea that minimal colostrum consumption (lactocrine deficiency) was responsible for long-term negative effects on reproductive performance (Bartol et al., 2013; Vallet et al., 2015).

Neither specific lactocrine-active MbFs in colostrum, nor critical periods for their delivery during early postnatal life are well defined. Moreover, beyond maternal lactocrine effects on postnatal developmental programming, it is important to recognize that other maternal-neonatal interactions can affect developmental outcomes (Algers

1993). For example, maternal care in rodents is important for neural development and affects adult responses to stress, as well as memory and spatial learning (Liu *et al.* 1997, Caldji *et al.* 1998, Liu *et al.* 2000). Whether eliminating some nursing behaviors, as can occur by gavage or bottle feeding, affects neonatal porcine uterine development attributed to lactocrine signaling is unknown.

E. Neonatal Gut Closure and the Lactocrine Programming Window

The neonatal gut is permeable to large macromolecules for some time after birth, with the length of time being variable and species-specific (Pacha 2000). It was suggested that this is primarily due to paracellular transport. This occurs when tight junctions that define the luminal and basolateral compartments of enterocytes are not intact, thereby allowing for transport of macromolecules across intestinal epithelium and into the circulation via passive transport (Pacha 2000, Anderson & Van Itallie 2009). Macromolecules can also cross the intestine via receptor-mediated endocytosis and transcytosis (Wada & Lonnerdal 2014). In pigs, cessation of intact macromolecule uptake, termed gut closure, occurs between 24-48 hours after birth, corresponding to the transition of colostrum to mature milk (Leece 1973, Westrom *et al.* 1984, Klobasa *et al.* 1987, Xu 1996).

To define the window for optimal transmission of MbFs, effects of timing and duration of nursing from birth on uterine and cervical development were examined. Gilts that nursed continuously from 0 h (birth) or 30 min of age had similar uterine and cervical MMP9 and TIMP1 protein expression at PND 2 (Ho *et al.* 2017). These proteins were undetectable at PND 2 when nursing was delayed for 12 h from birth, or when a

lactocrine-null condition was imposed from birth by milk-replacer feeding. However, when duration of nursing from birth was increased to 12 h, uterine and cervical MMP9 protein expression at PND 2 was similar to that observed for gilts nursed continuously from birth (Ho *et al.* 2017). In the porcine cervix, a single oral dose of colostrum at birth increased cell proliferation at 12 h postnatal, suggesting that the first ingestion of colostrum is consequential in term of initiating the postnatal uterine developmental program (Camp *et al.* 2014). Together, data can be interpreted to suggest that a window of opportunity for lactocrine transmission of MbFs is open from birth to 12 h postnatal in the pig .

F. Development of the Neonatal Porcine Uterus

The mammalian female reproductive tract develops from the Müllerian duct during fetal life as reviewed elsewhere (Spencer *et al.* 2012). Temporospatially unique expression of patterning genes is necessary to support antero-posterior segmentation and radial patterning of the developing Müllerian duct into structurally and functionally unique parts of the female reproductive tract including the uterine tubes, uterus, cervix and anterior vagina (Bartol *et al.* 2006). Essential to this process is homeobox-leucine zipper protein (HOX) 13 expression in the vagina, *HOXA11* in the cervix, *HOXA10* in the uterus, and *HOXA9* in the oviduct (Bartol *et al.* 2006). In addition to homeobox genes, Wingless-related integration site (WNT) and Hedgehog signaling are also necessary for proper female reproductive tract development. These gene products regulate events associated with cellular differentiation and cell fate (Bartol *et al.* 2006, Spencer *et al.* 2012). In mammals, uterine epithelial *WNT7A* is required to maintain stroma expression of *WNT5A*, *WNT4*, *HOXA10* and *HOXA11* (Bartol *et al.* 2006, Spencer *et al.* 2012, Cooke

et al. 2013). Additionally, Hedgehog signaling is active in the female reproductive tract with Indian Hedgehog and Patched receptors expressed in porcine uterine tissues at birth, possibly regulating WNT expression (Bartol *et al.* 2006).

In domestic animals, humans, and rodents, uterine development is completed postnatally (Cooke *et al.* 2013). At birth in the pig, the uterus consists histologically of a simple, columnar luminal epithelium supported by an undifferentiated mesenchyme and underlying, rudimentary myometrium (Bartol *et al.* 1993, Spencer *et al.* 1993). Between birth and PND 3, endometrial luminal epithelium (LE) differentiates into nascent glandular epithelium (GE) that proliferates and begins to penetrate the uterine stroma (Bartol *et al.* 1993, Tarleton *et al.* 1998, Masters *et al.* 2007). As adenogenesis proceeds, glandular differentiation is marked by estrogen receptor 1 (ESR1) expression in nascent GE (Tarleton *et al.* 1999). By PND 14, uterine glands increase in number within the endometrial stroma, and appear as coiled, tubular structures (Spencer *et al.* 1993). Endometrial glands have reached the inner adluminal layer of the myometrium and begun to branch by PND 28-42 By PND 56 endometrial folds are present (Spencer *et al.* 1993). By PND 120 the porcine uterus is functionally mature as it is able to support pregnancy with progesterone supplementation (Bartol *et al.* 1993).

Similar to rodents (Bigsby & Cunha 1985, Branham & Sheehan 1995) and sheep (Bartol *et al.* 1988), porcine uterine morphogenesis is ovary-independent during postnatal life until approximately PND 60 (Tarleton *et al.* 1998). Evidence that uterine growth and histogenesis continue on a positive trajectory between birth and PND 60 in gilts bilaterally ovariectomized at birth indicates that extraovarian factors support uterine

development during this period. Lactocrine transmission of MbFs may provide such support (Yan *et al.* 2006b, Bartol *et al.* 2008).

The idea that lactocrine transmission of MbFs could support early postnatal uterine development in the pig was borne from studies involving relaxin (RLX). A uterotrophic peptide hormone, RLX is present in porcine colostrum in nanogram quantities shortly after parturition, and declines to undetectable levels by lactation day 4 (Frankshun *et al.* 2011). In the neonatal pig, RLX was undetectable in serum prior to nursing at birth, and in pigs fed RLX-free milk replacer (Yan *et al.* 2006b). When pigs were allowed to nurse normally, serum RLX levels approached 200 pg/ml on PND 0 and 1, but were undetectable on and after PND 2 (Yan *et al.* 2006b). Treatment of gilts from PND 12 to PND 14 with exogenous RLX increased uterine weight, protein content, and uterine luminal epithelial height at PND 14 (Yan *et al.* 2006a). Collectively, data were interpreted to indicate that milk-borne RLX, present in large quantities in colostrum, can be absorbed into the circulation of nursing offspring when ingested within two days of birth, and supports endometrial development in the neonatal pig (Bartol *et al.* 2009).

It is likely that lactocrine programming of porcine female reproductive tract tissues begins with first ingestion of colostrum over a relatively narrow widow of time shortly after birth. A single feeding of colostrum, but not milk replacer, at birth supported normal cervical cell proliferation when evaluated at 12 h postnatal (Camp *et al.* 2014). When colostrum consumption was delayed for 0.5 h from birth, or limited to 12 h from birth, uterine expression of developmental markers MMP9 and TIMP1 at PND 2 was similar to that observed for uterine tissues obtained from gilts nursed continuously from birth (Ho *et al.* 2017). Imposition of a lactocrine-null state for two days from birth by

feeding milk replacer in lieu of nursing altered the neonatal uterine transcriptome on PND 2 (Miller *et al.* 2013, Rahman *et al.* 2016) and inhibited uterine gland genesis by PND 14 (Miller *et al.* 2013). Further, nursing for as little as 1 h from birth was sufficient to establish passive immunity in newborn piglets (Coalson and Lecce, 1973). Together, these studies indicate that lactocrine effects begin at birth, and that the porcine lactocrine programming window may be closed by 12 h postnatally. Whether lactocrine effects on neonatal porcine uterine development are detectable at 12 h of age is unknown.

G. Insulin-like Growth Factor (IGF) 1: Candidate MbF

IGF1 (7.6 kDa peptide) is a member of the insulin family of hormones and mediates growth and development (Baker *et al.* 1993). This peptide hormone is found in mammary secretions of many species including pigs (Simmen *et al.* 1988, Simmen *et al.* 1990, Donovan *et al.* 1994, Donovan & Odle 1994), tammar wallabies (Ballard *et al.* 1995), cows (Campbell & Baumrucker 1989), horses (Hess-Dudan *et al.* 1994), rats (Donovan *et al.* 1991b), sheep (Simmen *et al.* 1988), and humans (Nagashima *et al.* 1990, Donovan *et al.* 1991a, Eriksson *et al.* 1993). In the pig, similar to other MbFs (Frankshun *et al.* 2009), milk-borne IGF1 levels are greatest in colostrum (50-500 ng/ml) and decline 10- to 100-fold during the transition to mature milk (Simmen *et al.* 1988).

When administered orally to neonates, IGF1 is a potent mitogen that can act both locally within the gastrointestinal tract and systemically by entering the circulation to target other neonatal tissues. In neonatal pigs, oral IGF1 supplementation increased intestinal cellular proliferation, intestinal enzyme activity, and ileal villus height (Houle *et al.* 1997, Houle *et al.* 2000). In neonatal rats, oral IGF1 supplementation increased body, brain, and liver weights, suggesting that IGF1 exerts these systemic effects after

entering the circulation (Philipps *et al.* 1997). Consistently, radiolabeled IGF1 was detected in plasma one hour after oral administration to pigs at birth (Xu & Wang 1996). Another study in neonatal rats showed that oral administration of insulin results in a large drop in blood glucose, suggesting insulin is absorbed by the neonatal gastrointestinal tract (Mosinger *et al.* 1959). Furthermore, oral IGF1 supplementation at birth increased cervical cell proliferation and markers of IGF1 signaling at 12 h in neonatal gilts (Camp *et al.* 2014). Whether oral IGF1, a candidate MbF, affects neonatal uterine development similarly is unknown.

H. Global Gene Expression Profiling: Next-Generation Sequencing

Next generation sequencing (NGS), also known as high throughput sequencing, deep sequencing, or massively parallel sequencing, first became commercially available in 2000 from Lynx technologies (Goldman & Domschke 2014). Now, 18 years later, NGS technologies are more automated and have advanced exponentially with regard to speed, price, and most importantly, accuracy (Goldman & Domschke 2014). In contrast to microarrays or real-time qPCR, the benefits of NGS methods are substantial and include genome-wide profiling, non-coding RNA characterization, and novel transcript identification at single nucleotide resolution (Mutz *et al.* 2013). Importantly, the low cost and high throughput characteristics of NGS allowed for extensive profiling of human genetic variation, with millions of polymorphisms described (Goldman & Domschke 2014).

For NGS studies presented in Chapters III and IV, the Illumina HiSeq 2500 (Illumina, Inc.) platform was used. Sample preparation and sequencing steps central to this process are briefly described here. Following conversion of RNA to cDNA, cDNAs

are fragmented, ligated to adapter sequences, and bound to primers fixed on a flow cell (Goodwin *et al.* 2016). Through PCR, bridge amplification occurs. Through this process the distal ends of hybridized templates bind with nearby primers, allowing for further amplification (Goodwin *et al.* 2016). Through several rounds of amplification, millions of clonal clusters are formed on the flow cell. Illumina sequencing used in studies described here in, is based on a sequencing-by-synthesis platform. In this system, polymerase is used and a base-specific, cleavable fluorophore marks the incorporation of a single nucleotide onto an elongating strand that is complementary to the template (Goodwin *et al.* 2016). Each deoxynucleotide triphosphate (dNTP) is blocked by a 3'OH-inactivating residue, so fragments in each cluster will only add one nucleotide at a time. After base incorporation and fluorescence imaging to identify the nucleotide, fluorescently labeled reversible terminators are cleaved and a new cycle begins, termed 'cyclic reversible termination' (Goodwin *et al.* 2016). Currently, Illumina platforms account for the largest market share for sequencing instruments (Goodwin *et al.* 2016).

There are challenges and limitations associated with analysis of RNAseq data when studies involve organisms with incomplete reference genomes. Compared to the human and mouse, some domestic animal species, including the pig, have genome assemblies of lesser quality that are incompletely annotated (Hekman *et al.* 2015). The porcine genome was fully sequenced as of 2012, but annotation remains incomplete (Gutierrez *et al.* 2015). When such draft genomes are utilized in a gene expression pipeline, assembly errors and misannotation can occur resulting in incorrect or unidentifiable transcript characterization (Hekman *et al.* 2015). Nevertheless, alternative approaches for transcript identification are constantly evolving. Options include

performing comparisons to a high-quality reference genome of a closely related species, and de-novo assembly of RNAseq reads (Hekman *et al.* 2015).

I. MicroRNAs in the Uterus

MicroRNAs are short non-coding RNAs that regulate post-transcriptional gene expression (Bartel 2009). Initially, miRNAs are transcribed in the nucleus as primary-miRNAs (pri-miRNAs), characterized by a stem-loop structure (Bartel 2004). Following cleavage by the microprocessor complex, consisting of Drosha and its cofactor DGCR8, pri-miRNAs become hairpin-like precursor-miRNAs or pre-miRNAs (Nguyen *et al.* 2015). After translocation to the cytoplasm, Dicer cleaves pre-miRNAs into double-stranded intermediates about 22 nucleotides long (Song & Rossi 2017). Mature, single-stranded miRNA is then loaded into the RNA-induced silencing complex (RISC) where it can bind to complementary target mRNAs, resulting in translational repression or, more often in mammals, mRNA destabilization (Eichhorn *et al.* 2014). A single miRNA can target multiple mRNAs (Lewis *et al.* 2005). MicroRNA effects on transcript stability and translational repression are recognized to be important in regulation of many if not most biological processes, including those necessary for female reproductive tract development and function (Nothnick 2016).

Dicer, a riboendonuclease necessary for miRNA production, is required for normal female reproductive tract development and fertility, suggesting that miRNAs themselves are key players regulating these processes (Gonzalez & Behringer 2009). Furthermore, miRNAs were identified in human and porcine endometrium, with expression varying during the menstrual cycle in humans (Kuokkanen *et al.* 2010, Revel *et al.* 2011, Sha *et al.* 2011) and during implantation (Su *et al.* 2014, Krawczynski *et al.*

2015a, Krawczynski *et al.* 2015b, Wang *et al.* 2016, Wang *et al.* 2017) and placentation (Bidarimath *et al.* 2015, Liu *et al.* 2015) in pigs. Additionally, miRNAs were implicated in multiple human uterine pathologies, including endometriosis (Burney *et al.* 2009, Jia *et al.* 2013, Cho *et al.* 2015), adenomyosis (Herndon *et al.* 2016), and uterine fibroids (Marsh *et al.* 2016).

Transcriptomic analysis of the neonatal porcine uterus identified age- and lactocrine-sensitive changes in gene expression between birth and PND 2 (Rahman *et al.* 2016). More than 3000 genes were differentially expressed in uteri from PND 2 as compared to PND 0 gilts. Gene enrichment and functional analyses revealed age-sensitive biological processes including WNT β-catenin, transforming growth factor-β (TGFβ), MMP, and ESR1 signaling pathways. On PND 2, more than 800 uterine genes were differentially expressed between nursed and replacer-fed (lactocrine-null) gilts. Gene enrichment and functional analyses identified lactocrine-sensitive biological processes including response to wounding, coagulation, cell adhesion, hedgehog, and ESR1 signaling pathways. Mechanisms regulating these age- and lactocrine-sensitive transcriptional changes in the neonatal porcine uterus are unknown. Whether miRNAs play a role in post-transcriptional regulation of gene expression in the neonatal porcine uterus remains to be determined.

J. The Periattachment Period of Early Pregnancy in the Pig

Reproductive performance and efficiency can be measured by documenting production of viable, healthy offspring. In eutherian mammals this requires uterine support. In all eutherian mammals, uterine gestation of conceptuses requires progesterone of ovarian origin produced by corpora lutea (Bazer 2015). Conceptus-induced events that

insure maintenance of a luteal source of progesterone define ‘maternal recognition of pregnancy’ (MRP) (Short 1969). Conceptus-produced signals responsible for MRP vary among species (Bazer 2015). In the pig, MRP involves inhibition of luteolysis induced by estrogens produced by periattachment stage conceptuses *in utero*. Conceptus-produced estrogens act on the maternal endometrium causing prostaglandin F2α (PGF2α) of endometrial origin to be secreted adluminally, in an exocrine manner, rather than systemically, in an endocrine manner (Bazer & Thatcher 1977). This transition from endocrine to exocrine secretion of PGF2α occurs between days 10 and 12 post-mating, and prevents the luteolysin, PGF2α, from entering the blood supply as necessary for luteolysis (Geisert *et al.* 1982). A second phase of conceptus-produced, estradiol-driven PGF2α secretion into the uterine lumen during days 15 to 25-30 of pregnancy is required to prolong progesterone secretion and maintain pregnancy (Geisert *et al.* 1990). Prostaglandin E2 synthase, produced by the trophoblast and endometrium, provides additional support for luteal maintenance (Waclawik *et al.* 2017). Genes expressed by porcine endometrium involved in prostaglandin synthesis and secretion during this period include aldo-keto reductase family 1 member B (*AKR1B1*) (Seo et al. 2014a), lysophosphatidic acid receptor 3 (*LPAR3*) (Seo et al. 2008), prostaglandin G/H synthase 1 (*PTGS1*) (Seo *et al.* 2014a), and solute carrier organic anion transporter family, member 2A1 (*SLCO2A1*) (Seo *et al.* 2014b).

In addition to and associated with MRP, uterine endometrial receptivity is maternally programmed, transient condition that evolves during the periattachment period of early pregnancy. Endometrial receptivity is necessary for successful conceptus attachment, placentation and maintenance of pregnancy. In pigs, establishment of a

receptive endometrium is associated with down-regulation of *ESR1*, and progesterone receptor (*PGR*) expression by uterine epithelial cells during the periattachment period (days 12-15 post-insemination) is required for establishment of pregnancy (Geisert *et al.* 1993, Geisert *et al.* 1994). Conceptus estrogens induce uterine luminal epithelial expression of keratinocyte growth factor (*KGF* or *FGF7*) (Ka *et al.* 2001, Ka *et al.* 2007) and endothelial differentiation gene 7 (*EDG7*) (Seo *et al.* 2008), as well as interferon-regulatory factor 2 and uteroferrin (*UF*) (Basha *et al.* 1979, Joyce *et al.* 2007). Other estrogen-sensitive genes expressed by the pig endometrium during the periattachment period include relaxin/insulin-like family peptide receptor 1 (*RXFP1*), and matrix metalloproteinase 9 (*MMP9*) (Chen *et al.* 2010), secreted phosphoprotein 1 (*SPP1* or osteopontin) (Garlow *et al.* 2002, White *et al.* 2005), and stanniocalcin 1 (*STC1*) (Song *et al.* 2009). Expression of these genes is thought to be important for migration, proliferation, and adhesion of trophectodermal epithelium to uterine luminal epithelial cells. Together, these events support processes required for establishment and maintenance of pregnancy. Disruption of the normal uterine developmental program during early neonatal life may affect such processes and compromise uterine capacity to support pregnancy.

Minimal colostrum consumption by newborn gilts is associated with reduced lifetime fecundity (Bartol *et al.* 2013, Vallet *et al.* 2015). Serum iCrit, an indirect measure of colostrum consumption in neonatal pigs (Vallet *et al.* 2013), was used to identify low and high iCrit gilts on their day of birth. Low iCrit was associated with reduced growth and increased age at puberty (Vallet *et al.* 2015). When tracked into adulthood, data for 799 gilts over four parities revealed that gilts with low birthday iCrit

values produced smaller litters as adults when compared to their high iCrit littermates, with no effect of parity (Vallet *et al.* 2015). Thus, neonatal colostrum consumption may be a predictor of uterine capacity in adult pigs. The extent to which disruption of normal lactocrine inputs by reduced colostrum consumption affects uterine endometrial programming and functional uterine capacity remains to be determined.

K. Significance

Maternal influence on offspring development begins during gestation and extends postnatally through lactation. Development of many tissues, including those of the female reproductive tract, is incomplete at birth. Disruption of the developmental program, as may occur by interruption of normal processes or exposure to disruptive signals that affect organizationally critical events, can affect tissue developmental trajectory with long-term consequences (Stockard 1921, Nathanielsz 2006, Bartol *et al.* 2008, Burggren & Reyna 2011).

It is well established in ungulate species, including sheep (Bartol *et al.* 1999) and cattle (Bartol *et al.* 1995), as well as in mice (Cooke *et al.* 2012, Filant *et al.* 2012), that transient disruption of uterine development from birth can result in extreme adult endometrial phenotypes and impaired uterine function in adulthood (Cooke *et al.* 2013). In the pig, newborn gilts exposed to estradiol valerate (EV) from PND 0 through PND 13 displayed reduced embryo survival as adults on pregnancy day 45 when compared to unexposed controls (Bartol *et al.* 1993). Adult, neonatally EV-treated gilts had reduced uterine luminal fluid protein content, increased retinol-binding protein (RBP), and decreased *KGF* gene expression levels at pregnancy day 12 (Tarleton *et al.* 2003). Consistently, some changes in gene expression observed in uteri from neonatally EV-

exposed gilts on PND 14 persisted to adulthood (Chen *et al.* 2010). Thus, as observed for other species (Cooke *et al.* 2013), short-term disruption of porcine uterine development during the early neonatal period can alter both the uterine developmental program and functional trajectory of adult uterine tissues.

Data for the pig show that disruption of normal lactocrine signaling from birth, by substitution of a porcine milk replacer for colostrum, altered patterns of uterine gene expression by PND 2 (Miller *et al.* 2013, Rahman *et al.* 2016) and inhibited uterine endometrial gland development by PND 14 (Miller *et al.* 2013). The observation that gilts consuming minimal amounts of colostrum on the day of birth displayed reduced fecundity in adulthood (Bartol *et al.* 2013, Vallet *et al.* 2015) provided strong support for the lactocrine hypothesis for maternal programming of adult uterine capacity to support pregnancy. The extent to which minimal lactocrine support at birth affects uterine development and/or uterine capacity and endometrial function during the periattachment period of early pregnancy remains undefined.

Results reported and discussed in this dissertation provide new information about how postnatal maternal lactocrine programming affects neonatal porcine uterine development with long-term consequences in adults. Experiments described here advance and extend studies designed to test the lactocrine hypothesis for maternal programming of uterine development. Information provided by these studies in the pig have broader implications, as lactocrine mechanisms are now documented to affect development of the bovine mammary gland (Soberon & Van Amburgh 2013), the murine central nervous system (Liu *et al.* 2014) and aspects of behavior in primates (Hinde *et al.* 2015).

L. Hypothesis and Objectives

The hypothesis underlying proposed research is that MbFs, delivered from mother to offspring via a lactocrine mechanism shortly after birth, support porcine neonatal uterine development and program endometrial functionality, as reflected by patterns of endometrial gene expression in pregnant adults. The overall goal of this research was to determine the role of maternal lactocrine programming on porcine uterine development in the neonate and adult. This information will be important to the design of husbandry practices aimed at optimizing reproductive performance of swine. Knowledge of acute and long-term effects of colostrum consumption on development could be used to improve human infant care and health.

Objectives of research described herein were to:

Objective 1 (Chapter II):

a) Determine acute effects of nursing vs milk replacer feeding on neonatal uterine development at 12 h postnatally.
b) Evaluate whether method of feeding a single dose of colostrum at birth, with or without supplemental IGF1, affects aspects of porcine uterine development at 12 h postnatally.

Objective 2 (Chapter III):

a) Determine short-term effects of age and nursing at birth on porcine uterine miRNA expression at postnatal day (PND) 2 using miRNA sequencing (miRNAseq).
b) Define affected miRNA-mRNA interactions and associated biological processes *in silico* using integrated target prediction analysis.

Objective 3 (Chapter IV):

a) Determine long-term effects of lactocrine-deficiency from birth on adult porcine endometrial mRNA and miRNA expression during the periattachment period of early pregnancy (pregnancy day 13).

b) Identify lactocrine-sensitive miRNA–mRNA interactions and associated biological processes *in silico*, using integrated target prediction analysis.

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CHAPTER II

Effects of Colostrum, Feeding Method, and Oral IGF1 on Porcine Uterine Development

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ABSTRACT

Nursing ensures lactocrine delivery of maternally-derived, milk-borne bioactive factors to offspring, which affects postnatal development of female reproductive tract tissues. Disruption of lactocrine communication for two days from birth (postnatal day = PND 0) by feeding milk replacer in lieu of nursing or consumption of colostrum alters porcine uterine gene expression globally by PND 2 and inhibits uterine gland genesis by PND 14. Here, objectives were to determine effects of: 1) nursing or milk replacer feeding from birth; 2) a single dose of colostrum or milk replacer and method of feeding; and 3) a single feeding of colostrum or milk replacer, with or without oral supplementation of IGF1, administered at birth on aspects of porcine uterine development at 12 h postnatally. Results indicate nursing for 12 h from birth supports rapid establishment of a uterine developmental program, illustrated by patterns of endometrial cell proliferation, expression of genes associated with uterine wall development and entry into mitosis, and establishment of a uterine MMP9/TIMP1 system. A single feeding of colostrum at birth increased endometrial cell proliferation at 12 h, regardless of method of feeding. Oral supplementation of IGF1 was sufficient to support endometrial cell proliferation at 12 h in replacer-fed gilts, and supplementation of colostrum with IGF1 further increased endometrial cell proliferation. Results that indicate lactocrine regulation of postnatal uterine development is initiated with the first ingestion of colostrum. Further, results suggest IGF1 may be lactocrine-active and support a 12 h bioassay which can be used to identify uterotrophic lactocrine activity.

INTRODUCTION

Nursing provides a conduit for lactocrine delivery of maternally derived, milk-borne bioactive factors (MbFs) to offspring where they can affect postnatal development as proposed by the lactocrine hypothesis (Yan *et al.* 2006b, Bartol *et al.* 2008). Data for the pig showed that imposition of a lactocrine-null condition from birth (postnatal day = PND 0), by gavage feeding a porcine milk replacer in lieu of nursing and colostrum (first milk) consumption, altered patterns of uterine gene expression (Rahman *et al.* 2016, George *et al.* 2017) and endometrial cell proliferation by PND 2 (Miller *et al.* 2013), and inhibited uterine gland development by PND 14 (Miller *et al.* 2013). Lactocrine deficiency, documented in nursed gilts that consumed minimal amounts of colostrum on the day of birth, had similar anti-uterotrophic effects when evaluated on PND 14 (Bartol *et al.* 2017). Moreover, adult gilts that were lactocrine deficient on their day of birth displayed reduced lifetime fecundity (Bartol *et al.* 2013, Vallet *et al.* 2015). Thus, disruption of lactocrine signaling from birth affects both the program and trajectory of porcine uterine development.

Evidence that lactocrine deficiency on the day of birth leads to permanent impairment of reproductive performance in adult female pigs, as reflected by reduced uterine capacity to support large, viable litters (Vallet *et al.* 2015), suggests that lactocrine signaling from birth affects the porcine uterine developmental program rapidly. Consistently, when colostrum consumption was delayed 0.5 h from birth or limited to 12 h from birth, markers of female reproductive tract (FRT) development, including uterine matrix metalloproteinase-9 (MMP9) and tissue inhibitor of metalloproteinase-1 (TIMP1), as well as cervical MMP9 expression levels on PND 2 were similar to those observed for nursed gilts that consumed colostrum continuously for 48 h from birth (Ho *et al.* 2017).

Further, a single feeding of colostrum at birth was sufficient to support normal levels of cervical cell proliferation at 12 h postnatal (Camp *et al.* 2014). These observations, taken together with data indicating that nursing for as little as one hour from birth is sufficient to establish passive immunity in newborn piglets (Coalson & Lecce 1973), suggest that lactocrine signals supportive of FRT development are likely to be communicated within 12 h of birth (Bartol *et al.* 2017).

To the extent that lactocrine signals communicated to nursing female piglets within 12 h of birth contribute to mechanisms that define the uterine organizational program and reproductive performance in adults, it is important that the biology of uterine development during this early neonatal period be understood. Further, given that the first 12 h of neonatal life may represent a critical period for lactocrine programming of uterine function (Bagnell *et al.* 2017), establishment of a model system for study of lactocrine-sensitive uterine organizational events specific to this period is important. Data specific to the developing porcine cervix during this neonatal period (Camp *et al.* 2014) provide a framework and rationale for establishment of a similar protocol specific to the uterus. In refining that protocol for this purpose, three studies were conducted linked by a common 12 h bioassay design. The objective of Study 1 was to determine whether lactocrine effects on specific aspects of the neonatal uterine organizational program could be detected at 12 h postnatally in gilts nursed from birth. The objective of Study 2 was to evaluate whether method of feeding a single dose of colostrum affects markers of uterine development at 12 h postnatally. Finally, Study 3 was conducted to determine if known, potentially lactocrine-active factors found in porcine colostrum, such as insulin-like

growth factor 1 (IGF1) (Simmen *et al.* 1990, Donovan *et al.* 1994, Donovan & Odle 1994), could alter uterine histology or patterns of cell proliferation by 12 h postnatally.

MATERIALS AND METHODS

Animals and Experimental Design

Gilts (*Sus scrofa domesticus*) were born and raised from 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 animals were reviewed and approved by the Rutgers Institutional Animal Care and Use Committee and conducted in accordance with the Guide for the Care and Use of Agricultural Animals in Agriculture Research and Teaching (McGlone *et al.* 2010). Three studies were conducted to establish a 12 h postnatal bioassay for studying lactocrine effects in the uterus, as outlined in Fig. 1. In all studies, care was taken to insure that treatments were balanced for potential effects of litter (n = 5 litters for Study 1 (Fig. 1A); n = 10 litters for Study 2 (Fig. 1B); and n = 6 litters for the Study 3 (Fig. 1C)). Treatment groups were normalized by number of offspring and male siblings were not removed. Gilts were included in the experiments when minimum body weight at birth was 1.3 kg based on studies indicating that lower body weight pigs exhibit retarded postnatal development (Rehfeldt & Kuhn 2006).

Study 1 was conducted to determine the effects of nursing from birth on postnatal uterine development at 12 h (Fig. 1A). Gilts (n = 6/group) were randomly assigned at birth to nurse *ad libitum* or to be gavage-fed a commercial porcine milk replacer (30 ml/kg BW/2h; Advance Liqui-Wean, Milk Specialties Co. Dundee, IL) until 12 h of age when uteri were collected. Endpoints examined included histology, cell proliferation, gene expression, and extracellular matrix (ECM) remodeling proteins. Study 2 was conducted to determine the effects of a single dose of colostrum or milk replacer and

method of feeding on postnatal uterine development at 12 h, as indicated by effects on histology, cell proliferation, and gene expression (Fig. 1B). Gilts (n = 5-6/group) were randomly assigned at birth to one of five treatment groups: 1) nursed *ad libitum* for the first hour after birth or provided a single volume (15 ml/kg BW) of either 2) colostrum by bottle-feeding, 3) colostrum by gavage-feeding, 4) replacer by bottle-feeding, or 5) replacer by gavage-feeding. Pooled porcine colostrum, collected at 0 h of lactation, or a commercially complete porcine milk replacer were used for bottle and gavage feeding groups. After the first feeding, gilts were gavage-fed the milk replacer (30 ml/kg BW) every two hours until 12 h of age when uteri were collected.

Study 3 was conducted to determine the effects of a single dose of colostrum or milk replacer feeding at birth with or without oral IGF1 on postnatal endometrial histoarchitecture and cell proliferation at 12 h (Fig. 1C). Given the small amount of uterine tissue available for analyses in the 12 h postnatal gilt, the focus of Study 3 was narrowed to measures of uterine histology and cell proliferation. At birth, gilts (n = 4–6/group) were gavage-fed a single volume (15 ml/kg BW) of either 1) colostrum collected at the onset of parturition (0 h lactation), 2) colostrum supplemented with recombinant human IGF1 (1 μg/ml; Prepro Tech, Rocky Hill, NJ, USA), 3) milk replacer alone, or 4) milk replacer supplemented with IGF1 (1 μg/ml). The dose of IGF1 used in these studies was based on the concentration of IGF1 found in porcine colostrum (Simmen *et al.* 1990). After the initial feeding, all gilts were gavage-fed milk replacer (30 ml/kg BW per 2 h) until 12 h of age when uteri were collected.

Histomorphometry, Immunohistochemistry, and Image Analysis

Uterine tissues, trimmed of connective tissue, were fixed in Xpress Molecular Fixative (Sakura Finetek, Torrance, CA, USA) and embedded in Paraplast Plus (Fisher Scientific, Hampton, NH, USA). For histomorphometry and immunohistochemistry (IHC), uterine cross sections were cut at 5 μm (4-6 sections/gilt) and mounted on SuperFrost Plus slides (Fisher Scientific, Hampton, NH, USA). Hematoxylin staining of uterine tissue sections was performed using Harris Modified Hematoxylin (Fisher Scientific, Hampton, NH, USA). Entire uterine sections were imaged and measurements taken using Aperio Imagescope (Leica Biosystems; Wetzlar, Germany). Endometrial thickness was measured as the distance from the base of the luminal epithelium (LE) to the interface of the endometrium with inner circular myometrium.
Stromal cell nuclei within an 800 μm^2 area per animal were counted using ImageJ (National Institute of Health, Bethesda, MD, USA).

For proliferating cell nuclear antigen (PCNA) immunostaining, uterine sections were deparaffinized, rehydrated, and subjected to heat-induced epitope retrieval in boiling sodium citrate buffer (pH 6). For evaluation of cell proliferation, sections were incubated overnight at 4°C with mouse anti-rat PCNA antibody (1:100; 0.91 μg/ml; clone PC10; Invitrogen, Carlsbad, CA, USA). Sections representing all animals from each treatment group were processed together using a VectaStain ABC elite kit (Vector Laboratories; Burlingame, CA, USA). Following incubation with secondary antibody and ABC reagent, color was developed using 0.1% 3,3'-diaminobenzidine substrate (Sigma-Aldrich, St. Louis, MO, USA). Negative controls were incubated with mouse primary antibody isotype IgG (1.06 μg/ml; Invitrogen, Carlsbad, CA, USA) in place of the

primary antibody. Images were obtained using a QImaging camera and analyzed using ImageJ Software (National Institute of Health, Bethesda, MD, USA).

PCNA Labeling Index

Images were converted to grayscale and analyzed using ImageJ Software (National Institute of Health, Bethesda, MD, USA) following techniques described in (Miller *et al.* 2013). Briefly, nuclei were identified as positive when staining intensity values generated by image analysis were within 25% of the highest possible intensity value (Miller *et al.* 2013). Grayscale images were converted to pseudocolor using ImageJ to better illustrate staining intensity. Positively, immunostained cells appear red-yellow on a black background. Labeling index (LI), for PCNA, expressed as a percentage, was determined for the stroma (St) and LE of uteri collected from gilts at 12 h of age. A minimum of 2000 uterine LE and St cells were counted for each gilt. The PCNA LI for St and LE was expressed as a percentage by dividing the number of PCNA-labeled cells counted in each category by the total number of cells (both labeled and unlabeled) and multiplied by 100 to generate LI values.

RNA Extraction, cDNA Synthesis, and Quantitative PCR (qPCR)

RNA was isolated from 30–40 mg of whole uterine tissue from one uterine horn/animal using the miRNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's protocol. The RNA concentration was determined using a NanoDrop Spectrophotometer and RNA integrity was evaluated using an Agilent 2100 Bioanalyzer (Applied Biosystems, Carlsbad, CA, USA). Samples with an RNA integrity number ≥7.0

were used for cDNA generation. Reverse transcription was performed using 2 µg of total uterine 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, Carlsbad, CA, USA).

Uterine expression of cell cycle and morphoregulatory transcripts including cyclin dependent kinase 1 (*CDK1*), *CDK2*, *CDK4*, cyclin B1 (*CCNB1*), wingless-type MMTV integration site family member 5A (*WNT5A*), *WNT7A*, homeobox A10 (*HOXA10*), vascular endothelial growth factor A (*VEGFA*), relaxin family peptide receptor 1 (*RXFP1*), and porcine cyclophilin A (*PPIA*; reference gene) were determined by qPCR.

Briefly, qPCR was performed using Power SYBR Green PCR Master Mix and universal thermal cycling parameters (40 cycles) indicated by the manufacturer on a StepOne Plus System (Applied Biosystems, Carlsbad, CA, USA). Primers were designed using Primer Quest software (Integrated DNA Technologies, Inc., Coralville, IA, USA) and synthesized by Sigma-Aldrich (St. Louis, MO, USA). Primer sequences (Table 1) were evaluated for quality by amplifying serial dilutions of the cDNA template. For each transcript, a 50 ng aliquot of cDNA representing each sample was run in triplicate. 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. Data were analyzed using the relative standard curve method for quantitation of gene expression as described by the manufacturer (ABI User Bulletin 2, 2001; Applied Biosystems, Carlsbad, CA, USA).

Protein Extraction and Immunoblot Analyses

Total uterine protein was extracted by homogenizing tissues (20 mg) in lysis buffer (1% Triton X-100, 10% glycerol, 150 mM Tris-HCl, 300 mM NaCl, and 1 mM MgCl2; pH 7.5). For each sample, individual homogenates were centrifuged (12,000g at 4°C for 10 min), and protein supernatants were removed and stored at −80°C. Protein concentrations were determined using a DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Uterine proteins (20 μg) were resolved on 12% total monomer, NuPAGE Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) under reducing conditions, followed by transfer onto nitrocellulose membranes (0.45 μm pore size; Bio-Rad Laboratories, Hercules, CA, USA). Relative abundance of uterine MMP9 (pro 92 kDa, active 84 kDa), MMP2 (pro 72 kDa, active 66 kDa), and TIMP1 (28 kDa) proteins were determined by immunoblotting and densitometry using actin (43 kDa) as the loading control (Ho *et al.* 2017).

Briefly, membranes were blocked with 10% nonfat milk powder (NFMP) in TBST buffer (25 mm Tris [pH 7.5], 0.14 mm NaCl, 3-mm KCl, and 0.05% Tween 20) for 1 h at room temperature. The membranes were incubated with either mouse anti-human MMP2 (1:1000; IM33; EMD Millipore, Billerica, MA, USA), mouse anti-human MMP9 (1:100; IM09L; EMD Millipore, Billerica, MA, USA), rabbit anti-human TIMP1 (1:1000; C20; Santa Cruz Biotechnology, Dallas, TX, USA), or goat anti-human actin antibody (1:3000, C11; Santa Cruz Biotechnology, Dallas, TX, USA) in TBST-5% NFMP overnight at 4°C. Antibodies directed against human MMP2, MMP9, and TIMP1 were validated for use in the neonatal pig uterus and cervix (Ho *et al.* 2017).

Blots were washed with TBST and incubated with infrared fluorescent dye-conjugated secondary antibodies (1:20,000; LI-COR Biosciences, Lincoln, NE, USA) in TBST-5% NFMP for 1 h at room temperature and proteins were visualized with the Odyssey Clx infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA). Positive controls for MMP2, MMP9, and TIMP1 immunoblots consisted of pregnant sow uterine tissue (gestation d 90), as described by (Ho *et al.* 2017). Negative controls consisted of primary antibody substitution with an irrelevant mouse IgG (for MMP2 and MMP9) or rabbit IgG (for TIMP1), in each case as appropriate. Incubation with secondary antibodies alone showed no evidence of cross reactivity with uterine proteins at expected molecular sizes for targeted proteins.

Protein signals were quantified densitometrically using ImageJ Software (National Institute of Health, Bethesda, MD, USA). All data were corrected for background signals and expressed as target protein to actin ratio. Densitometric analyses of total MMP2 (pro 72 kDa, active 66 kDa) and total MMP9 (pro 92 kDa, active 84 kDa) protein are reported. Immunoblot signals that were below background under identical exposure conditions were recorded as not detectable.

Statistical Analyses

Data were subjected to the analyses of variance using General Linear Model procedures (SAS 2013, Cary, NC, USA) and are presented as least squares means (LSM) ± standard error of the mean (SEM). Preplanned comparisons were performed for each study. For Study 1, statistical models considered the main effects of treatment (nursed vs replacer), and, where appropriate, cell compartment (luminal epithelium vs stroma), and

their interactions. For Study 2, statistical models considered the main effects of treatment (colostrum vs replacer), method of feeding colostrum (nursing vs bottle vs gavage), cell compartment (luminal epithelium vs stroma), and their interactions. For Study 3, statistical models considered the main effects of treatment (colostrum vs replacer), main effects of IGF1 (with vs without IGF1), cell compartment (luminal epithelium vs stroma), and their interactions, as appropriate.

RESULTS

Study 1: Effects of nursing on postnatal endometrial cell proliferation and uterine gene expression at 12 h

Pseudocolor images illustrating the effects of nursing for 12 h from birth on patterns of PCNA immunostaining, and histograms summarizing PCNA LI data for endometrial luminal epithelium and stroma are presented in Figure 2. Positively-stained cells are bright red-yellow against a blue/black background. Signal indicative of PCNA labeling above background was observed in all endometrial cell compartments. In comparison with gilts nursed for 12 h from birth, uterine wall development was retarded in replacer-fed gilts, as indicated by reduced PCNA staining (Fig. 2A and B). Mean endometrial PCNA LI was greater ($P < 0.001$) in nursed than in replacer-fed gilts in both stromal and epithelial cell compartments (Fig. 2C). Neither endometrial thickness (185 ± 24.5 μm) nor stromal cell nuclear density (30 ± 1 cell nuclei/area) were affected by treatment.

Effects of treatment on uterine expression of cell cycle, morphoregulatory, and lactocrine-sensitive gene expression are shown in Figure 2. With regard to cell cycle transcripts, imposition of a lactocrine-null state from birth did not affect uterine *CDK2* or *CDK4* expression (Fig. 2D and 2E). However, expression of both *CCNB1* (Fig. 2F, $P < 0.01$) and *CDK1* (Fig. 2G, $P < 0.001$) was greater in nursed than replacer-fed gilts at 12 h. For uterine morphoregulatory and lactocrine-sensitive gene expression, *WNT5A* (Fig. 2H, $P < 0.02$), *HOXA10* (Fig. 2J, $P = 0.03$), *VEGFA* (Fig. 2K, $P = 0.01$) and *RXFP1* (Fig. 2L, $P < 0.01$) was greater in nursed than replacer-fed gilts at 12 h. Uterine expression of *WNT7A* (Fig. 2I) did not differ between nursed and replacer-fed gilts.

Study 1: Effects of nursing on postnatal uterine MMP2, MMP9, *and* TIMP1 *expression at 12 h*

A representative immunoblot (Fig. 3A) illustrating relative abundance of uterine MMP2, MMP9, TIMP1, and actin proteins at 12 h in nursed and replacer-fed gilts is shown. Treatment did not affect uterine levels of proMMP2 (72 kDa) or MMP2 (66 kDa), which were detected in all tissues (Fig. 3A and 3B). Immunoreactive proMMP9 (92 kDa) and MMP9 (84 kDa) levels were greater (Fig. 3B, $P < 0.001$) at 12 h in nursed as compared to replacer-fed gilts (Fig. 3A and 3B). TIMP1 (28 kDa) was detected in uterine tissues at 12 h in nursed but not in replacer-fed gilts (Fig. 3A and 3B).

Study 2: Effects of a single dose of colostrum or milk replacer and method of feeding on postnatal endometrial histoarchitecture, cell proliferation, and uterine gene expression at 12 h

Figure 4 shows the effects of a single dose of colostrum or milk replacer and method of feeding on postnatal endometrial histoarchitecture at 12 h. Neither treatment nor method of feeding affected endometrial thickness (Fig. 4A). However, stromal cell nuclear density was greater (Fig. 4B, $P = 0.01$) in gilts fed colostrum, by either bottle or gavage, when compared to replacer-fed gilts. Within the colostrum-fed groups, gilts fed colostrum by either bottle or gavage had greater stromal cell nuclear density ($P < 0.001$) compared to those that nursed for the first feeding (Fig. 4B).

Effects of a single dose of colostrum or milk replacer and method of feeding on postnatal endometrial PCNA immunostaining and LI at 12 h are illustrated in Figure 4.

Uterine PCNA immunostaining was more intense in gilts fed a single dose of colostrum (Fig. 4C-E) compared to those fed milk-replacer (Fig. 4F and G). Endometrial stromal (Fig. 4H) and epithelial (Fig. 4I) PCNA LI were greater ($P < 0.001$) in gilts fed a single dose of colostrum by either bottle or gavage when compared to replacer-fed gilts. In lactocrine-null gilts, PCNA immunostaining was similar regardless of the method of feeding (Fig. 4F and G). In nursed gilts, uterine PCNA immunostaining was more intense (Fig. 4C) compared to those fed colostrum via bottle or gavage (Fig.4D and E). However, evaluation of PCNA LI within colostrum-fed groups, revealed no effect of method of feeding in the stromal cell compartment (Fig. 4H), and a slight increase in nursed as compared with other colostrum-fed groups in the epithelium (Fig. 4I; $P = 0.07$).

Effects of treatment and method of feeding on uterine expression of cell cycle transcripts are depicted in Figure 5. Uterine expression of *CDK2* (Fig. 5A) was similar in gilts fed a single dose of colostrum or replacer. However, within colostrum-fed groups, an effect of method of feeding was identified, with greater *CDK2* expression observed in nursed gilts than those fed via bottle or gavage (Fig. 5A, $P = 0.03$). No effects were identified for *CDK4* (Fig. 5B) or *CDK1* (Fig. 5D). Uterine *CCNB1* expression was moderately lower (Fig. 5C; $P = 0.08$) in colostrum- as compared with replacer-fed gilts, with no effect of method of feeding identified.

Effects of a single dose of colostrum or milk replacer and method of feeding on postnatal uterine morphoregulatory and lactocrine-sensitive gene expression at 12 h are depicted in Figure 5. Relative expression of *WNT5A* (Fig. 5E), *WNT7A* (Fig. 5F), *HOXA10* (Fig. 5G), *VEGFA* (Fig. 5H), and *RXFP1* (Fig. 5I) were not affected by treatment (colostrum vs milk-replacer). Within colostrum-fed groups, *WNT7A* expression

was greater in nursed gilts as compared with those fed via bottle or gavage (Fig. 5F, P = 0.05). A treatment by method of feeding interaction was identified for *HOXA10* expression (Fig. 5G, P < 0.05).

Study 3: Effects of a single dose of colostrum or milk replacer, with or without oral IGF1, on postnatal endometrial histoarchitecture and cell proliferation at 12 h.

Endometrial thickness was not affected by a single feeding of colostrum or milk replacer, with or without oral IGF1 (Fig. 6A). However, stromal cell nuclear density was greater (P < 0.005) in colostrum- than with milk replacer-fed gilts (Fig. 6B). Oral supplementation with IGF1 did not affect stromal cell nuclear density in either treatment group (Fig. 6B).

Images illustrating effects of a single feeding of colostrum or milk replacer, with or without oral IGF1, on PCNA immunostaining patterns, and histograms depicting PCNA LI data for uterine tissues at 12 h are shown in Figure 6. Uterine wall development was advanced in gilts fed a single oral dose of IGF1, as reflected by the state of development of nascent uterine glands along the uterine lumen. This was not as well defined in gilts fed milk-replacer alone (Fig. 6C-F). Endometrial PCNA immunostaining was more intense at 12 h in response to a single feeding of colostrum (Fig. 6C), colostrum with IGF1 (Fig. 6D) or replacer with IGF1 (Fig. 6F) as compared to gilts fed replacer alone (Fig. 6E). Mean PCNA LI data for endometrial stroma (Fig. 6G) and epithelium (Fig. 6H) are shown. Main effects of treatment and IGF1 were identified for both cell compartments (P < 0.05). Compared to replacer-fed gilts, a single feeding of

colostrum increased PCNA LI ($P < 0.05$). Similarly, a single oral dose of IGF1 increased PCNA LI in comparison to gilts fed colostrum or milk replacer alone ($P < 0.003$). A treatment by IGF1 interaction was identified for the stromal cell compartment (Fig. 6G, $P < 0.002$). This is illustrated by increased PCNA LI observed in replacer-fed gilts supplemented with IGF1 (Fig. 6G).

DISCUSSION

Previous studies in the pig showed that nursing is necessary for normal development of porcine uterine (Miller *et al.* 2013, Rahman *et al.* 2016, George *et al.* 2017, Ho *et al.* 2017), cervical (Camp *et al.* 2014), and testicular (Rahman *et al.* 2014) tissues between birth and PND 2. Present results confirm and extend those findings for the uterus by showing that lactocrine effects on aspects of uterine development are detectable as early as 12 h postnatally. Indeed, a single dose of colostrum at birth was sufficient to affect postnatal uterine development at 12 h, regardless of method of feeding colostrum. Further, results complement findings for the cervix (Camp *et al.* 2014), indicating that oral IGF1 is bioactive during this period, as reflected by increased uterine cell proliferation. In fact, supplementation of milk-replacer with IGF1 rescued the lactocrine-null phenotype as reflected by PCNA immunostaining patterns. Collectively, results establish the utility of this experimental model system for identification of lactocrine-active factors affecting female reproductive tract development.

At the start of lactation, colostrum is rich in immunoglobulins and MbFs (Coalson & Lecce 1973, Klobasa *et al.* 1987, Farmer & Quesnel 2009). However, over time its composition changes with the transition to mature milk. In the pig, high concentrations of solids and protein in colostrum decrease linearly within the first 12 h postnatally (Coalson & Lecce 1973, Klobasa *et al.* 1987, Farmer & Quesnel 2009). Macromolecule uptake by the gastrointestinal tract occurs within the first 24-48 h after birth, corresponding to the transition from colostrum to mature milk (Leece 1973, Klobasa *et al.* 1987). Gilts that ingest minimal amounts of colostrum during the first day of postnatal life, display altered patterns of neonatal uterine wall development by PND 14

(Bartol *et al.* 2017), and reduced adult uterine capacity to support large, viable litters, reflecting permanent impairment of reproductive performance (Vallet *et al.* 2015). These observations support the idea that lactocrine-sensitive organizational events associated with uterine development within 12-24 h of birth are critical determinants of uterine programming and functional uterine capacity.

Consistent with endometrial histology observed on PND 2 (Miller *et al.* 2013), endometrial thickness and stromal cell nuclear density were similar in nursed and replacer-fed gilts at 12 h postnatally. However, imposition of a lactocrine-null state resulted in decreased endometrial stromal and luminal epithelial cell proliferation, as reflected by PCNA LI, similar to effects of treatment observed on PND 2 (Miller *et al.* 2013). Cell proliferation is regulated by cell cycle-associated gene products (Malumbres & Barbacid 2009). The cell cycle is defined by four phases: G1, S (DNA synthesis), G2, and M (mitosis) (Malumbres & Barbacid 2009). Cyclins and CDKs associated with each phase determine cellular progression through the cycle and are implicated in other roles, such as transcription and DNA damage repair (Lim & Kaldis 2013). Thus, expression patterns of specific cyclins and CDKs are indicative of successful cell division or cell cycle arrest. Mechanistically, CDK4 is activated during G1 phase, in preparation for DNA replication, and CDK2 is involved in G1/S DNA damage checkpoint, as well as S/G2 transitions (Malumbres & Barbacid 2009). Only CDK1 is essential for cell cycle progression and mitosis in most cell types (Malumbres & Barbacid 2009). Therefore, it was not unexpected that uterine expression of *CDK2* and *CDK4* were not lactocrine-sensitive. However, uterine expression of *CCNB1* (Cyclin B1) and *CDK1* increased in nursed as compared to replacer-fed gilts postnatally at 12 h. Considering that Cyclin B1-

CDK1 complexes are necessary for cellular entry into mitosis, this lactocrine effect suggests that mitogenic factors in colostrum support uterine cell division and ultimately, proliferation, as indicated by the increase in PCNA LI reported here.

Communication between stroma and epithelium is required for uterine morphogenesis (Cooke *et al.* 2013). Previous *in situ* hybridization studies showed that a Hoxa/Wnt expression axis develops in the porcine uterus between birth and PND 14 (Bartol *et al.* 2006). Later studies showed that nursing supports specific expression patterns of other markers of uterine development, including ESR1, VEGFA, and RXFP1 (Chen *et al.* 2011, Miller *et al.* 2013). As the uterus develops, these morphoregulatory transcripts are temporospatially expressed (Bartol *et al.* 2006, Chen *et al.* 2011, Miller *et al.* 2013). In the present study, nursing increased postnatal uterine expression of *WNT5A* and *HOXA10* transcripts at 12 h, consistent with stromal expression that increased between birth and PND 14 (Bartol *et al.* 2006). At 12 h, uterine *WNT7A* expression was unaffected by nursing. Studies in mice showed that *WNT7A* is required for development of the uterine Hoxa/Wnt expression axis which, in turn, is necessary for proper radial patterning of the female reproductive tract (Miller & Sassoon 1998). Uterine *VEGFA* expression was unaffected by nursing for two days from birth (Chen *et al.* 2011, Miller *et al.* 2013). However, uterine expression of *VEGFA* increased in nursed as compared to replacer-fed animals at 12 h, suggesting lactocrine-mediated transcription occurs prior to PND 2. Postnatal uterine expression of *RXFP1* increased in nursed versus replacer-fed gilts at 12 h. However, nursing from birth and relaxin administration decreased uterine expression of *RXFP1* on PND 2 (Chen *et al.* 2011, Miller *et al.* 2013). This apparent inconsistency may indicate that lactocrine transmission of milk-borne relaxin (Frankshun

et al. 2011) may be insufficient to down-regulate uterine RXFP1 expression by 12 h postnatally. While serum relaxin in nursed pigs is elevated within 24-48 h of birth (Yan *et al.* 2006b), the extent to which relaxin and other MbFs are transmitted to nursing pigs prior to 24 h is unknown.

In addition to stromal–epithelial cell communication, cell-extracellular matrix interactions are required to support development of uterine glands (Hu *et al.* 2004). The MMP/TIMP system regulates synthesis and degradation of the ECM, necessary for tubulogenesis and branching morphogenesis in several tissues (Werb & Chin 1998). Analysis of the porcine uterine transcriptome between birth and PND 2 identified age- and lactocrine-sensitive transcripts within the plasminogen-activating network, including MMPs and TIMPs (Rahman *et al.* 2016). Further, a recent study determined that nursing for 12 h from birth was sufficient for the expression of uterine MMP9 and TIMP1, as well as cervical MMP9, at levels equivalent to those observed in gilts nursed for 48 h from birth (Ho *et al.* 2017). Consistently, nursing from birth until 12 h is required for normal uterine expression of MMP9 and TIMP1. Similar to previous reports (Chen *et al.* 2011, Ho *et al.* 2017), uterine MMP2 expression was not lactocrine-sensitive. Results reinforce observations that nursing for 12 h from birth is sufficient to provide lactocrine support of the developing neonatal porcine uterus, effects of which are detectable by 12 h.

Studies on the postnatal cervix showed that a single feeding of colostrum, but not milk replacer, was sufficient to support cervical cell proliferation at 12 h (Camp *et al.* 2014). Data presented here extend these findings by showing that a single feeding of colostrum at birth increased stromal cell nuclear density, as well as endometrial stromal

and epithelial cell proliferation at 12 h. These responses were not reflected by associated changes in endometrial thickness or uterine expression of cell cycle or morphoregulatory transcripts. By contrast, as described above, nursing for 12 h from birth increased uterine expression of some cell cycle-related and morphoregulatory genes at 12 h postnatal. It is important to note that mRNA measurements do not always correlate with protein expression. One possible explanation for this is that transcription is a relatively early, rapid event and mRNAs are typically less stable than proteins (Vogel & Marcotte 2012). In this regard, it is not surprising that for an acute treatment, defined by a single feeding of colostrum at birth, only differences in PCNA immunostaining patterns were identified at 12 h. Thus, as reported previously (Ho *et al.* 2017), duration of nursing from birth is important to support normal uterine expression patterns of lactocrine-sensitive gene products. It will be important to determine the extent to which lactocrine signaling is required to support uterine development within this 12 h window.

In addition to nursing, other methods of feeding (colostrum delivery) and related maternal-neonatal interactions have potential to affect development (Curley & Champagne 2016). Here, methods of feeding were evaluated for colostrum-fed groups. For the majority of postnatal endpoints examined at 12 h, no effects of feeding method were identified. For colostrum-fed gilts, main effects of feeding method (nursed vs bottle- and gavage-fed) were identified only for endometrial stromal cell nuclear density and uterine *CDK2* and *WNT7A* expression. This may reflect differences in the quantity or quality of colostrum consumed in a single nursing bout compared to that delivered in a bottle or gavage feeding (15 ml/kg BW/h) (Houle *et al.* 2000). Colostrum consumption during nursing varies substantially (Devillers *et al.* 2011), and can be influenced by

multiple factors including birth weight, birth rank, litter size (Devillers *et al.* 2007, Devillers *et al.* 2011), and teat position (Wu *et al.* 2010). Still, the preponderance of data suggests that method of feeding colostrum does not affect postnatal porcine uterine development overtly at 12 h. Nevertheless, it will be important to consider potential effects of feeding method in future studies of this kind. Evidence of lactocrine effects on both patterns of neonatal uterine wall development and lifetime fecundity in nursed gilts that consumed minimal amounts of colostrum from birth, as defined by birth day immunoglobulin immunocrit (Vallet *et al.* 2015, Bartol *et al.* 2017), provide support for the lactocrine hypothesis unbiased by feeding method.

An earlier study showed that IGF1, present in high concentrations in porcine colostrum (Simmen *et al.* 1988), increased cervical cell proliferation by 12 h postnatal when supplemented orally in milk replacer at birth (Camp *et al.* 2014). Here, in contrast to data for the cervix (Camp *et al.* 2014), results showed positive effects of IGF1 on endometrial cell proliferation when supplemented in either milk replacer or colostrum. Moreover, effects of IGF1 were additive when administered in colostrum. Collectively, data support the idea that peptide growth factors such as IGF1 can be lactocrine-active when administered at birth. Results also suggest that strategic supplementation of milk replacer with lactocrine-active peptides could enable rescue of an aberrant, lactocrine-deficient developmental program (Bagnell *et al.* 2017, Bartol *et al.* 2017).

Studies in multiple species, including rhesus macaques (Hinde *et al.* 2015), rodents (Nusser & Frawley 1997, Liu *et al.* 2014), and marsupials (Nicholas *et al.* 1997, Trott *et al.* 2003), as well as the pig (Vallet *et al.* 2015), indicate the importance of nursing, as well as the quality and quantity of colostrum consumed, on lactocrine

programming of developing tissues. Prior to this report, examination of lactocrine effects on neonatal porcine uterine development focused on the period between birth and PND 2 (Chen *et al.* 2011, Miller *et al.* 2013, Rahman *et al.* 2016, George *et al.* 2017, Ho *et al.* 2017). However, evidence that minimal colostrum consumption on the day of birth affects patterns of uterine wall development with lasting consequences in adulthood (Vallet *et al.* 2015, Bartol *et al.* 2017) indicates lactocrine effects on the uterine organizational program occur within 24 h postnatally. Data presented here support this concept. Evidence that, regardless of method, a single feeding of colostrum at birth supports normal patterns of endometrial cell proliferation a 12 h suggests that lactocrine regulation of postnatal uterine development is initiated with the first ingestion of colostrum.

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Table 1: Primer sequences for quantitative real-time PCR.

Gene Symbol	Gene Name	Accession #	Forward and Reverse Primers
CDK1	Cyclin dependent kinase 1	NM_001159304.2	**F:** GGGTCAGCTCGCTACTCAAC **R:** AAGTTTTTGACGTGGGATGC
CDK2	Cyclin dependent kinase 2	NM_001285465.1	**F:** TTGCTGAGATGGTGACCCG **R:** GGGTCCCAAGAGTCCGAAAG
CDK4	Cyclin dependent kinase 4	NM_001123097.1	**F:** GGCCAGAATCTACAGCTACCAG **R:** TCCACAGGTGTTGCATACGT
CCNB1	Cyclin B1	NM_001170768.1	**F:** CCAACTGGTTGGTGTCACTG **R:** GCTCTCCGAAGAAAATGCAG
WNT5A	Wingless-type MMTV integration site family member 5A	CA_997683	**F:** GGAGCACAGCCTCTCTGCAG **R:** GGTCCTGATACAAGTGGCATAGTTT
WNT7A	Wingless-type MMTV integration site family member 7A	CA_997684	**F:** CACCACCAAGACCTGCTGG **R:** TCCTTGAGCACGTAGCCCA
HOXA10	Homeobox A10	AF_281156	**F:** CGGCCGGAAGAAGCA **R:** AGAAACTCCTTCTCCAGCTCCA
VEGFA	Vascular endothelial growth factor A	AF_318502	**F:** AAGATCCGCAGACGTGTAAA **R:** CACATCTGCAAGTACGTTCG
RXFP1	Relaxin family peptide receptor 1	CA_994862	**F:** GCATCACTTTGAGGCAGAGACA **R:** CCTCGGCAAAGACATTGCAT
VEGFA	Vascular endothelial growth factor A	AF_318502	**F:** AAGATCCGCAGACGTGTAAA **R:** CACATCTGCAAGTACGTTCG
PPIA	Porcine cyclophilin A	AU_058466	**F:**TTATAAAGGTTCCTGCTTTCACAGAA **R:** TGCCATTATGGCGTGTGAAG

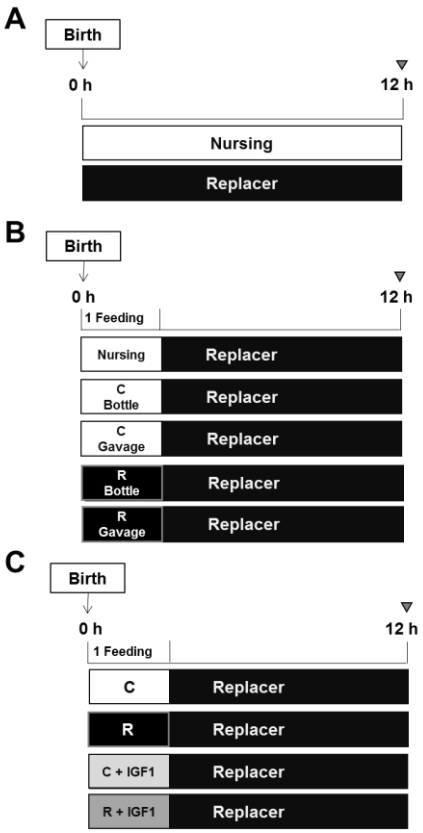

Figure 1: Experimental designs for (A) Study 1; (B) Study 2; and (C) Study 3. (A) Study 1: Gilts were allowed to either nurse ad libitum or were fed milk replacer (R) from birth (0 h) to 12 h postnatal. (B) Study 2: Gilts were randomly assigned at birth to one of five treatment groups: 1) nursed ad libitum for the first hour after birth or provided a single volume (15 ml/kg BW) of either 2) colostrum, bottle-fed; 3) colostrum, gavage-fed; 4) replacer, bottle-fed; or 5) replacer, gavage-fed. Pooled porcine colostrum, collected at 0 h of lactation, or a commercially complete porcine milk replacer were used for the first feeding in bottle and gavage-fed groups. Thereafter, all gilts were gavage-fed milk replacer until 12 h of age. (C) Study 3: Gilts were gavage-fed a single dose of colostrum (C) or replacer (R) at birth with or without oral IGF1 followed by replacer feeding by gavage until 12 h postnatal when uteri were collected indicated by arrowheads; n = 4–6/group.

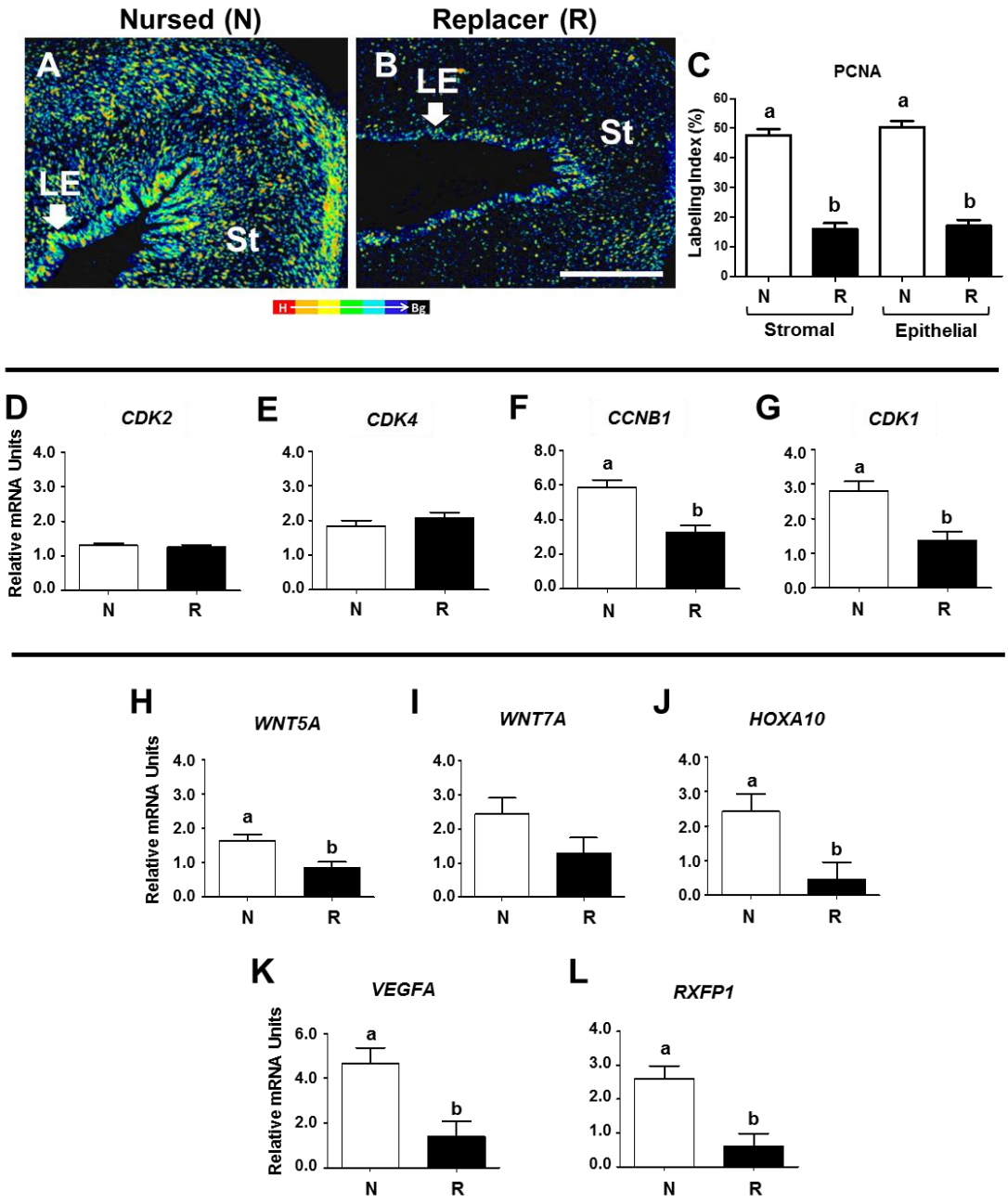

Figure 2: Effects of nursing on postnatal uterine development at 12 h. Pseudocolored images illustrate PCNA labeling patterns in luminal epithelium (LE) and stroma (St) for uterine tissues collected at 12 h postnatal from (A) nursed and (B) replacer-fed gilts. Signal intensity is indicated by color (bottom legend: H, high, to Bg, background). Positively, immunostained cells appear red-yellow on a black background; scale bar indicates 100 μm. (C) Endometrial PCNA labeling indices for stromal and epithelial cell

compartments are shown. Histograms demonstrate the effects of nursing on postnatal uterine expression of cell cycle transcripts (D) CDK2, (E) CDK4, (F) CCNB1, (G) CDK1, as well as morphoregulatory and lactocrine-sensitive transcripts (H) WNT5A, (I) WNT7A, (J) HOXA10, (K) VEGFA, and (L) RXFP1 at 12 h. Data were normalized to cyclophilin gene expression. White bars denote nursed and black bars denote replacer-fed groups. Data are presented as LSM ± SEM; n = 5-6/group. Different letters indicate differences between nursed and replacer-fed gilts at 12 h postnatal ($P < 0.05$).

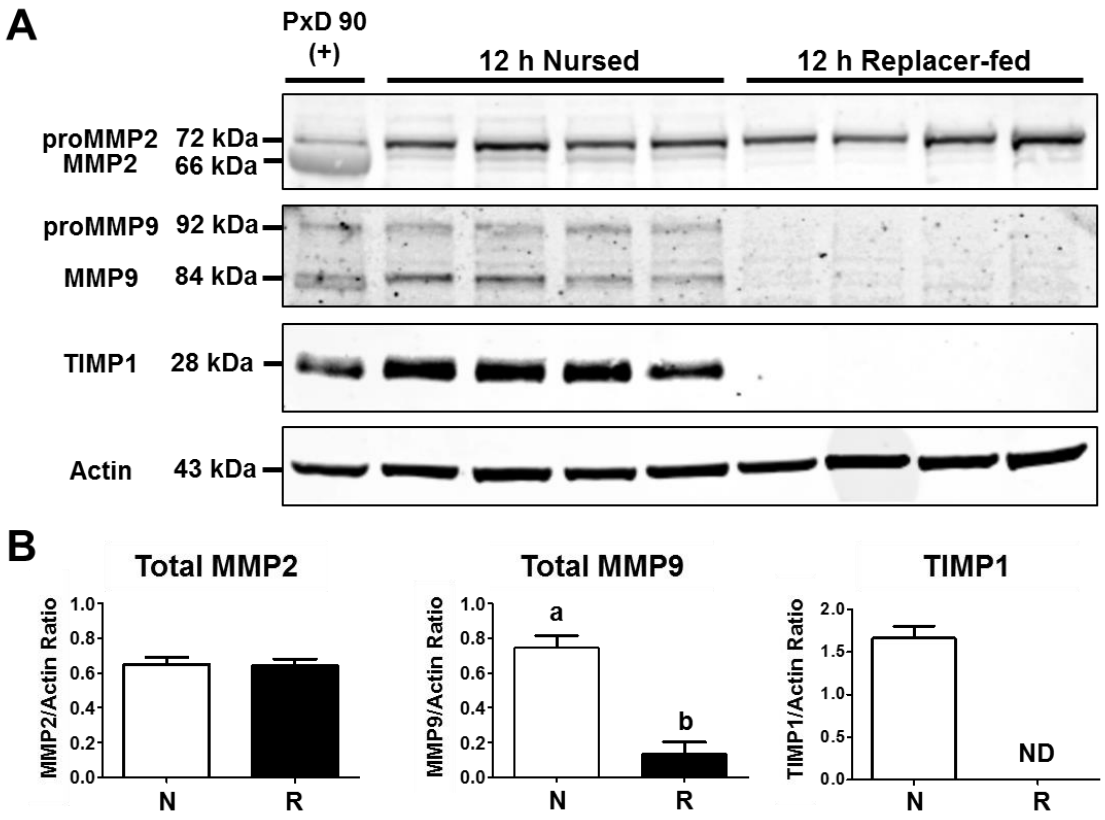

Figure 3: Effects of nursing on postnatal uterine MMP2, MMP9, and TIMP1 protein expression at 12 h. Representative immunoblots (A) are shown. Immunoreactive bands for proMMP2 (72 kDa) and MMP2 (66 kDa), proMMP9 (92 kDa) and MMP9 (86 kDa), as well as for TIMP1 (28 kDa) are indicated. Positive (+) control consisted of uterine tissue from a sow on pregnancy day 90 (PxD 90), as described in Materials and Methods. Densitometric data (B) for the relative expression of total MMP2 (72 + 66 kDa), total MMP9 (92 + 84 kDa), and TIMP1 in relation to actin (43 kDa) are presented as LSM ± SEM; n = 8/group. Signal below the detection range is marked as non-detectable (ND). Different letters indicate differences between nursed and replacer-fed gilts at 12 h ($P < 0.05$).

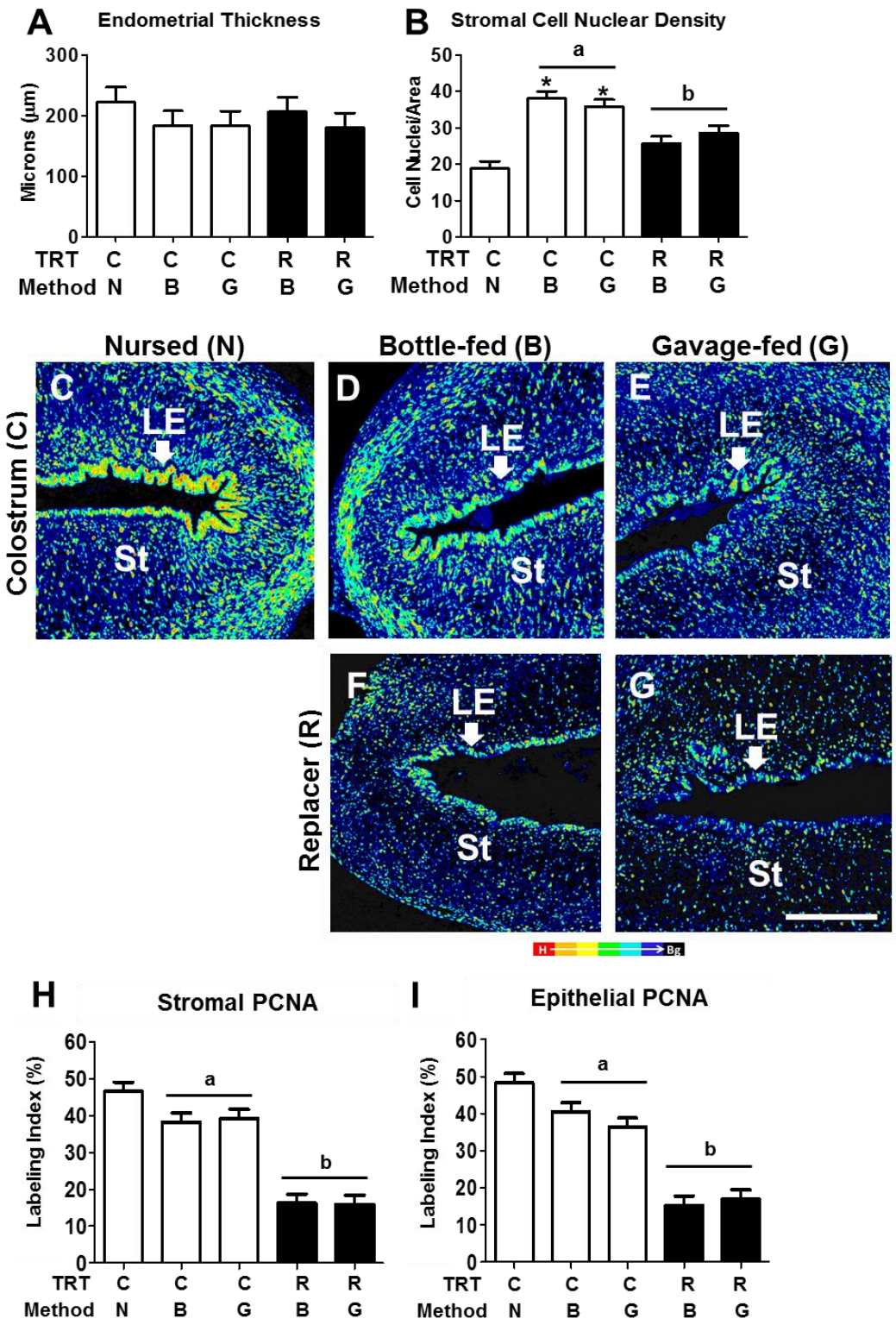

Figure 4: Effects of a single dose of colostrum (C) or milk replacer (R) and method of feeding [nursing (N), bottle-fed (B), and gavage (G)] on postnatal uterine development at

12 h. Data for (A) endometrial thickness and (B) stromal cell nuclear density at 12 h are shown. Pseudocolored images illustrate PCNA labeling patterns in luminal epithelium (LE) and stroma (St) for postnatal uterine tissues collected at 12 h from (C) colostrum, nursed, (D) colostrum, bottle-fed, (E) colostrum, gavage-fed, (F) replacer, bottle-fed, and (G) replacer, gavage-fed gilts. Signal intensity is indicated by color (bottom legend: H, high, to Bg, background). Positively, immunostained cells appear red-yellow on a black background; scale bar indicates 100 μm. Endometrial PCNA labeling indices for (H) stromal and (I) epithelial cell compartments are shown. Data are expressed as LSM ± SEM; n = 5/group. Different letters denote differences between colostrum-fed and replacer-fed gilts ($P \leq 0.01$). Within colostrum-fed groups, asterisks indicate an effect of method of feeding (nursing vs bottle- or gavage-fed groups; $P < 0.001$).

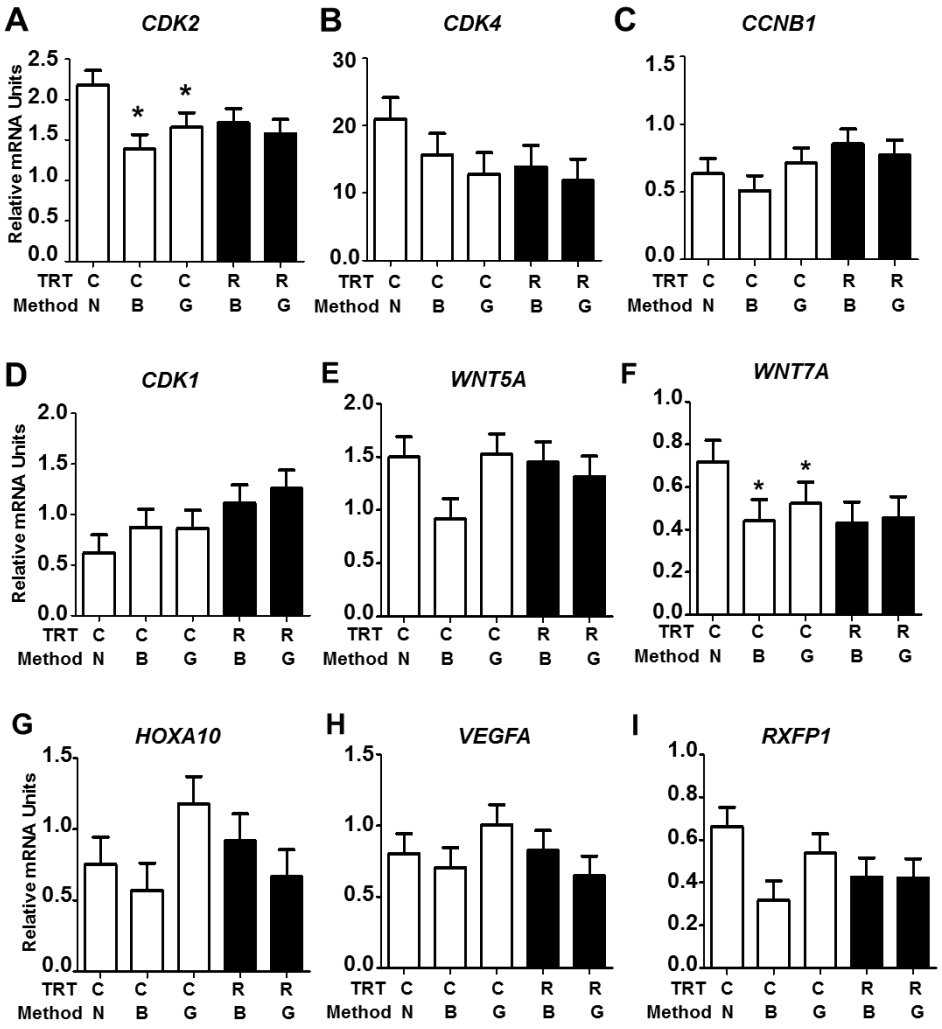

Figure 5: Effects of a single dose of colostrum (C) or milk replacer (R) and method of feeding [nursing (N), bottle-fed (B), and gavage (G)] on postnatal uterine gene expression at 12 h. Cell cycle transcript expression for (A) CDK2, (B) CDK4, (C) CCNB1, and (D) CDK1, as well as morphoregulatory and lactocrine-sensitive transcript expression for (E) WNT5A, (F) WNT7A, (G) HOXA10, (H) VEGFA, and (I) RXFP1 at 12 h are shown. Data were normalized to cyclophilin gene expression and are presented as LSM ± SEM; n = 5/group. Within colostrum-fed groups, asterisks indicate an effect of method of feeding (nursing vs bottle- or gavage-fed groups: CDK2, $P = 0.03$; WNT7A, $P < 0.05$). A treatment by method of feeding interaction was identified for HOXA10 ($P < 0.05$).

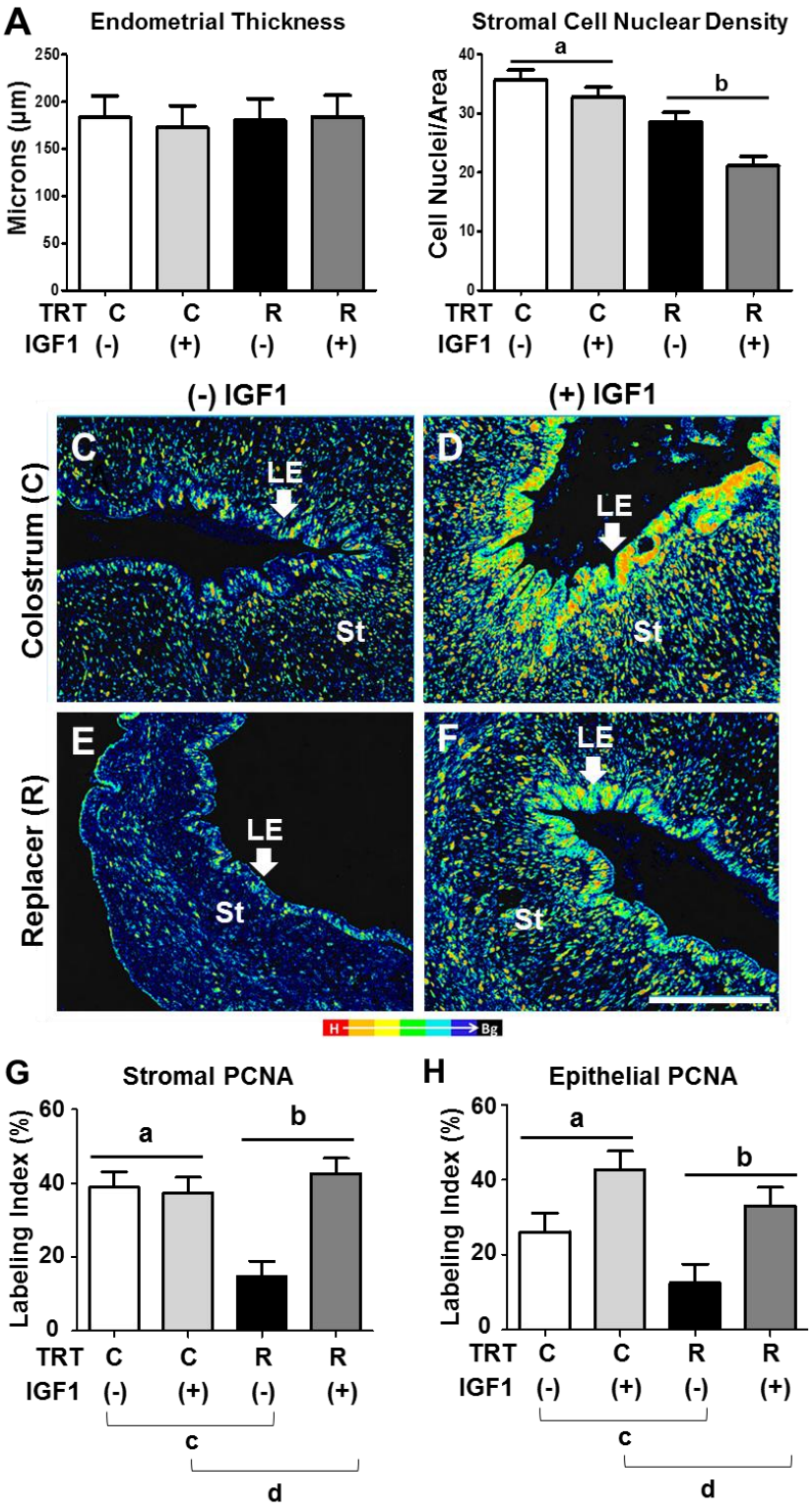

Figure 6: Effects of a single dose of colostrum (C) or milk replacer (R), with (+) or without (−) oral IGF1, on postnatal endometrial histology and PCNA immunostaining

patterns at 12 h. Data for endometrial thickness (A) and stromal cell nuclear density (B) are shown. Pseudocolored images illustrate PCNA labeling patterns in luminal epithelium (LE) and stroma (St) for uterine tissues collected at 12 h from gilts fed a single dose of (C) colostrum, without IGF1, (D) colostrum, with IGF1, (E) milk replacer, without IGF1, or (F) milk replacer, with IGF1. Signal intensity is indicated by color (bottom legend). Positively, immunostained cells appear red-yellow on a black background; scale bar indicates 100 μm. Endometrial PCNA labeling indices for (G) stromal and (H) epithelial cell compartments are shown. Data are presented as LSM ± SEM; n = 6/group. Letters a and b indicate differences between colostrum-fed and replacer-fed groups ($P < 0.05$). Letters c and d indicate differences between gilts fed colostrum or replacer with and without IGF1 ($P < 0.05$). See text for detailed descriptions of these relationships.

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CHAPTER III

Defining Age- and Lactocrine-Sensitive Elements of the Neonatal Porcine Uterine MicroRNA-mRNA Interactome

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ABSTRACT

Factors delivered to offspring in colostrum within two days of birth support neonatal porcine uterine development. The uterine mRNA transcriptome is affected by age and nursing during this period. Whether uterine microRNA (miRNA) expression is affected similarly is unknown. Objectives were to: 1) determine effects of age and nursing on porcine uterine miRNA expression between birth and postnatal day (PND) 2 using miRNA sequencing (miRNAseq) and; 2) define affected miRNA-mRNA interactions and associated biological processes using integrated target prediction analysis. At birth (PND 0), gilts were euthanized, nursed *ad libitum* or gavage-fed milk replacer for 48 h. Uteri were collected at birth or 50 h postnatal. miRNAseq data were validated using quantitative real-time PCR (qPCR), and targets were predicted using an established mRNA database generated from the same tissues. For PND 2 versus PND 0 comparisons, 31 differentially expressed (DE) miRNAs were identified for nursed, and 42 DE miRNAs were identified for replacer-fed gilts. Six DE miRNAs were identified for nursed versus replacer-fed gilts on PND 2. Target prediction for inversely correlated DE miRNA-mRNA pairings indicated 20 miRNAs targeting 251 mRNAs in nursed, versus 29 miRNAs targeting 585 mRNA in replacer-fed gilts for PND 2 versus PND 0 comparisons, and 5 miRNAs targeting 81 mRNAs for nursed versus replacer-fed gilts on PND 2. Biological processes predicted to be affected by age and nursing included cell-to-cell signaling, cell morphology, and tissue morphology. Results indicate novel age- and lactocrine-sensitive miRNA-mRNA relationships associated with porcine neonatal uterine development between birth and PND 2.

INTRODUCTION

One of the defining characteristics of mammals is lactation. Nursing provides a conduit for delivery of both nutrients and milk-borne bioactive factors (MbFs) from mother to offspring in colostrum (first milk) via a lactocrine mechanism. Colostral MbFs can affect neonatal development as proposed in the lactocrine hypothesis (Yan *et al.* 2006b, Bartol *et al.* 2008). In pigs and other mammals, female reproductive tract development, initiated prenatally, continues postnatally (Cooke *et al.* 2013). Data for the pig show that disruption of normal lactocrine signaling from birth (postnatal day = PND 0), by substitution of a porcine milk replacer for colostrum, altered patterns of uterine gene expression by PND 2, and inhibited uterine endometrial gland development by PND 14 (Miller *et al.* 2013). The observation that gilts consuming minimal amounts of colostrum on their day of birth, as reflected by low immunoglobulin immunocrit ratio (Bartol *et al.* 2013), displayed reduced live litter size over four parities as adults (Bartol *et al.* 2013, Vallet *et al.* 2015) provided strong support for the lactocrine hypothesis for maternal programming of uterine development and function.

Recently, RNA sequencing (mRNAseq) of the neonatal porcine uterus revealed both age- and lactocrine-sensitive changes in gene expression (Rahman *et al.* 2016). More than 3000 genes were differentially expressed in uteri at PND 2 as compared to PND 0. Lactocrine effects were also pronounced on PND 2, when more than 800 genes were differentially expressed by uterine tissues in nursed as compared to replacer-fed gilts. Mechanisms responsible for such age- and lactocrine-sensitive transcriptional changes in the neonatal uterus are unknown.

One mechanism by which transcriptomic changes can occur is through regulation by microRNAs (miRNAs). MicroRNAs are short non-coding RNAs (18-25 nucleotides

in length) that regulate post-transcriptional gene expression through translational repression and/or mRNA destabilization and degradation (Bartel 2004). MicroRNAs target mRNAs, and a single miRNA can have multiple mRNA targets (Lewis *et al.* 2005). Moreover, disruption of miRNA processing affects biological processes governing development and function of the uterus (Nothnick 2016). In pigs, miRNAs were identified in adult endometrium (Su *et al.* 2014, Bidarimath *et al.* 2015, Krawczynski *et al.* 2015b) and in placental tissues (Wessels *et al.* 2013, Krawczynski *et al.* 2015b, Liu *et al.* 2015) during early pregnancy. Endometrial (Cordoba *et al.* 2015) and placental (Li *et al.* 2015) miRNA-mRNA interactions during pregnancy were also characterized. Little is known about miRNA expression in the developing uterus, or the roles of miRNAs in regulation of uterine gene expression during the perinatal period. Ideally, such studies should integrate miRNA and mRNA expression profiles generated from the same tissues. With these observations in mind, objectives of the present study were to: 1) determine effects of age and nursing on the porcine uterine miRNA transcriptome between birth and PND 2 using miRNA sequencing (miRNAseq); and 2) define uterine miRNA-mRNA interactions and associated age- and lactocrine-sensitive biological processes *in silico* using integrated target prediction analysis.

MATERIALS AND METHODS

Animals and Experimental Design

Gilts (*Sus scrofa domesticus*) were born and raised from 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 animals 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). Consideration was given to ensure that sows nursed litters of similar size and that treatments were balanced for potential effects of litter (n = 8).

At birth, gilts (n = 12) were assigned randomly to be either: 1) sacrificed on PND 0, prior to nursing (n = 4); 2) nursed *ad libitum* from birth through 48 h of age (PND 2N, n = 4); or 3) gavage-fed a nutritionally-complete, commercial pig milk replacer (30 mL/kg BW/2 h; Advance Liqui-Wean MSC Specialty Nutrition; Carpentersville, IL, USA) from birth through 48 h of age (PND 2R, n = 4) (Houle *et al.* 2000). Gilts were euthanized and uterine tissues were collected on either PND 0 or 50 h of age. Uteri were trimmed of associated tissues and uterine wet weights (mg) were recorded. Uterine tissue samples were stored in RNAlater (Life Technologies, Carlsbad, CA, USA) and stored at -80°C until total RNA was extracted.

Uterine RNA Isolation and Analysis

Total RNA (including miRNA and mRNA) was isolated from 50-60 mg of whole uterine tissue from one uterine horn/animal using the miRNeasy Mini Kit (Qiagen Inc.,

Valencia, CA, USA) following manufacturer's protocol. RNA quantity was determined using a Qubit® 2.0 Fluorometer (Invitrogen; Carlsbad, CA, USA) and RNA integrity was evaluated using an Agilent 2100 Bioanalyzer (Applied Biosystems; Carlsbad, CA, USA). Samples with an RNA integrity number (RIN) $\geq$ 8.0 were used for library preparation for miRNA sequencing (miRNAseq).

Preparation of miRNA Libraries

miRNAseq was performed at the Genomic Services Laboratory, HudsonAlpha Institute for Biotechnology (Huntsville, AL, USA). Total RNA (500 ng) from each uterine sample was used for RNA library preparation using the NEBNext® Small RNA Library Prep Set for Illumina (New England Biolabs Inc., Ipswich, MA, USA) according to the manufacturer's protocol. Briefly, adapters were ligated to total RNA, multiplex primers were hybridized, and reverse transcription was accomplished using SuperScript III RT (Life Technologies, Grand Island, NY, USA) for 1 h at 50°C. Bar codes with uniquely indexed primers were attached to each cDNA library and amplified through six PCR cycles. Following PCR amplification, purification was done using the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA, USA). Size selection of the libraries was then performed using a 3% dye-free agarose gel on the Pipin prep instrument (Sage Science, Beverly, MA, USA). Post size-selected miRNA library concentration was assessed using a Qubit® 2.0 Fluorometer and library quality was determined using a DNA High Sense chip on 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 1.25 nM and equimolar amounts of each sample were pooled prior to sequencing.

miRNA Sequencing and Data Analysis

miRNAseq was performed using an Illumina HiSeq 2500 instrument (Illumina Inc., San Diego, CA, USA) at 50bp Single End condition, generating approximately 15 million reads per sample. Quality control checks on raw sequence data from each sample were performed using FastQC (Babraham Bioinformatics, London, UK). Raw reads were imported on a commercial data analysis platform Avadis NGS (Strand Scientifics, CA, USA). Adapter trimming was done to remove ligated adapters from the 3' end of the sequenced reads with only one mismatch allowed; poorly aligned 3' ends were also trimmed. Sequences shorter than 15 nucleotides in length were excluded from further analysis. Trimmed reads with low qualities (base quality score less than 30, alignment score less than 95, mapping quality less than 40) were removed. Filtered reads were used to extract and count miRNAs which were annotated with miRBase release 18 database (Griffiths-Jones 2004, Griffiths-Jones *et al.* 2006, Griffiths-Jones *et al.* 2008, Kozomara & Griffiths-Jones 2011, Kozomara & Griffiths-Jones 2014). Reads were grouped according to their respective identifiers followed by quantification of miRNA abundance (Robinson & Oshlack 2010). Differentially expressed miRNAs, based on fold change (≥ ± 2.0), were identified with respect to effects of neonatal age (PND 2N versus PND 0; PND 2R versus PND 0) and nursing (PND 2N versus PND 2R). Probability values for each differentially expressed miRNA were estimated by z-score calculations using a false discovery rate of 0.05. Data were subjected to principal component analysis and

hierarchical clustering created with Spearman correlation coefficient. Relative fold change in miRNA abundance was illustrated using volcano plots generated for each comparison using R Programming (GNU General Public License; www.r-project.org).

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

TaqMan® Advanced miRNA assays (Life Technologies) were used for qPCR validation of the miRNAseq data. The same RNA used to generate cDNA libraries for miRNAseq was also used to validate results by qPCR. Uterine RNA from individual animals was pooled to create PND 0, PND 2N, and PND 2R samples. For qPCR validation, miRNAs were selected at random from the population of miRNAs that increased or decreased in at least one of the three conditions. Reverse transcription of total RNA samples (5 ng) was done using gene-specific primers and the TaqMan® Advanced MicroRNA cDNA Synthesis kit. Primer pairs specific to each miRNA and TaqMan® Fast Advanced PCR Master Mix were used for amplification per manufacturer's recommendation. Primers were evaluated for quality by amplifying serial dilutions of the cDNA template. Control qPCR reactions included substitution of water 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.

Geometric means of qPCR CT (cycle threshold) values for miRNAs with high, medium, and low reads (ssc-miR-21, ssc-miR-101, and ssc-miR-127 respectively) were used for normalization as described by Maalouf (Maalouf *et al.* 2014). Expression of reference miRNAs did not change significantly between samples from PND 0, PND 2N,

or PND 2R gilts, as determined using data from miRNAseq and qPCR analyses. Data generated by qPCR were analyzed using the ΔΔCT method as described by Applied Biosystems (ABI User Bulletin 2, 2001). Pearson correlation coefficients were determined to compare miRNA expression fold-change results obtained by miRNAseq and qPCR.

Integrated Target Prediction Analysis

Human orthologs of mRNAs were identified using NCBI BLAST and a custom computer program written in Python (www.python.org) using NCBI modules within Biopython (Cock *et al.* 2009), as described elsewhere (Rahman *et al.* 2016). Human orthologs of miRNAs were identified using miRBase release 21 database (Griffiths-Jones 2004, Griffiths-Jones *et al.* 2006, Griffiths-Jones *et al.* 2008, Kozomara & Griffiths-Jones 2011, Kozomara & Griffiths-Jones 2014). Relationships between differentially expressed miRNAs and their respective differentially expressed mRNA targets were determined using Qiagen's Ingenuity Pathway Analysis MicroRNA Target Filter (IPA, Qiagen Redwood City, CA; www.qiagen.com/ingenuity). Differentially expressed uterine mRNA data, generated from the same tissues, were reported previously (Rahman *et al.* 2016) and can be found in the GEO repository under series accession number GSE72388. Gene enrichment and functional annotation analyses were conducted using the Database for Annotation, Visualization, and Integrated Discovery (DAVID 6.7; http://david.abcc.ncifcrf.gov/) (Huang da *et al.* 2009b, Huang da *et al.* 2009a) and IPA to identify enriched biological functions among differentially expressed gene transcripts, including miRNAs and mRNAs.

RESULTS

Effects of Age and Nursing on Neonatal Uterine miRNA Expression

Uterine miRNA data for PND 0, PND 2N, and PND 2R treatments grouped independently according to principal component analysis and Spearman correlation (Fig. 1, A-B). By examining age (PND 2N versus PND 0; PND 2R versus PND 0) and nursing (PND 2N versus PND 2R), differentially expressed miRNAs (≥ 2-fold, $P < 0.05$) were determined in neonatal porcine uteri. Expression analyses are illustrated as volcano plots (Fig. 2, A-C). Results indicated 31 differentially expressed miRNAs on PND 2N when compared with PND 0 and all of these miRNAs decreased with age (Fig. 2A). When gilts were fed milk replacer for two days from birth, 42 miRNAs were differentially expressed on PND 2R as compared with PND 0 (Fig. 2B). Of these differentially expressed miRNAs, one was increased and 41 were decreased on PND 2R compared to PND 0 (Fig. 2B). A total of six miRNAs were differentially expressed in the uteri of nursed (PND 2N) as compared to replacer-fed (PND 2R) gilts (Fig. 2C). Of these differentially expressed miRNAs, three were increased and three were decreased on PND 2N as compared to PND 2R (Fig. 2C). Differentially expressed miRNAs in each comparison and their respective fold changes are presented in Supplemental Tables S1-3. Quantitative real-time PCR results for six miRNAs to validate miRNAseq data are shown in Fig. 2, D-F. Positive correlations between miRNAseq and qPCR results were identified ($r = 0.76$, $P < 0.01$).

To examine the overlap of differential miRNA expression, a Venn diagram was created to categorize expression domains through a three-way comparison (Fig. 3). Overall, 62 miRNA species were differentially expressed (Fig. 3). There was a single

differentially expressed miRNA species (miR-184) that was common to each group (Fig. 3). Comparison of differentially expressed miRNAs in PND 2N versus PND 0 revealed 16 uniquely expressed, 13 miRNAs were shared with PND 2R versus PND 0 and one was shared with PND 2N versus PND 2R (miR-296-5p; Fig. 3). For PND 2R versus PND 0, 27 differentially expressed miRNAs were uniquely expressed and one was common with PND 2N versus PND 2R (miR-345-5p; Fig. 3). A total of three differentially expressed miRNAs were uniquely expressed between nursed and replacer groups on PND 2 (Fig. 3). Altogether, common and unique differentially expressed uterine miRNAs were identified between treatments.

Integrated Target Prediction Analyses

Based on the previous results, it was hypothesized that a portion of the uterine transcriptomic changes observed due to age and nursing were regulated by differentially expressed miRNAs. Therefore, integrated miRNA-mRNA analyses were conducted, via Qiagen's Ingenuity Pathway Analysis (IPA) miRNA Target Filter, to explore differentially expressed uterine miRNAs and mRNA targets between treatments. Figure 4 summarizes the workflow and output for IPA data integration and bioinformatics analyses. Table 1A-C lists the differentially expressed miRNAs and top five differentially expressed mRNA targets. Supplementary Tables S4-S6 list all differentially expressed mRNA targets for each comparison.

With respect to pigs nursed from birth (PND 2N versus PND 0), there were 31 differentially expressed miRNAs and 3,283 differentially expressed mRNA transcripts. Of the 31 differentially expressed miRNAs, 20 had 251 predicted mRNA targets (Fig. 4,

Table 1A). Relative abundance of all of these miRNAs decreased, and their respective mRNA targets increased, on PND 2 in nursed gilts (Fig. 4, Table 1A).

For pigs fed replacer from birth (PND 2R versus PND 0), there were 42 differentially expressed miRNAs and 4662 differentially expressed mRNA transcripts (Fig. 4). Of these 42 miRNAs, 29 were predicted to target 585 mRNA transcripts (Fig. 4). Relative abundance of all but one of these miRNAs decreased (Fig. 4). The single miRNA that increased (miR-345-5p) on PND 2 in replacer-fed gilts was predicted to have 103/585 differentially expressed mRNA targets (Table 1B). The other 28 differentially expressed miRNAs and the number of predicted mRNA targets are presented in Table 1B.

For nursed versus replacer-fed gilts on PND 2 (PND 2N versus PND 2R), there were six differentially expressed miRNAs and 896 differentially expressed mRNA transcripts. Five of the six lactocrine-sensitive miRNAs were predicted to target 81 mRNAs (Fig. 4). These five included miR-184, miR-296-5p, miR-345-5p, miR-490-3p, and miR-582 (Fig. 4; Table 1C). The number of predicted mRNA targets and the top five differentially expressed mRNAs are presented in Table 1C.

Integrated Functional Annotation Analyses

Enriched biological processes in neonatal porcine uteri associated with mRNAs targeted by miRNAs affected by age in nursed gilts (PND 2N versus PND 0) as identified by DAVID are shown in Table 2A. Functional annotation by DAVID analysis revealed terms including: ‘defense response’, ‘inflammatory response’, ‘response to wounding’, ‘immune response’, and ‘cellular homeostasis’ (Table 2A). Selected functional

annotation categories identified by IPA for inversely correlated, differentially expressed transcripts (miRNAs and mRNAs) in neonatal porcine uteri between PND 2N versus PND 0 are shown in Figure 5A and Supplementary Table S7. MicroRNA-mRNA interactions were predicted to be involved with multiple biological processes including 'cellular movement', 'cell-to-cell signaling and interaction', 'cellular function and maintenance', and 'tissue morphology' (Fig. 5A).

Enriched biological processes in neonatal porcine uteri associated with mRNAs targeted by miRNAs affected by age in replacer-fed gilts (PND 2R versus PND 0) as identified by DAVID are shown in Table 2B. Functional annotation by DAVID analysis revealed terms including: 'cell-cell signaling', 'cellular ion homeostasis', and 'cellular homeostasis' (Table 2B). Selected functional annotation categories identified by IPA for inversely correlated, differentially expressed transcripts (miRNAs and mRNAs) in neonatal porcine uteri between PND 2R versus PND 0 are shown in Figure 5B and Supplementary Table S8. MicroRNA-mRNA interactions were predicted to be involved with multiple biological processes similar to the PND 2N versus PND 0 comparison, including: 'cell morphology', 'cellular growth and proliferation', 'tissue morphology', and 'cell-to-cell signaling and interaction' (Fig. 5B). However, the miRNAs involved and the mRNA targets were distinct (Supplementary Table S8).

Enriched biological processes in neonatal porcine uteri associated with mRNAs targeted by miRNAs affected by nursing (PND 2N versus PND 2R) as identified by DAVID are shown in Table 2C. Functional annotation by DAVID analysis identified categories of interest, including: 'cellular ion homeostasis', 'chemical homeostasis', and 'cellular component morphogenesis' (Table 2C). Further investigation of lactocrine-

sensitive miRNAs and their mRNA targets by comparison of PND 2N versus PND 2R uterine transcripts by IPA identified ‘cell-to-cell signaling and interaction’, ‘organ, organismal, tissue, and cellular development’, and ‘cell, organ, and tissue morphology’ (Fig. 5C; Supplementary Table S9).

For the three miRNAs unique to the PND 2N versus PND 2R comparison, two (miR-490-3p and miR-582) were predicted to target 12 mRNAs. Biological processes associated with these miRNA-mRNA relationships, identified by DAVID analysis, included: ‘organ morphogenesis’ and ‘muscle tissue morphogenesis’ (data not shown). Using IPA, functional annotation categories previously observed, such as ‘cell, organ, and tissue morphology’, ‘organ, organismal, tissue, and cellular development’, as well as ‘reproductive system development and function’ were identified (Supplementary Table S10).

Predicted miRNA-mRNA Uterine Interactome Networks Affected by Age and Nursing

Ingenuity Pathway Analysis (IPA) enables illustration of miRNA-mRNA interactome networks. Shown here are examples for ‘Cell-to-Cell Signaling and Interaction’ networks illustrating the size, directionality (positive or negative fold-change) and overlap for each comparison (Fig. 6). Additional examples for the ‘Tissue Morphology’ network are provided in Supplementary Figure S1. In each case, up-regulated transcripts are shown in red and down-regulated transcripts are green.

Interactome networks presented here depict important dynamic relationships associated with effects of age and nursing on the uterine transcriptome. The skeletons of interactome networks are identical, illustrating overlap in response domains (Fig. 6).

These relationships are seen readily in inset details (Fig. 6A-C). Note, the number of affected elements in 'Cell-to-Cell Signaling and Interaction' networks (denoted by red and green) differs between experimental groups (Fig. 6A-C), as does the direction (up or down) and degree (color intensity) of change for specific elements of each interactome. For example, *FOXA2*, a predicted miRNA target, is unchanged between PND 0 and PND 2N (Fig. 6A inset), upregulated on PND 2 compared to PND 0 in replacer-fed gilts (Fig. 6B inset), and downregulated in nursed as compared to replacer-fed gilts on PND 2 (Fig. 6C inset). Similar complex relationships were identified for other interactome networks (Supplemental Figures S1, S5-S7, and other data not shown).

DISCUSSION

Through lactocrine mechanisms, bioactive factors are delivered from mother to offspring as a consequence of nursing and support neonatal development (Yan *et al.* 2006b, Menzies *et al.* 2007, Bartol *et al.* 2008, Liu *et al.* 2014, Hinde *et al.* 2015). Recently, both age and lactocrine effects on the neonatal porcine uterine transcriptome were defined (Rahman *et al.* 2016). Using the same tissues, present results illustrate similar effects on miRNA expression profiles associated with uterine development in nursed as compared to replacer-fed gilts between birth and PND 2. Further, integrated target prediction analyses conducted *in silico* extended previous mRNAseq findings (Rahman *et al.* 2016) by revealing novel age- and lactocrine-sensitive miRNA-mRNA interactions and biological processes associated with porcine neonatal uterine development. Results suggest that miRNAs may contribute to regulation of uterine gene expression post-transcriptionally between birth and PND 2 and that imposition of a lactocrine-null state by replacer-feeding during this period dysregulates the normal uterine developmental program at transcriptional and, potentially, post-transcriptional levels.

The first few days of neonatal life encompass a period of organizational transition for the porcine endometrium. Differentiation of uterine glandular epithelium (GE) from luminal epithelium (LE) is marked by onset of estrogen receptor (ESR1) expression in nascent GE, evident by 24 h postnatal (Bartol *et al.* 2013). Transition of the endometrium from a non-proliferative to a proliferative state is associated with progression of morphogenetic events supportive of uterine gland development by PND 3 (Bartol *et al.* 1993, Spencer *et al.* 1993, Masters *et al.* 2007). The fact that uterine histogenesis

proceeds normally prior to PND 60 in gilts ovariectomized at birth (Bartol *et al.* 1993, Tarleton *et al.* 1998) emphasizes the importance of extra-ovarian postnatal uterotrophic support. Nursing for 12 h from birth is necessary to support development of porcine uterine and cervical tissues to PND 2 (Ho *et al.* 2016), and imposition of a lactocrine-null state from birth by milk replacer feeding altered patterns of endometrial cell proliferation and cell compartment-specific gene expression during this period (Miller *et al.* 2013). Present data extend earlier observations (Miller *et al.* 2013, Rahman *et al.* 2016), indicating that nursing and maternally derived, lactocrine-active factors constitute one source of such uterotrophic support.

Neonatal uterine miRNAs expressed at PND 0, PND 2N, and PND 2R clustered independently, as indicated by principal component analysis, indicating distinct miRNA response domains for each condition. Characteristics of age- and lactocrine-sensitive response domains can be defined in terms of size, directionality (positive or negative fold-change) and overlap. For age comparisons (PND 2N versus PND 0 and PND 2R versus PND 0), absolute domain size was smaller in nursed (31 differentially expressed miRNAs) as compared to replacer-fed gilts (42 differentially expressed miRNAs) by PND 2. Moreover, with the exception of a single miRNA in PND 2R gilts, relative uterine miRNA expression decreased from birth to PND 2 in both groups. Consequently, as supported by results of *in silico* target prediction analysis, imposition of a lactocrine-null condition in replacer-fed gilts resulted in a larger number of differentially expressed miRNAs with a larger number of predicted mRNA targets on PND 2. While overlap in differential uterine miRNA expression for age comparisons was substantial, the majority of differentially expressed miRNAs were unique to each domain (PND 2N versus PND 0

and PND 2R versus PND 0). These miRNA relationships and differences in the size of mRNA target pools (251 for nursed versus 585 for replacer-fed) suggest substantial divergence in transcriptomic signatures by PND 2 between these two conditions, with potential to effect very different organizational trajectories (Miller *et al.* 2013, Rahman *et al.* 2016). The size of the response domain for uterine miRNA expression on PND 2 in nursed versus replacer-fed gilts (six differentially expressed miRNAs) was smaller than those defined for both age comparisons. This undoubtedly reflects the fact that the age component is absent from this comparison. While overlap was observed in all domains, three of six differentially expressed miRNAs were unique to the PND 2N versus PND 2R domain, indicating a lactocrine-specific response.

A few miRNAs identified here, including miR-296-5p, miR-345-5p, and miR-582, are expressed in adult uterine and placental tissues (Kuokkanen *et al.* 2010, Bidarimath *et al.* 2015, Krawczynski *et al.* 2015b, Li *et al.* 2015, Liu *et al.* 2015). However, none were identified in neonatal uterine tissues prior to this report. Two miRNAs for which uterine expression was both unique and lactocrine-sensitive on PND 2 included miR-582, which increased, and miR-345-5p, which decreased in nursed as compared to replacer-fed gilts. These miRNAs were also expressed differentially in adult human endometrium (Kuokkanen *et al.* 2010). Specifically, expression of both miR-582-5p and miR-345 decreased in late proliferative as compared to mid-secretory endometrium (Kuokkanen *et al.* 2010). Authors proposed that these miRNAs could function to suppress endometrial cell proliferation during the secretory phase of the menstrual cycle (Kuokkanen *et al.* 2010). In the pig, imposition of a lactocrine-null state for two days from birth reduced epithelial cell proliferation by PND 2 in both LE and GE

(Miller *et al.* 2013). Thus, lactocrine effects on neonatal uterine miR-582 and miR-345-5p expression observed here may be affecting events associated with regulation of endometrial cell proliferation and nascent uterine gland development.

Uterine expression of miR-451, which decreased due to age in both nursed and replacer-fed gilts, increased in estrogen-treated, ovariectomized mice (Nothnick & Healy 2010). Additionally, wild-type mice that received endometrial fragments from miR-451 deficient mice displayed increased uterine expression of fibrinogen alpha chain precursor and fewer endometriotic lesions (Nothnick *et al.* 2014). This was suggested to reflect effects on the biochemistry of cell adhesion. The extent to which miR-451 may be regulating similar organizationally important processes in the neonatal porcine uterus remains to be determined.

The fact that miRNA-mRNA interactions have functional consequences for mRNA stability and translational efficiencies (Bartel 2004, Lewis *et al.* 2005, Kuokkanen *et al.* 2010) emphasizes the importance of defining such interactions. In the pig, miRNA-mRNA interactions are implicated in uteroplacental and pregnancy biology. Unique uterine miRNA signatures were defined during implantation (Su *et al.* 2014, Bidarimath *et al.* 2015, Krawczynski *et al.* 2015a, Krawczynski *et al.* 2015b) and in pregnancy (Wessels *et al.* 2013, Liu *et al.* 2015). Integrated analysis of miRNA-mRNA networks in placental tissues of Large White and Qingping sows revealed mRNAs and miRNAs associated with onset of labor, as well as a subset of genes that may play a role in regulation of gestation length (Li *et al.* 2015). Endometrial mRNAs and miRNAs in pregnant sows were also associated with extreme prolificacy phenotypes (Cordoba *et al.*

2015). Present results now implicate miRNA-mRNA interactions in neonatal porcine uterine development.

Potential mRNA targets and related interactions were identified here using DAVID and IPA. Results generated using DAVID provided information regarding predicted mRNA targets of differentially expressed miRNAs (Huang da *et al.* 2009a). Prediction of miRNA-mRNA interactions and their potential functions required use of IPA. This program incorporates miRNA and mRNA data, fold change values, and experimentally validated miRNA-mRNA interactions from miRecords (Xiao *et al.* 2009), TarBase (Vlachos *et al.* 2015), and predicted interactions from TargetScan (Agarwal *et al.* 2015). Use of both DAVID and IPA enabled objective assessment of miRNA-mRNA interactions within this complex dataset.

Present results, taken together with data for the neonatal porcine uterine mRNA transcriptome (Rahman *et al.* 2016), implicate many biological processes associated with uterine development between birth and PND 2. Among enriched biological processes associated with miRNA-mRNA interactions related to effects of age and nursing were those likely to be involved with cytodifferentiative and morphogenetic events affecting development of the neonatal uterine wall (Yan *et al.* 2006a). Biological processes including ‘cell-to-cell signaling and interaction’, ‘cellular assembly and organization’, ‘cellular function and maintenance’, and ‘cell morphology and tissue morphology’ were determined to be age- and lactocrine-sensitive. IPA analyses of miRNA-mRNA interactions unique to nursing (PND 2N versus PND 2R) identified similar biological processes. Consistently, previous RNAseq analyses (Rahman *et al.* 2016) identified similar processes for the mRNA transcriptome alone. Observations are consistent with

data indicating that events associated with imposition of a lactocrine-null state for two days from birth set the stage for significant changes in endometrial development that are evident by PND 14 in the neonatal pig (Miller *et al.* 2013).

Careful evaluation of related miRNA–mRNA networks showed that, while some elements of these networks are shared, others are unique to nursed and lactocrine-null domains. Interactome networks show the size of the response domain, number of affected elements, direction (up or down) and degree of expression (color intensity) for each element of the domain of comparison. For example, the number of elements for 'Cell-to-Cell Signaling and Interaction' is the same for each comparison, while the number of affected elements and direction of predicted effects are comparison-specific. By way of illustration, expression of *FOXA2,* implicated as a mediator of murine uterine gland genesis (Jeong *et al.* 2010) and a target for miR-184, is predicted to be unaffected by age in nursed gilts, up-regulated in replacer-fed gilts between birth and PND 2, and down-regulated on PND 2 in nursed as compared to replacer-fed gilts. These predicted effects on *FOXA2* expression are likely to reflect complex miRNA-mRNA interactions affected by age and nursing. The miRNA-mRNA interactions identified here remain to be proven functionally.

Robust computational models used to predict miRNA targets notwithstanding, precise identification of targeted transcripts remains challenging (Agarwal *et al.* 2015). While imperfect, the *in silico* approach to miRNA target prediction taken here benefits from the fact that expression profiling of both miRNA and mRNA populations was conducted on the same uterine tissues (Rahman *et al.* 2016). Complimentary miRNA and mRNA data generated through these efforts, used in concert with *in silico* target

prediction analyses, will support studies designed to define functional miRNA-mRNA interactions.

Results of miRNAseq analyses identified both age- and lactocrine-sensitive miRNAs in the neonatal porcine uterus between birth and PND 2. Integrated target prediction analyses extended previous mRNAseq findings by revealing novel age- and lactocrine-sensitive miRNA-mRNA interactions predicted to regulate biological processes associated with porcine neonatal uterine development during this period. Results reinforce the importance of nursing from birth and lactocrine signaling on establishment of an optimal uterine developmental program (Miller *et al.* 2013, Rahman *et al.* 2016).

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Table 1. Integrated target prediction analysis[1] for differentially expressed uterine miRNAs and mRNA targets between (A) PND 2N versus PND 0, (B) PND 2R versus PND 0, and (C) PND 2N versus PND 2R as identified by Ingenuity Pathway Analysis.

(A) PND 2N versus PND 0

miRNA	# of Targeted mRNAs[2]	Top 5 Differentially Expressed Target mRNAs
let-7d-5p	30	*AGXT2, PTPRO, SLC10A2, DAPK2, DUSP9*
miR-106a	26	*ERICH3, PTPRO, ZBTB38, DAPK2, ESR1*
miR-1343	26	*C14orf105, PLEKHB1, LRRN2, SRRM4, PTPRO*
miR-135	22	*GABRG1, CXCL10, STMN4, FAM25A, HNRNPA3*
miR-136	17	*ATP5F1, SRRM4, FAM229B, SLC7A3, TMEM232*
miR-146a-5p	19	*CCL8, CAMP, S100A12, SEPT14, CNTF*
miR-184	10	*PLEKHB1, BST2, CX3CR1, F5, C21orf62*
miR-193a-5p	16	*C14orf105, NRXN1, THEMIS2, CPXM2, FXYD3*
miR-205	11	*WDR77, C17orf97, ZBTB38, AFF3, CXorf21*
miR-206	32	*CXCL11, CCL2, FXYD3, SRRM4, BRI3BP*
miR-210	7	*CAPN9, SF3B3, PCYT1B, CEND1, ATXN10*
miR-221-3p	14	*BBOX1, GABRG1, SEPT14, NRXN1, CXCL11*
miR-296-5p	42	*CXCL10, HRASLS5, SERPINA1, FXYD3, SRRM4*
miR-339-5p	29	*GABRG1, NRXN1, RBM3, UNC45B, PTPRO*
miR-374a-5p	13	*CCL8, GABRG1, CCL2, ZBTB38, AFF3*
miR-451	5	*CXorf21, BATF, NR5A2, KIAA0101, FAM159B*
miR-455-3p	20	*XCL1, MS4A2, CAMK2N1, ANKRD34C, CBLN2*
miR-486	11	*BUB1B, CAMK2N1, AFF3, VTCN1, INMT*
miR-504	26	*UNC45B, ZBTB38, SAA4, FEZ1, EPHA8*
miR-708-5p	28	*C14orf105, PLEKHB1, SKA1, COL17A1, SRRM4*

(B) PND 2R versus PND 0

miRNA	# of Targeted mRNAs[2]	Top 5 Differentially Expressed Target mRNAs
miR-106a	35	*VCL, DAPK2, PTH, SCRT2, PTPRD*
miR-10a-5p	32	*ATP5F1, RBM3, OPALIN, SKA1, UNC45B*
miR-129a-5p	19	*AFF3, CALM1, AUTS2, SF3B3, CAMK2N1*
miR-129b	30	*SNTN, CFAP61, VNN2, BPI, KLK15*
miR-135	39	*KALRN, OPALIN, STMN4, SLC9A4, PTPRD*
miR-149	61	*VCL, FAM216B, CAPN8, TDRD3, MURC*
miR-181b	34	*KALRN, TRIM64/TRIM64B, THBS4, CCL8, AGT*
miR-184	17	*FOXA2, BST2, DLX3, SCRT2, C21orf62*
miR-185	54	*KALRN, UNC45B, LRRC38, GPX6, SOX5*
miR-193a-5p	30	*VCL, KLK4, DIO2, THEMIS2, C14orf105*
miR-196a	18	*CALM1, SCRT2, PDE11A, PABPC1L2A, INMT*
miR-206	38	*CWC15, SOX5, CXCL11, CCL2, CALM1*
miR-221-5p	22	*BBOX1, CXCL11, ACTC1, SEPT14, RBP2*
miR-30b-3p	70	*TRIM64/TRIM64B, VCL, AGT, SNTN, PATE3*
miR-331-5p	15	*SNTN, HR, INMT, FAM19A3, GEN1*
miR-335	19	*KALRN, NPAS4, GBP4, NOS1, LSM1*
miR-345-5p	103	*CRISPLD2, PIEZO2, GRIK3, HSPA12A, EZR*
miR-34c	59	*CAV3, WDR77, HOXA13, VCL, DAPK2*

miR-361-3p	72	*GGT1, TMEM242, FAM92B, ACTC1, NPAS4*
miR-362	14	*TBXAS1, ANGPTL7, PTPRD, F5, ANKRD34B*
miR-451	11	*MEGF6, CRELD2, GDAP1, CXorf21, FAM159B*
miR-486	18	*AFF3, BUB1B, CAMK2N1, INMT, UBTF*
miR-504	41	*SAA4, KALRN, SAA2-SAA4, UNC45B, FAM196A*
miR-505	23	*TDRD3, MPL, ASCL4, LZTFL1, NOS1*
miR-615	14	*BPIFB4, CFAP61, LRRN2, ITSN1, SESN2*
miR-628	22	*FAM229B, SKA1, TMEM242, CALM1, CRYBA4*
miR-671-5p	34	*WDR77, OPALIN, VNN2, PTPRD, SF3B3*
miR-769-3p	19	*VCL, GPX6, DIO2, NPAS4, SCRT2*
miR-9843-3p	32	*MYH7, SOCS2, EED, PTPRD, C17orf80*

(C) PND 2N versus PND 2R

miRNA	**# of Targeted mRNAs[2]**	**Top 5 Differentially Expressed Target mRNAs**
miR-184	4	*FOXA2, LIPG, NOS1, SIRPA*
miR-296-5p	49	*RAB11FIP5, HEYL, GPR37L1, ADCY2, OXCT1*
miR-345-5p	20	*GALNTL6, OXCT1, GRIK3, PCDH10, HSPA12A*
miR-490-3p	6	*KALRN, CLEC18A/CLEC18C, SP5, SLC5A3, MAGI1*
miR-582	7	*KALRN, PBK, TTN, AGTPBP1, YTHDF3*

[1]For case where miRNA expression is down-regulated and mRNA expression is upregulated or miRNA expression is up-regulated and mRNA expression is down-regulated.

[2]Individual miRNAs can target overlapping target populations of mRNAs.

Table 2. Top 10 enriched biological processes in neonatal porcine uteri associated with miRNA-mRNA interactions between (A) PND 2N versus PND 0, (B) PND 2R versus PND 0, and (C) PND 2N versus PND 2R as identified by DAVID functional annotation analysis.

(A) PND 2N versus PND 0

Functional Terms of Overrepresented Biological Processes[a]	Enrichment Score[b]
Defense response (24)	4.8
Inflammatory response (15)	3.7
Response to wounding (19)	3.3
Immune response (22)	3.2
Acute-phase response (5)	2.6
Locomotory behavior (11)	2.3
Cellular homeostasis (15)	2.2
Behavior (15)	2.2
Response to calcium ion (5)	2.1
Chemotaxis (8)	2.1

(B) PND 2R versus PND 0

Functional Terms of Overrepresented Biological Processes[a]	Enrichment Score[b]
Vitamin metabolic process (10)	3.2
Cellular chemical homeostasis (25)	2.9
Ion homeostasis (26)	2.8
Cell-cell signaling (34)	2.7
Cellular ion homeostasis (24)	2.7
Fat-soluble vitamin metabolic process (6)	2.4
Chemical homeostasis (29)	2.4
Cellular metal ion homeostasis (15)	2.4
Cellular homeostasis (27)	2.4
Response to temperature stimulus (9)	2.3

(C) PND 2N versus PND 2R

Functional terms of Overrepresented Biological Processes[a]	Enrichment Score[b]
Cell differentiation in hindbrain (3)	2.9
Regulation of neurotransmitter levels (4)	2.5
Neurotransmitter metabolic process (3)	2.4
Neuron differentiation (7)	2.0
Cytosolic calcium ion homeostasis (4)	1.9
Transmission of nerve impulse (6)	1.8
Regulation of neuron differentiation (4)	1.8
Regulation of membrane potential (4)	1.7
Chemical homeostasis (7)	1.7
Cellular ion homeostasis (6)	1.7

[a] Values within parentheses indicate the number of annotated mRNAs targeted by miRNAs that are involved with the corresponding functional term.

[b] Enrichment scores were calculated by taking the geometric mean of the p-values associated with the differentially expressed transcripts involved in the corresponding annotation cluster (in $-\log_{10}$ scale).

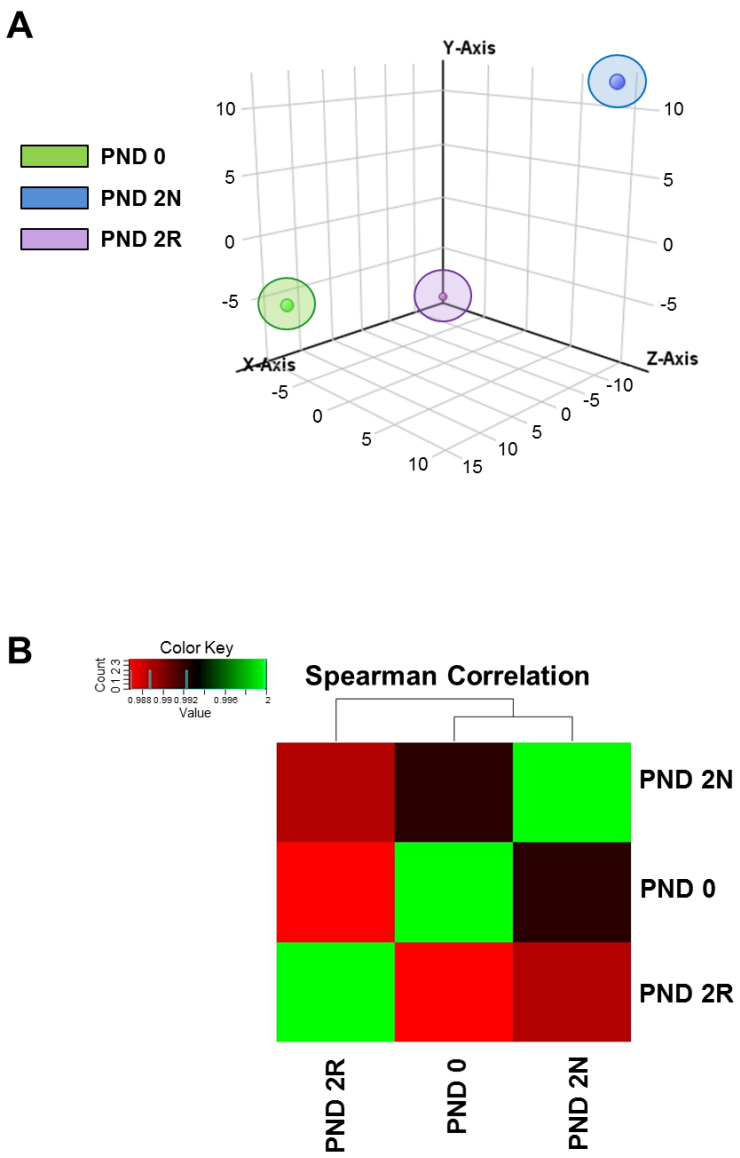

Figure 1. (A) Principal component analysis (PCA) plot and (B) Spearman correlation heat map from miRNAseq analysis. A) PCA plot of pooled neonatal porcine uterine samples showing the clustering of expressed miRNAs for PND 0 (green), PND 2N (blue), and PND 2R (purple). B) Heat map of pairwise correlations between pooled samples based on the Spearman correlation coefficients. Light green represents highest correlations and bright red represents lowest correlations.

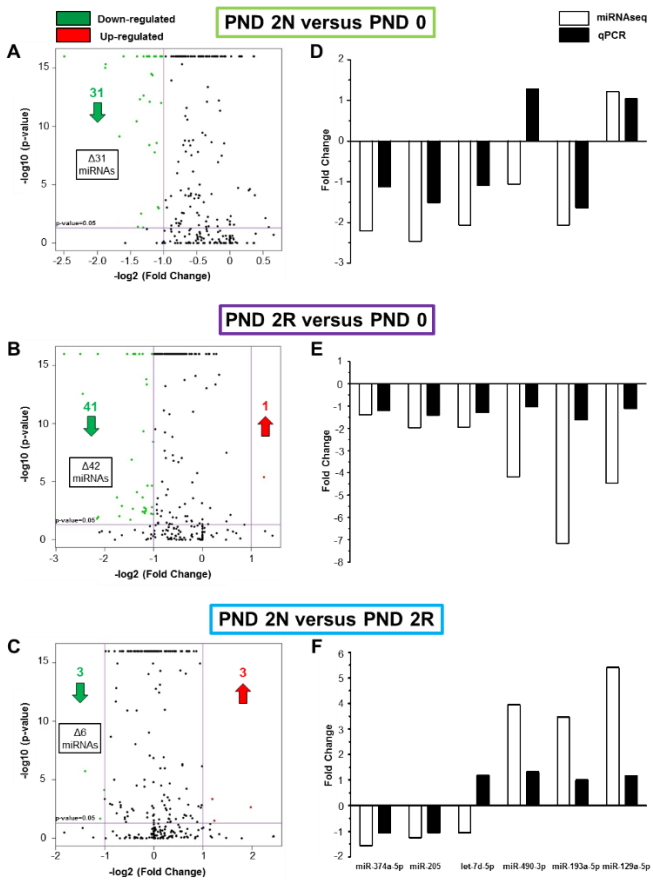

Figure 2. Volcano plots (left) demonstrating differences in uterine expression of miRNAs between (A) PND 2N versus PND 0, (B) PND 2R versus PND 0, and (C) PND 2N versus PND 2R. Up-regulated miRNAs are indicated in red (fold-change ≥ 2) and down-regulated miRNAs are denoted in green. Black indicates miRNAs that did not change between groups. The horizontal line indicates $P = 0.05$. For each comparison, the total number of differentially expressed miRNAs is given (Δ value). The number of miRNAs that were down-regulated (green) or up-regulated (red) are shown. Results of qPCR validation (right) for six miRNAs identified by miRNAseq for (D) PND 2N versus PND 0; (E) PND 2R versus PND 0; (F) PND 2N versus PND 2R. White bars indicate miRNAseq fold change; black bars indicate qPCR fold change. An overall positive correlation between miRNAseq and qPCR results was identified ($r = 0.76$, $P < 0.01$).

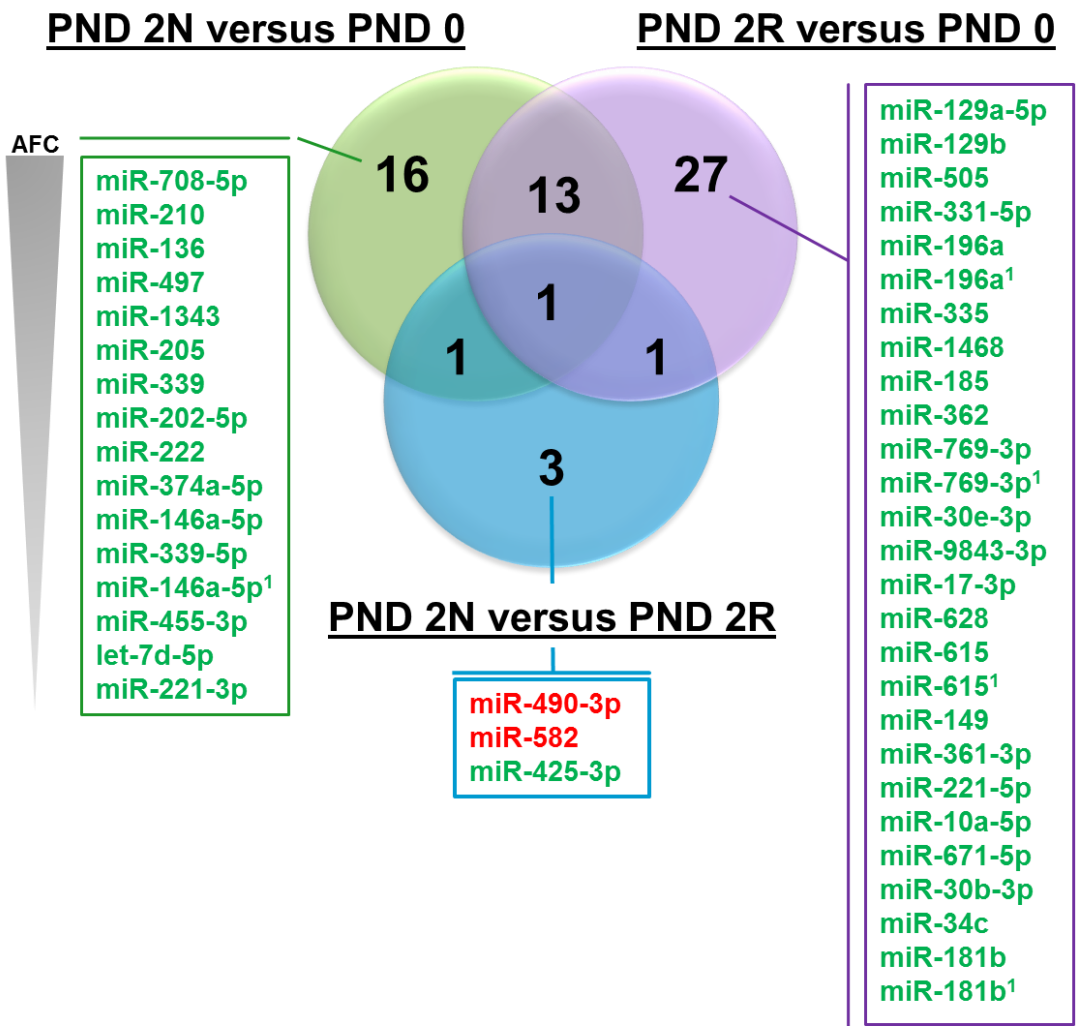

Figure 3. Venn diagram illustrating unique and overlapping differential miRNA expression domains for the three group comparisons [PND 2N versus PND 0 (green); PND 2R versus PND 0 (purple); PND 2N versus PND 2R (blue)]. Values indicate the number of differentially expressed miRNAs associated with unique and overlapping domains. Differentially expressed miRNAs determined to be unique to each comparison are listed in descending order for absolute fold change (AFC > 2-fold; red = up-regulated, green = down-regulated). Superscript 1 indicates miRNAs with same name found at different genomic loci.

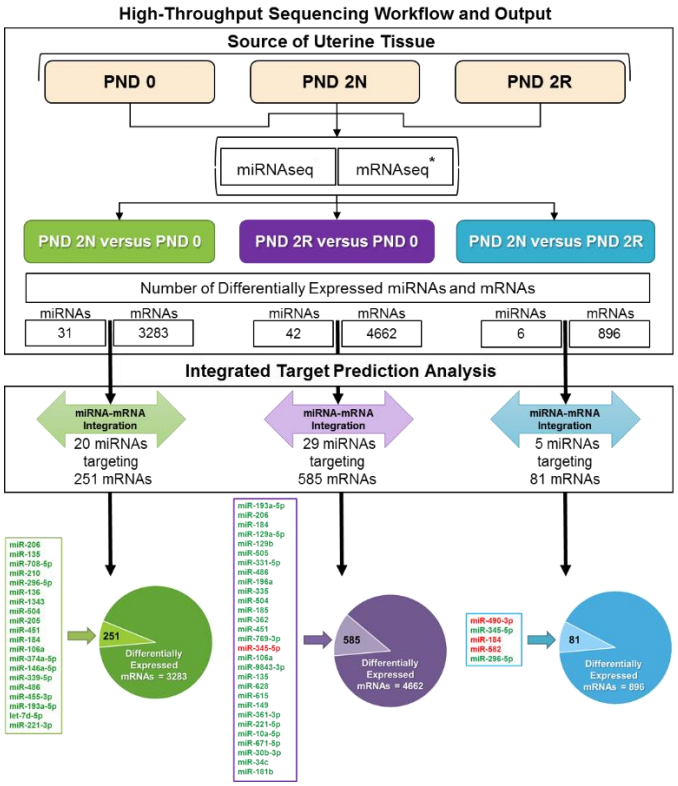

Figure 4. High-throughput sequencing workflow and output. Uterine tissues were obtained from gilts 1) at birth on PND 0, before nursing; 2) after nursing *ad libitum* from birth through 48 h of age (PND 2N); or 3) after gavage-feeding a commercial pig milk replacer for 48 h (PND 2R). Total uterine RNA was isolated and both small RNAs and mRNAs were sequenced as described in Materials and Methods. Asterisk indicates mRNAseq data for uterine mRNAs as described in Rahman et al (Rahman *et al.* 2016). The numbers of differentially expressed miRNAs and mRNAs for each comparison are presented. Integrated target prediction analysis was conducted using IPA as described in Materials and Methods. The numbers of differentially expressed miRNAs targeting differentially expressed mRNAs are presented for each comparison. Individual miRNAs targeting mRNAs are listed (bottom; absolute fold change > 2; red = up-regulated, green = down-regulated).

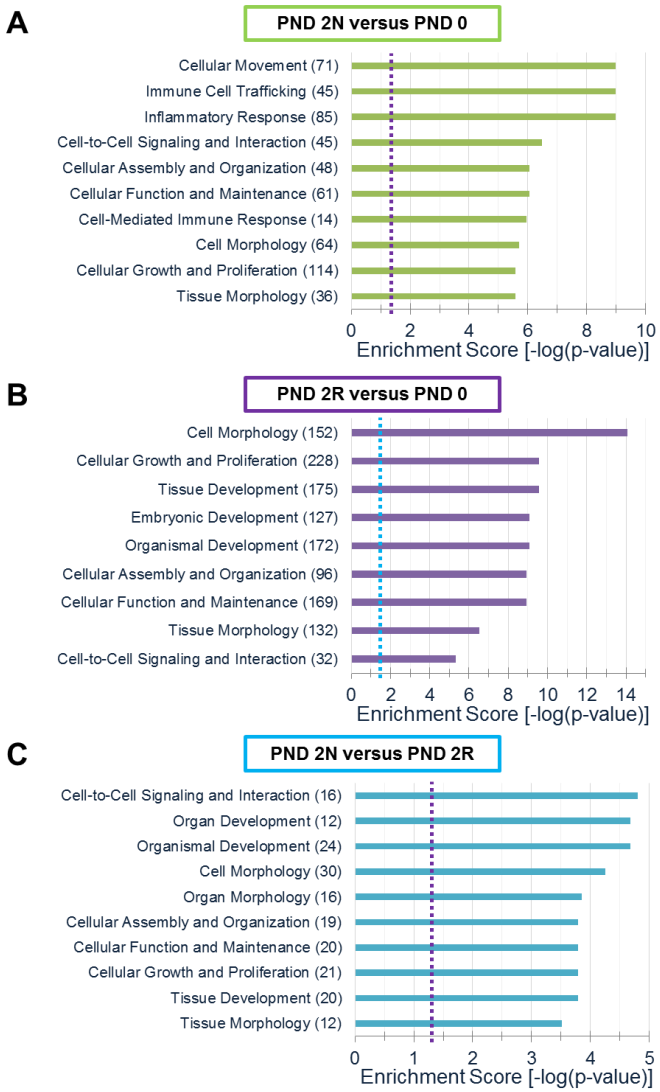

Figure 5. Selected functional annotation categories for differentially expressed transcripts (miRNAs and mRNAs) associated with miRNA-mRNA interactions between: (A) PND 2N versus PND 0; (B) PND 2R versus PND 0; and (C) PND 2N versus PND 2R as identified by IPA. Values within parentheses indicate the number of annotated mRNAs targeted by miRNAs that are involved in the corresponding functional term. Enrichment scores were calculated by taking the geometric mean of the P-values associated with differentially expressed transcripts (in $-\log_{10}$ scale). Dashed vertical line indicates $P = 0.05$.

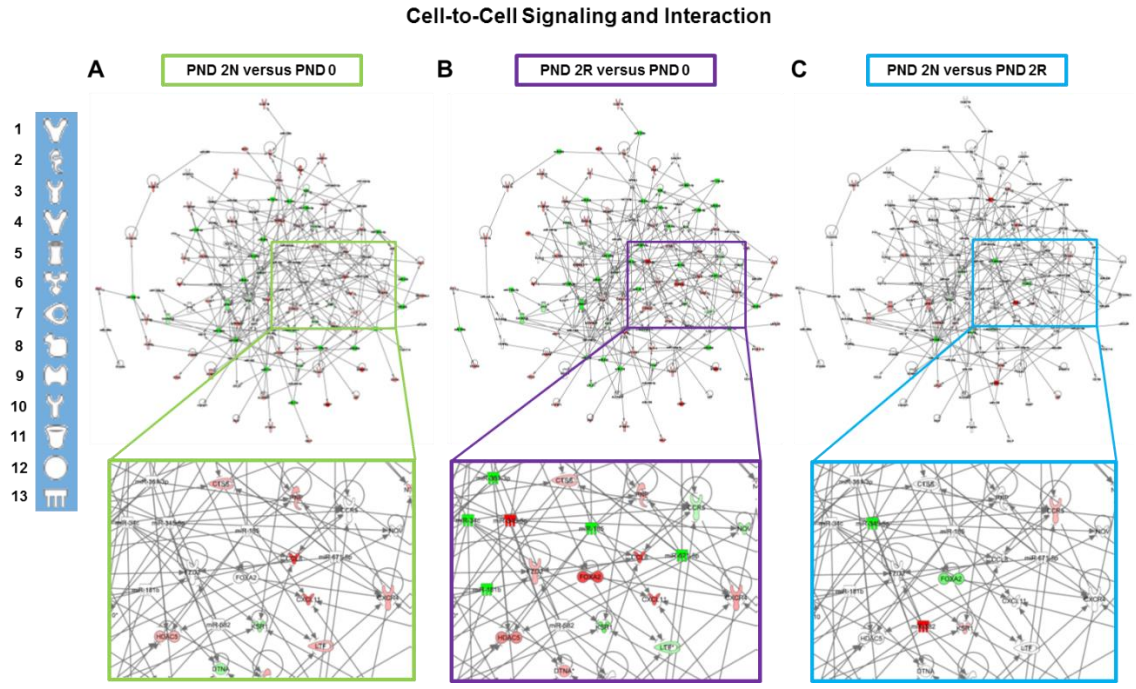

Figure 6. Integrated uterine miRNA-mRNA interactomes illustrating effects of: (A) PND 2N versus PND 0; (B) PND 2R versus PND 0; and (C) PND 2N versus PND 2R on cell-to-cell signaling and interaction. Red denotes increased and green denotes decreased transcript expression ($P < 0.05$). Color intensity indicates degree of change. Inset provides an opportunity to study detailed relationships within the interactome network. IPA Legend Key (left): 1) cytokine/growth factor; 2) enzyme; 3) G-protein coupled receptor; 4) growth factor; 5) ion channel; 6) kinase; 7) peptidase; 8) phosphatase; 9) transcription regulator; 10) transmembrane receptor; 11) transporter; 12) other; and 13) mature miRNA.

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CHAPTER IV

Long-Term Effects of Lactocrine-Deficiency on the Global Endometrial Porcine mRNA and MicroRNA Transcriptome at Pregnancy Day 13

ABSTRACT

Reproductive performance of female pigs that do not receive sufficient colostrum from birth is permanently impaired. Whether such lactocrine deficiency, reflected by low serum immunoglobulin immunocrit (iCrit), affects patterns of endometrial gene expression during the periattachment period of early pregnancy is unknown. Here, objectives were to determine effects of low iCrit at birth on the adult endometrial transcriptome on pregnancy day (PxD) 13. At birth, gilts were assigned to high (n = 8) or low (n = 7) iCrit groups. Adult high and low iCrit gilts were bred (PxD 0) and euthanized on PxD 13 when reproductive tissues and fluids were collected. The endometrial transcriptome was defined for each group using mRNAseq and microRNAseq. Raw reads were mapped to the *Sus scrofa* 11.1 genome build, and mature microRNAs were annotated using miRBase 21. Differential expression was defined based on fold change ($\geq \pm 1.5$). Lactocrine deficiency from birth did not affect corpora lutea number, uterine horn length, uterine wet weight, embryo recovery, or uterine luminal fluid estrogen content on PxD 13. However, mRNAseq revealed 1157 differentially expressed endometrial mRNAs in high versus low iCrit gilts. Affected genes were associated with aspects of solute transport, endometrial receptivity, and immune response. Six differentially expressed endometrial microRNAs included five predicted to target 62 differentially expressed mRNAs, affecting similar biological processes. Thus, lactocrine deficiency from birth can alter uterine developmental trajectory with lasting effects on endometrial responses to pregnancy as reflected at the level of the transcriptome on PxD 13.

INTRODUCTION

The mammalian female reproductive tract is incompletely developed at birth (postnatal day = PND 0) (Cooke *et al.* 2013). Data for ungulates (Bartol *et al.* 1988, Bartol *et al.* 1999, Gray *et al.* 2001b) and mice (Cooke *et al.* 2012, Filant *et al.* 2012) showed that transient disruption of neonatal uterine development can alter the postnatal developmental program with lasting consequences for adult uterine structure and function. Thus, neonatal uterine tissues are developmentally plastic. Moreover, conditions necessary to support an optimal uterine developmental trajectory are not well defined. Disruption of such conditions, resulting in a sub-optimal developmental trajectory, can compromise the ability of adult uterine tissues to support pregnancy and, for polytocous species, to produce large, viable litters.

Maternal support of offspring development does not end at birth, but extends into the postnatal period through factors communicated from mother to nursing young in first milk (colostrum) via a lactocrine mechanism (Bagnell *et al.* 2017, Bartol *et al.* 2017). In the pig (*Sus scrofa*), studies designed to test the lactocrine hypothesis for maternal programming of postnatal uterine development showed that imposition of a lactocrine-null state for two days from birth by feeding milk replacer in lieu of nursing altered uterine gene expression on PND 2 (Miller *et al.* 2013, Rahman *et al.* 2016, George *et al.* 2017), and stunted endometrial gland development by PND 14 (Miller *et al.* 2013). Observations indicated a requirement for lactocrine support of the neonatal uterine developmental program.

Lactocrine deficiency from birth, defined as suboptimal delivery of milk-borne bioactive factors (MbFs) to nursing young, also occurs under non-experimental

conditions. In the pig, lactocrine deficiency can occur naturally through maternal agalactia, mastitis, or competition among nursing young for teat position (Wu *et al.* 2010, Kraeling & Webel 2015, Vallet *et al.* 2015). The serum immunoglobulin immunocrit (iCrit) assay (Vallet *et al.* 2013), used to monitor passive transfer of maternal immunoglobulin to nursing piglets, provides an indirect method for assessing the relative amount of colostrum acquired by neonates on their day of birth. In pigs, minimal colostrum consumption on the day of birth (PND 0), reflected by low serum iCrit values, not only altered patterns of uterine wall development in neonates by PND 14 (Bartol *et al.* 2017), but also reduced live litter size through four parities in lactocrine-deficient piglets that reached adulthood (Vallet *et al.* 2015). Results provided compelling evidence indicating that reproductive performance of adult, neonatally lactocrine-deficient female pigs is permanently impaired (Vallet *et al.* 2015).

Factors that disrupt uterine receptivity to implantation of blastocysts during the periattachment period can compromise reproductive performance (Bazer *et al.* 2011). In the pig, conceptus elongation and trophoblast attachment to the uterine luminal epithelium occurs starting on PxD 13, and is completed by PxD 18 (Burghardt *et al.* 1997). During this period uterine secretions support developing conceptuses, and functional changes in the endometrium facilitate implantation (Geisert *et al.* 2017, Waclawik *et al.* 2017). These events contribute to establishment of an embryotrophic intrauterine environment that evolves as pregnancy progresses.

Significant changes in patterns of endometrial gene expression occur in response to pregnancy and the presence of conceptuses *in utero*. Studies of the porcine endometrial transcriptome revealed effects of pregnancy on both mRNA (Samborski *et al.* 2013a,

Samborski *et al.* 2013b) and microRNA (miRNA; (Bidarimath *et al.* 2015, Cordoba *et al.* 2015, Krawczynski *et al.* 2015a, Wang *et al.* 2016, Wang *et al.* 2017) populations. MicroRNAs are approximately 22 nucleotide RNAs that inhibit gene expression post-transcriptionally through mRNA destabilization/degradation and translational repression (Bartel 2004). Thus, efforts to predict miRNA-mRNA interactions through paired expression profiling are important functionally.

Given documented (Vallet *et al.* 2015), negative effects of lactocrine deficiency from birth on maternal capacity to produce large, viable litters in adulthood, it was hypothesized that sub-optimal lactocrine support, reflected by minimal serum iCrit at birth, would affect patterns of endometrial gene expression during the periattachment period of early pregnancy. Therefore, objectives of this study were to determine effects of lactocrine deficiency from birth on the adult porcine endometrial transcriptome, including predicted miRNA-mRNA interactions, on PxD 13.

MATERIALS AND METHODS

Animals and Experimental Design

Gilts (*Sus scrofa domesticus*) were born and raised from a herd of crossbred (Duroc, Landrace, and Yorkshire genetics) pigs at the U. S. Meat Animal Research Center (USMARC) in Clay Center, Nebraska. All procedures involving animals were reviewed and approved by the USMARC Institutional Animal Care and Use Committee and conducted in accordance with the Federation of Animal Science Societies (2010) guidelines for the care and use of agricultural animals in research.

Gilts and sows up to fourth parity were observed for estrous behavior and mated by artificial insemination using semen from maternal line boars from several commercial sources according to standard operating procedures at USMARC. On day 110 of gestation, dams were moved into farrowing crates and allowed to farrow naturally. If farrowing did not occur by day 116 of gestation, gilts were injected with Estrumate (1 ml; Intervet Merck Animal Health, Madison, NJ, USA) to induce farrowing, which is standard operating procedure at USMARC.

The experimental design is illustrated in Figure 1. On the day of birth (PND 0), piglets were processed (body weight, ear notch, tail dock, needle teeth clipped, penicillin and iron dextran injection) using standard operating procedures and a jugular blood sample was collected from each gilt offspring. As previously described (Vallet *et al.* 2013), clotted blood samples were centrifuged at 1,000 x g for 10 min. Equal amounts (50 ul) serum and 40% (wt/vol) ammonium sulfate in distilled water were mixed and the precipitated sample was loaded into a hematocrit centrifuge tube. Samples were centrifuged at full speed (12,000 x g) for 10 min. The immunocrit (iCrit) ratio was

calculated by taking length of the precipitate divided by the length of the diluted serum in the tube. Within each litter, gilts with an iCrit of 0.05 or lower (low iCrit; n = 7) were matched with a littermate of similar birth weight and an iCrit of greater than 0.10 (high iCrit; n = 8), and both members of each pair were tagged. If the high immunocrit gilt in each pair did not survive, another suitable littermate gilt with a high immunocrit was identified. If the low immunocrit gilt within each pair did not survive, the high littermate gilt was released from the experiment. Overall, serum iCrit was greater ($P < 0.001$) in high (0.115 ± 0.01) as compared to low (0.033 ± 0.01) iCrit groups. Care was taken to ensure experimental groups were balanced for potential effects of litter. Pigs from a total of 8 litters were used. If litter size exceeded 12 piglets per litter, male piglets were cross-fostered to other litters.

On day 15 of age, all gilts received creep feed and were weaned on approximately day 24. At weaning, gilts were housed in the USMARC nursery for 4 weeks and fed the USMARC standard nursery ration. After 4 weeks, gilts were moved to finishing barns and fed standard USMARC grower and finisher diets. At 170 days of age, gilts were moved to the gilt breeding area and were observed daily for estrous behavior. Following one estrous cycle of normal duration (17-23 days), gilts were mated at estrus by artificial insemination, and mated again 24 h later if still in estrus. On day 13 of pregnancy (PxD 13), gilts were humanely slaughtered at the USMARC abbatoir and reproductive tracts were collected. Corpora lutea were counted as an indicator of ovulation rate. Uteri were trimmed free of the broad ligament and uterine lengths (cm) and wet weights (g) were recorded.

To confirm pregnancy and collect uterine flushings, a clamp was placed at the cervix and an incision was made in the uterus at the tip of the uterine horn near the uterotubal junction. Using a syringe and a blunt needle, 0.9% saline (20 ml) was injected into the uterine lumen from the posterior end of each uterine horn, and the saline bolus was massaged through the length of the uterus until it emerged from the incised end. The saline flushing from each uterine horn was collected into a 100 mm plastic petri dish, and each flushing was examined for the number of conceputuses present. After flushing, each uterine horn was opened longitudinally, and endometrial tissue was collected from approximately the middle of the horn. Endometrial samples were frozen in liquid nitrogen and stored at -80°C until total RNA was extracted.

Conceptuses were removed from each uterine luminal flushing (ULF), and the flushings from each gilt were combined and centrifuged (2100 x g for 20 min at 4°C). Aliquots of pooled ULF were then frozen at -80 C until analysis. Estradiol-17 beta concentrations were determined in ULF samples from each pregnant gilt using a commercially available ELISA (EIA-2693, DRG International, Inc.; Springfield, NJ, USA) previously reported for use in swine (Rak *et al.* 2015) ELISA sensitivity ranged between 2000 and 9.714 pg/ml for estradiol-17 beta. ULF samples were assayed in duplicate within one assay with an intra-assay coefficient of variation of 3.68%. Total ULF estradiol content was calculated by multiplying ULF estradiol concentration values for each gilt by values for corresponding ULF recovery volumes.

Data for number of corpora lutea, uterine length and weight, conceptus number, and ULF estradiol content were analyzed statistically using General Linear Model procedures (SAS 2013, Cary, NC, USA). Data were subjected to analyses of variance

considering the main effect of PND 0 iCrit. Results are presented as least squares means (LSM) ± standard error of the mean (SEM).

Endometrial RNA Isolation and Analysis

Total RNA (including miRNA and mRNA) was isolated from 100 mg of endometrial tissue (50 mg/uterine horn) for each sample using the miRNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's protocol. RNA quantity was measured using a Qubit® 2.0 Fluorometer (Invitrogen; Carlsbad, CA, USA) and RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Applied Biosystems; Carlsbad, CA, USA). Samples with an RNA integrity number (RIN) $\geq$ 7.5 were used for library preparation for mRNA sequencing (mRNAseq) and miRNA sequencing (miRNAseq).

Preparation of mRNA and miRNA Libraries

All mRNAseq and miRNAseq procedures were performed at the Genomic Services Laboratory, HudsonAlpha Institute for Biotechnology, as previously described (Rahman *et al.* 2016, George *et al.* 2017). Briefly, total RNA (500 ng) from each endometrial sample was used for mRNA and miRNA library preparation. For mRNAseq, polyadenylated (polyA) RNAs were isolated using NEBNext Magnetic Oligo d(T)25 Beads (New England BioLabs, Inc., Ipswich, MA, USA). After polyA selection, RNA was fragmented and primed for first-strand synthesis using the NEBNext First Strand Synthesis Module, followed by second-strand synthesis using the NEBNext Second Strand Synthesis Module. Samples followed standard library preparation protocol using

NEBNext DNA Library Prep Master Mix Set for Illumina with slight modifications, as previously described (Rahman *et al.* 2016). For miRNAseq, the NEBNext Small RNA Library Prep Set for Illumina (New England Biolabs Inc., Ipswich, MA, USA) was followed according to the manufacturer's protocol.

mRNA and miRNA library quantities were assessed using a Qubit® 2.0 Fluorometer and library quality was determined using a DNA High Sense chip on Agilent 2100 Bioanalyzer. Further library quantification was completed using the qPCR-based KAPA Biosystem Library Quantification kit (Kapa Biosystems Inc., Woburn, MA, USA). For mRNAseq, each library was diluted to a final concentration of 12.5 nM. For miRNAseq, individual sample libraries were diluted to a final concentration of 1.25 nM. Equimolar amounts of individual barcoded samples from each group were pooled prior to sequencing.

mRNA and miRNA Sequencing and Data Analysis

Paired-end mRNAseq, at over 25 million reads per sample, was performed using a HiSeq 2500 (Illumina, Inc., San Diego, CA, USA) following the manufacturer's protocol and as previously described (Rahman *et al.* 2016). Reads were mapped to the Sscrofa 11.1 build of the porcine genome (National Center for Biotechnology Information [NCBI]) using TopHat v1.4.0 (Trapnell *et al.* 2009) with default settings that allowed only two mismatches per read. Alignment and filtering of mapped reads were conducted as previously described (Rahman *et al.* 2016). Reads were grouped according to their respective identifiers. Normalized gene expression was quantified using the trimmed mean of M-values method (Robinson & Oshlack 2010). Human orthologs of unannotated

differentially expressed genes (DEGs) were identified using the Reciprocal Best Hits method (Tatusov *et al.* 1997), whereby genes, g_1 and g_2, in the pig and human genome respectively, are deemed orthologs if g_2 is the best hit when g_1 is queried in a BLAST search against the database of human genes and g_1 is the best hit when g_2 is queried in a BLAST search against the database of pig genes.

miRNAseq was conducted using an Illumina HiSeq 2500 instrument (Illumina Inc., San Diego, CA, USA) at 50bp Single End condition generating approximately 15 million reads per sample. Quality control checks, adapter trimming, and read filtering were performed as previously described (George *et al.* 2017). Filtered reads were used to extract and count miRNAs which were annotated with miRBase release 21 database (Griffiths-Jones 2004, Griffiths-Jones *et al.* 2006, Griffiths-Jones *et al.* 2008, Kozomara & Griffiths-Jones 2011, Kozomara & Griffiths-Jones 2014). Reads were grouped according to their respective identifiers followed by quantification of miRNA abundance (Robinson & Oshlack 2010).

Differentially expressed mRNAs and miRNAs, based on fold change ($\geq \pm 1.5$), were identified with respect to neonatal immunocrit (high iCrit versus low iCrit). Probability values for each differentially expressed mRNA and miRNA were estimated by z-score calculations using a false discovery rate of 0.05. Data were subjected to principal component analysis and hierarchical clustering created with Spearman correlation coefficient. Relative fold change in mRNA abundance was illustrated using volcano plots generated for each comparison using R Programming (GNU General Public License; www.r-project.org).

For mRNAseq data, gene enrichment analyses were performed using Database for Annotation, Visualization, and Integrated Discovery (DAVID) (Huang da *et al.* 2009b, Huang da *et al.* 2009a) and Protein Analysis Through Evolutionary Relationships (PANTHER) (Mi *et al.* 2017). Ingenuity Pathway Analysis (IPA) software (Qiagen Redwood City, CA; www.qiagen.com/ingenuity) was used for functional annotation and related biological pathway analyses. Relationships between differentially expressed miRNAs and their respective differentially expressed mRNA targets were determined using Qiagen's Ingenuity Pathway Analysis MicroRNA Target Filter (IPA, Qiagen Redwood City, CA; www.qiagen.com/ingenuity), as described elsewhere (George *et al.* 2017).

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Validation of mRNAseq data was done by qPCR using the same RNA used to generate cDNA libraries. Endometrial RNA from individual pigs in each group were pooled to create high and low iCrit samples. Reverse transcription (2 µg/sample) was performed on a Peltier Thermal Cycler-200 (Bio-Rad Laboratories, Inc.) using the SuperScript III First-Strand Synthesis System (Life Technologies). Quantitative RT-PCR (50 ng/sample) was conducted using SYBR Green and universal thermal cycling parameters (40 cycles), as indicated by the manufacturer on a StepOne Plus System (Applied Biosystems/Life Technologies).

Primers were designed using Primer Quest software (Integrated DNA Technologies, Inc.) and synthesized by Sigma-Aldrich. Primer sequences (Table 1), directed to the porcine genome, were evaluated for quality by amplifying serial dilutions

of the cDNA template. To ensure specific amplification, control qPCR reactions included substitution of water only, in place of primers and template. Dissociation curves for primer sets were assessed to ensure that no amplicon-dependent amplification occurred.

Nine endometrial genes chosen for qPCR validation included those for which relative expression, determined by RNAseq, increased or decreased more than 1.5-fold, or were unchanged in high as compared to low iCrit gilts on PxD 13. These genes included serum amyloid A 3 (*SAA3*), solute carrier family 5 member 1 (*SLC5A1*), S100 calcium-binding protein A12 (*S100A12*), mucin 4 (*MUC4*), retinol binding protein 4 (*RBP4*), secreted phosphoprotein 1 (*SPP1*), fibroblast growth factor 7 (*FGF7*), leukemia inhibitory factor (*LIF*), and solute carrier family 24 member 4 (*SLC24A4*). Porcine cyclophilin A (*PPIA*) was used for normalization as the endogenous reference gene. Data generated by qPCR were analyzed using the ΔΔCT method as described by Applied Biosystems (ABI User Bulletin 2, 2001). Pearson correlation coefficient was determined to compare mRNA expression fold-change results obtained by mRNAseq and qPCR.

RESULTS

Ovarian and Uterine Measurements on PxD 13

When high and low iCrit gilts were compared on PxD 13, no differences in corpora lutea number (15.5 ± 1 CL), uterine length (129.93 ± 5.99 cm), uterine wet weights (861.67 ± 41.65 g), embryo recovery (7.07 ± 1 embryos), or total recoverable ULF estradiol content (10.8 ± 3.6 ng) were observed.

Effects of Lactocrine Deficiency on Endometrial mRNA Expression on PxD 13

Principal component analysis for mRNAseq data indicated high and low iCrit groups clustered independently (Figure 2). Effects of lactocrine deficiency at birth, as reflected by serum iCrit, on endometrial gene expression at PxD 13 are illustrated in Figure 3A. A total of 1157 endometrial mRNAs were differentially expressed (≥ 1.5-fold, $P < 0.05$) in high as compared to low iCrit gilts on PxD 13 (Fig. 3A). Of these differentially expressed mRNAs, 562 decreased and 595 increased in the endometrium of high versus low iCrit gilts (Fig. 3A). The top 10 most highly expressed endometrial mRNAs in high as compared to low iCrit gilts on PxD 13 included *NPY, ACOD1, CTRL, TGM3, TNIP3, FAM151A, CRISP3, NLRC4, FGA,* and *ACSBG1*.

Results of technical validation of mRNAseq data by qPCR are shown in Fig. 3B. Expression of nine transcripts including *SAA3, SLC5A1, S100A12, MUC4, RBP4, SPP1, FGF7, LIF,* and *SLC24A4* was evaluated. A positive correlation between mRNAseq and qPCR data was identified ($r = 0.91$, $P < 0.001$).

Gene Enrichment Analyses of Differentially Expressed mRNAs

Differentially expressed endometrial mRNAs in high as compared to low iCrit gilts on PxD 13 were categorized by enriched biological processes and pathways using DAVID, Reactome, IPA, and Panther (Figs. 4-5). The top ten enriched GO terms identified by DAVID functional annotation analysis included multiple terms related to immune response, such as ‘defense response’, ‘response to external stimulus’, ‘regulation of leukocyte migration’, and ‘inflammatory response’ (Fig. 4A). Other enriched GO terms identified by DAVID included ‘ion transport’, ‘nitrogen compound transport’, ‘reproduction’, and ‘reproductive process’.

Figure 4B illustrates enriched biological pathways within the differentially expressed mRNA dataset as identified by Reactome. Pathways affected by neonatal immunocrit on PxD 13 in the endometrium included those related to ECM remodeling, such as ‘collagen degradation’, ‘activation of matrix metalloproteinases’, ‘molecules associated with elastic fibres’, and ‘degradation of the extracellular matrix’. Other enriched pathways included ‘G alpha (i) signaling events’, ‘peptide ligand-binding receptors’, ‘chemokine receptors bind chemokines’, ‘O-linked glycosylation of mucins’, and ‘ligand-gated ion channel transport’.

Selected functional annotation categories identified by IPA for differentially expressed mRNAs in the endometrium of high versus low iCrit gilts on PxD 13 are shown in Figure 4C. Predominant terms specific to differential endometrial mRNA expression were related to cellular function and development. With regard to cellular function, enriched categories included ‘cell-to-cell signaling and interaction’, ‘cellular function and maintenance’, ‘cellular movement’, ‘immune cell trafficking’, and ‘cellular growth and proliferation’. Enriched developmentally-related categories included ‘tissue

morphology', 'endocrine and reproductive system development and function', and 'embryonic and organismal development'.

Figure 5 illustrates enriched GO biological processes for differentially expressed mRNAs on PxD 13 as identified by Panther. Processes sensitive to lactocrine deficiency included 'cellular process', 'reproduction', 'biological regulation and adhesion', 'response to stimulus,' as well as 'developmental, metabolic, and immune system' processes. Across analyses, overall top enriched biological processes on PxD 13 were associated with immune response, ECM remodeling, cell function, and reproduction/development.

Selected Differentially Expressed Endometrial mRNAs

Selected differentially expressed mRNAs with known or postulated roles in implantation are presented in Table 2. The gene family with the most abundant, lactocrine-sensitive elements involved solute carrier (SLC) membrane transporters. For this group, 20 SLC transcripts increased and 18 decreased in the endometrium of high as compared to low iCrit gilts (data not shown). Other lactocrine-sensitive, differentially expressed endometrial transcripts included those involved in prostaglandin signaling (*AKR1B1*, *PTGS1*, and *SLCO2A1*), immune response (*S100A8, CXCL10, LIF, IL1A*, and *ITGAM*), and endometrial receptivity (*ACOD1, STC1, LPAR3, DKK1*, and *BMP2*). Additionally, a number of endometrial transcripts related to uterine remodeling and proteolysis were lactocrine-sensitive. These included matrix metalloproteinases (*MMP12, MMP7, MMP8,* and *MMP13*), mucins (*MUC20, MUC6*, and *MUC13*), ADAMs (a disintegrin and metalloproteinase; *ADAM23, ADAM2, ADAM7*, and *ADAMTS19*), and

keratin 23 (*KRT23*; Table 1). With regard to the keratin (KRT) gene family, expression of eight transcripts (*KRT79, KRT14, KRT20, KRT23, KRT35, KRT5, KRT36,* and *KRT80*) increased, and one transcript (*KRT6A*) decreased in the endometrium of high as compared to low iCrit gilts (data not shown).

Effects of Lactocrine Deficiency on Endometrial miRNA Expression on PxD 13

Differentially expressed endometrial miRNAs (≥1.5-fold, $P < 0.05$) in high as compared to low iCrit gilts were determined via miRNAseq and are listed in descending order in Table 3. A total of six porcine miRNAs were differentially expressed and increased in the endometrium of high versus low iCrit gilts on PxD 13. Differentially expressed miRNAs included miR-129a-3p, miR-7135-5p, miR-371-5p, miR-338, miR-365-5p, and miR-144. Of those, five of the porcine miRNAs identified had human orthologs. No differential miRNA expression events greater than 2-fold were identified.

Integrated Target Prediction Analyses

The workflow and output for miRNA-mRNA data integration and bioinformatic analyses are illustrated in Figure 6. Procedures enabled integration of data for 1157 mRNAs and six miRNAs determined to be differentially expressed by endometrium obtained from high and low iCrit gilts on PxD 13. Relationships between differentially expressed miRNAs and respective mRNA targets were limited to inverse pairings (i.e., miRNA increased and mRNA targets decreased) and those transcripts with human orthologs identified by IPA. Thus, a total of five differentially expressed miRNAs were predicted to target 62 differentially expressed mRNAs (Fig. 6). Table 4 lists the five

differentially expressed miRNAs and predicted differentially expressed mRNA targets as identified by IPA. Illustration of predicted miRNA-mRNA interactions are presented as an interactome network in Figure 7. Only inverse relationships are presented, where endometrial miRNAs increased and associated mRNA targets decreased in expression between high and low iCrit gilts on PxD 13. Increased transcripts in high as compared to low iCrit gilts are shown in red and those that decreased are in green. Note, several differentially expressed mRNAs are targeted by more than one miRNA. Further, interactions between mRNA transcripts are also presented.

Enriched biological processes associated with endometrial mRNAs targeted by miRNAs between high and low iCrit gilts on PxD 13 as identified by DAVID are listed in Table 5. MicroRNA-mRNA interactions were predicted to be involved with similar processes observed for mRNAseq data alone, including those related to development and regulation of cellular functions, such as cell motility. Due to the limited number of predicted miRNA-mRNA interactions, enriched biological processes were not observed for Reactome and Panther analyses of mRNA targets.

DISCUSSION

The lactocrine hypothesis for maternal programming of uterine development posits that disruption of lactocrine signaling from birth can alter the neonatal uterine developmental program and trajectory with long-term consequences for reproductive performance in adults (Bartol *et al.* 2008). Consistently, data for neonates showed that imposition of a lactocrine-null condition from birth, by milk replacer-feeding, altered patterns of porcine uterine wall development and global patterns of uterine gene expression by PND 2 (Miller *et al.* 2013, Rahman *et al.* 2016, George *et al.* 2017) with overt, negative effects on endometrial histogenesis by PND 14 (Miller *et al.* 2013). Likewise, lactocrine deficiency from birth, reflected by low serum iCrit, inhibited uterine wall development by PND 14 (Bartol *et al.* 2017), as reflected by reduced endometrial gland genesis and immunostaining for proliferating cell nuclear antigen. Further, in a study involving 799 female pigs followed over four parities, lactocrine deficiency from birth was associated with reduced average preweaning growth rate, increased age at puberty and, critically, reduced live litter size with no effect of parity (Vallet *et al.* 2015). Present data support the idea that permanent impairment of reproductive performance in lactocrine-deficient, adult female pigs is associated with alterations in endometrial function, reflected by differential endometrial gene expression patterns between high and low iCrit gilts during the periattachment period of early pregnancy. Results establish a long-term effect of neonatal lactocrine programming on adult endometrial function in the pig.

Establishment and maintenance of an embryotrophic uterine environment requires that the adult, periattachment stage endometrium integrate systemic signals of maternal,

as well as local signals of conceptus origin (Waclawik *et al.* 2017). Results indicating that lactocrine deficiency from birth did not affect uterine horn length or wet weight, number of corpora lutea, number of conceptuses, or total recoverable ULF estradiol content, provide support for the idea that lactocrine deficiency did not affect adult uterine morphology, and that systemic and local signals driving endometrial function were similar in low and high iCrit gilts on PxD 13. Evidence of differential gene expression patterns between these groups likely reflects differences in the capacity of these endometrial tissues to integrate maternal and conceptus signals as necessary to insure development of an optimally embryotrophic uterine environment.

Lactocrine deficiency from birth had substantial effects on patterns of endometrial gene expression, as defined by the transcriptome in adult gilts on PxD 13. Results enabled definition of the size (number of affected transcripts), directionality (increased and decreased expression) and, to the extent that the porcine genome is annotated (Gutierrez *et al.* 2015), identification of individual transcriptomic elements of the endometrial domain of response associated with lactocrine disruption of the neonatal uterine developmental program (Rahman *et al.* 2016, Bagnell *et al.* 2017, Bartol *et al.* 2017, George *et al.* 2017). Elements of this response domain are, by definition, lactocrine-sensitive. Sub-domains of response, defined by biological processes identified using DAVID, Reactome, IPA, and Panther, included some that were similar to those described for the adult porcine endometrium in response to pregnancy on days 12 and 14 (Samborski *et al.* 2013a, Samborski *et al.* 2013b). Examples included defense response, proteolysis, and cell adhesion. Elements of the adult endometrial, lactocrine-sensitive transcriptome not grouped into sub-domains informatically, but that could affect

endometrial functionality and conceptus-endometrial interactions during the periattachment period, were also defined as discussed below.

Genes involved in epithelial cell transport mechanisms

During pregnancy, uterine epithelial secretory products, as well as factors delivered to the uterine lumen from maternal plasma, support conceptus development and implantation (Bazer *et al.* 2012a). Components of uterine luminal fluid, including glucose, fructose, and amino acids (Zavy *et al.* 1982), require specific epithelial transporter proteins to facilitate their movement across cellular compartments and into the uterine lumen (Vallet *et al.* 2014). One such class of mammalian transporters is the solute carrier (SLC) superfamily. Members of this superfamily were implicated in porcine placental function (Vallet *et al.* 2014, Steinhauser *et al.* 2016, Steinhauser *et al.* 2017). In the present study, several differentially expressed SLC transcripts were identified in the endometrium of high as compared to low iCrit gilts. Included were sodium-dependent glucose transporters *SLC5A1*, *SLC5A9, SLC5A4,* and *SLC5A8* (Wright 2013), expression of which was greater in high iCrit gilts. Estrogen-sensitive expression of *SLC5A1* was documented for porcine endometrial luminal epithelium on PxD 12-13 (Steinhauser *et al.* 2017). This suggested that locally produced, conceptus-derived estrogens induce endometrial expression of this glucose transporter. Glucose, which must be transported actively from maternal blood into the uterine lumen and across placental membranes (Goldstein *et al.* 1980), is necessary for porcine conceptus survival.

Fructose is also present in porcine uterine luminal fluids during early pregnancy, and uterine luminal fluid fructose concentrations increase between PxD 11 and 15

(Steinhauser *et al.* 2016). Fructose is produced when glucose is reduced to sorbitol through the polyol pathway via the action of enzymes AKR1B1 and SORD. Porcine endometrial expression of both enzymes was documented for uterine luminal epithelium from PxD 13-17 (Steinhauser *et al.* 2016). Here, endometrial *AKR1B1* expression increased in high as compared to low iCrit gilts on PxD 13, while *SORD* was expressed consistently in both groups. Collectively, data can be interpreted to suggest that lactocrine deficiency from birth could affect porcine endometrial glucose transport and metabolism during the periattachment period of early pregnancy.

Genes involved in prostaglandin synthesis and secretion

The enzyme AKR1B1 also acts as a prostaglandin F synthase (Seo *et al.* 2014a). Expression of *AKR1B1* by porcine endometrial explants increased in response to conceptus secretory products including estrogens and IL1B, while treatment with an inhibitor of AKR1B1 activity decreased PGF2α production *ex vivo* (Seo *et al.* 2014a). This supports a role for AKR1B1 in endometrial production of PGF2α and events associated with maternal recognition of pregnancy in the pig (Bazer & Johnson 2014). Increased endometrial expression of *PTGS1*, an enzyme involved in prostaglandin E2 and F2α synthesis (Seo *et al.* 2014a), was also observed in high iCrit gilts on PxD 13. Further, endometrial expression of the prostaglandin transporter *SLCO2A1* differed between high and low iCrit gilts. Implicated in regulation of uterine luminal prostaglandin influx during maternal recognition of pregnancy in the pig, SLCO2A1 expression was localized to basolateral membranes of uterine luminal epithelium and blood vessels during pregnancy (Seo *et al.* 2014b). Thus, lactocrine disruption of neonatal uterine development may

affect patterns of adult endometrial prostaglandin biosynthesis and secretory dynamics during early pregnancy.

Genes related to immune response

Mammalian implantation was described as an inflammatory process, involving immune responses required for conceptus survival (Griffith *et al.* 2017). Results of pathway analyses indicated that immunological processes associated with implantation were lactocrine sensitive. Some of these included regulation of leukocyte migration, immune cell trafficking and immune system processes. Specific endometrial transcripts that increased in high as compared to low iCrit gilts on PxD 13 included *S100A8*. A calcium binding protein, S100A8 was implicated in regulation of endometrial inflammatory response to infection in post-partum cows (Swangchan-Uthai *et al.* 2013), and increased in the porcine endometrium on PxD 12 (Wang *et al.* 2016). Another lactocrine-sensitive element of the endometrial transcriptome, *CXCL10* was implicated in immune cell recruitment to the endometrium during implantation in pigs (Han *et al.* 2017). Other mediators of immune function associated with the periattachment period (Fouladi-Nashta *et al.* 2008, Samborski *et al.* 2013a, Samborski *et al.* 2013b) for which expression differed between high and low iCrit gilts included *LIF*, *IL1A*, and *ITGAM*. Porcine endometrial expression of *LIF* was reported from PxD 12-18 and LIF protein content in uterine luminal flushings peaked on PxD 12 (Blitek *et al.* 2012). In mice, endometrial glandular LIF expression, is required for implantation (Stewart *et al.* 1992) and stimulates uterine luminal epithelial secretion of IL1A and prostaglandin E synthase during the peri-implantation period of pregnancy (Fouladi-Nashta *et al.* 2008).

Genes related to endometrial receptivity

In the pig, one marker of endometrial receptivity to conceptus signals and implantation is down-regulation of progesterone receptor expression in luminal and glandular epithelium (Geisert *et al.* 1994). The associated up-regulation of ESR1 expression enables endometrial responsiveness to conceptus estrogens starting on PxD 12 (Geisert *et al.* 1993). In turn, conceptus estrogens stimulate transcription of genes identified here to be lactocrine-sensitive, including *AKR1B1* (Seo *et al.* 2014a), *LPAR3* (Seo *et al.* 2008), and *STC1* (Song *et al.* 2009), the products of which can affect cell growth and adhesion, solute transport, and dynamics of prostaglandin synthesis and secretion. Other lactocrine-sensitive transcripts include *ACOD1* (also known as *IRG1*; (Cheon *et al.* 2003)), *DKK1* (Li *et al.* 2008), and *BMP2* (Lee *et al.* 2007), all documented to mediate uterine receptivity and implantation in mice.

Factors affecting endometrial mucin expression and distribution are central to development of a receptive endometrium (Geisert *et al.* 2015). Here, endometrial expression of several mucin transcripts was determined to be lactocrine-sensitive. Included were *MUC20*, *MUC6*, and *MUC13*, not identified previously as products of the porcine endometrium. Mucins, which can be secreted (including MUC2 and MUC6) or transmembrane in nature (including MUC1, MUC4, MUC13, and MUC20), function to protect and stabilize epithelia (Linden *et al.* 2008, Kufe 2009). Transmembrane Muc13 was localized to the apical surface of rat uterine luminal epithelium during implantation, where it was suggested to facilitate blastocyst attachment (Poon *et al.* 2014). Similarly, endometrial *MUC13* increased during the period associated with conceptus elongation in

cattle (Forde *et al.* 2013). Both MUC1 (Bowen *et al.* 1996) and MUC4 (Ferrell *et al.* 2003), not identified to be lactocrine-sensitive, regulate conceptus invasiveness and affect uterine epithelial receptivity to conceptus interactions in the pig.

Genes involved in implantation-related uterine remodeling and proteolysis

Matrix metalloproteinases (MMPs) function in extracellular matrix remodeling, and are required for successful implantation during pregnancy (Curry & Osteen 2003). Differential endometrial expression of *MMP7*, *MMP8*, *MMP12*, and *MMP13* was identified between high and low iCrit gilts on PxD 13. In the pig, increased endometrial expression of *MMP7*, *MMP12*, and *MMP13* was associated with early pregnancy on day 12 (Samborski *et al.* 2013b). Another category of metalloproteinases found to be lactocrine-sensitive included members of the ADAM gene family (*ADAM23, ADAM2, ADAM7*, and *ADAMTS19*). ADAM family gene products function in cell-cell adhesion, cellular signaling, and cleavage of transmembrane cell-surface proteins (Weber & Saftig 2012), all processes associated with conceptus-endometrial interactions during the periattachment period (Geisert *et al.* 2015). Disregulation of endometrial mucin and metalloproteinase expression could be expected to affect adult reproductive performance as predicted by the lactocrine hypothesis (Bartol *et al.* 2017).

Evidence presented here for porcine endometrial expression of keratin gene transcripts *KRT79, KRT14, KRT20, KRT35, KRT5, KRT36*, and *KRT80* is novel. Moreover, expression profiles for all of these transcripts reflected lactocrine programming effects on PxD 13. While specific functions of endometrial keratins are unknown, these proteins serve as major structural elements of epithelial cells, where they

act as a protective scaffold against mechanical and chemical stressors (Coulombe & Omary 2002). Evidence of increased porcine endometrial *KRT23* expression on PxD 12 (Samborski *et al.* 2013b) suggested a role for this keratin during the periattachment period. Data indicating reduced endometrial expression of eight different keratins in lactocrine-deficient gilts on PxD 13 suggests that functional studies of these gene products during the periattachment period are warranted.

Endometrial miRNA and miRNA-mRNA interactions

MicroRNAs target mRNAs and regulate their stability (Bartel 2004). A lactocrine-sensitive uterine miRNA-mRNA interactome was recently described for the neonatal pig (George *et al.* 2017). Two of six lactocrine-sensitive endometrial miRNAs, miR-338 and miR-144, were described for human chorioamniotic tissues and implicated in regulation of prostaglandin synthesis (Montenegro *et al.* 2009, Li *et al.* 2016). Actions of miR-338 were proposed to be mediated through phospholipase transcript *PLA2G4B* (Montenegro *et al.* 2009). Here, phospholipase A2 family members *PLA2G2D* and *PLA2G3* were determined to be lactocrine-sensitive. However, functional roles for the five annotated, lactocrine-sensitive miRNAs, predicted to target 62 mRNAs in the adult porcine endometrium on PxD 13 are unknown.

Data for the uterus, generated in rodent and ungulate models, including cattle, sheep and pigs, indicate that targeted disruption of uterine development from birth can alter the neonatal developmental program with lasting, and typically negative consequences for uterine phenotype and function in adulthood (Cooke *et al.* 2013). Data for the pig indicate that lactocrine effects on the neonatal porcine uterine developmental

program occur rapidly, within the first 12 to 24 hours of birth (George *et al.* 2017, Ho *et al.* 2017). Given that lactocrine deficiency from birth predicts permanent impairment of reproductive performance in female pigs (Vallet *et al.* 2015), observations suggest that the first 12 to 24 hours of neonatal life define a critical period for lactocrine programming of porcine uterine development and function (Bagnell *et al.* 2017, Bartol *et al.* 2017). It is now important to define epigenetic lactocrine programming mechanisms regulating uterine development (Bartol *et al.* 2013, Bartol *et al.* 2017).

Epigenetic mechanisms implicated in developmental programming include DNA methylation, post-translational histone modifications, and miRNA-mediated effects on transcript stability and repression of translation during critical organizational periods (Hoffman *et al.* 2017). Data for the pig, indicating lactocrine effects on aspects of the neonatal uterine miRNA-mRNA interactome by PND 2 (George *et al.* 2017), implicated miRNAs as elements of the lactocrine programming story. Nothing is known about roles for DNA methylation or post-translational histone modifications in this regard. Interrogation of neonatal porcine transcriptomic datasets on PND 2 (Rahman *et al.* 2016, George *et al.* 2017) for evidence of lactocrine-sensitive uterine expression of genes that code for enzymes regulating these processes, such as DNA methyltransferases and histone modifying enzymes, will be instructive. To the extent that the epigenome connects genotype to phenotype, definition of lactocrine mechanisms mediating development and, ultimately, tissue function will be important for understanding developmental origins of mammalian health and disease, including reproductive performance. Evidence for the pig indicates that adequate colostrum consumption within

24 hours of birth is required for establishment of a normal uterine developmental program and optimal reproductive performance in adults.

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Table 1. Primer sequences for quantitative real-time PCR validation of mRNAseq

Gene Symbol	Gene Name	Accession #	Primer Sequences
SAA3	Serum amyloid A 3	DQ367410.1	**F:** CTCAAGGAAGCTGGTCAAGG **R:** GGACATTCTCTCTGGCATCG
SLC5A1	Solute carrier family 5 member 1	NC_010456.5	**F:**GGCTGGACGGAAGTATGGTGT **R:**ACAACCACCCAAATCAGAGC
S100A12	S100 calcium-binding protein A12	NM_001160272.1	**F:**GGCATTATGACACCCTTATC **R:** GTCACCAGGACCACGAAT
MUC4	Mucin 4	NM_001206344	**F:**AGGATGCCCAATGGCTCTACT **R:** AAGGAGGCTGGTTCCGTTGAT
RBP4	Retinol binding protein 4	NM_214057.1	**F:**CCCCAGAAGTGCAGAAAATTGTGAG **R:**AGGGCTGAAGGGGTTAATGGAAGTT
SPP1	Secreted phosphoprotein 1	NM_214023	**F:**CTCATTGCTCCCATCATAGGTCTTG **R:**CAAGAGAAGGACAGTCAGGAGACGA
FGF7	Fibroblast growth factor 7	AF052657.1	**F:**CTGCCAAGTTTGCTCTACAG **R:**TCCAACTGCCAGGGTCCTGAT
LIF	Leukemia inhibitory factor	NM_214402	**F:**CCTTTCCATCACTCCTGTCAA **R:** CTGGGCTGTGTAGTAGAGAATAAA
SLC24A4	Solute carrier family 24 member 4	NC_010449.5	**F:**TCAAAATGGGAGACACGAGAG **R:** CCACCAGGATGTCAAACACG
PPIA	Porcine cyclophilin A	AU_058466	**F:**TTATAAAGGTTCCTGCTTTCACAGAA **R:**TGCCATTATGGCGTGTGAAG

Table 2. Selected differentially expressed endometrial mRNAs in high versus low iCrit gilts on PxD 13 with known or postulated roles in implantation.

Function	Entrez ID	Gene ID	FC	Gene Name
Epithelial Cell Transport Mechanisms	397113	*SLC5A1*	2.53	Solute carrier family 5 member 1
	100522082	*SLC5A9*	2.35	Solute carrier family 5 member 9
	397376	*SLC5A4*	1.9	Solute carrier family 5 member 4
	100524807	*SLC5A8*	1.72	Solute carrier family 5 member 8
Prostaglandin Synthesis and Secretion	396816	*AKR1B1*	2.5	Aldo-keto reductase family 1 member B
	397541	*PTGS1*	1.77	Prostaglandin G/H synthase 1
	100144510	*SLCO2A1*	-1.5	SC organic anion transporter member 2A1
Immune Response	100127488	*S100A8*	2.84	S 100 calcium binding protein A8
	494019	*CXCL10*	2.66	C-X-C motif chemokine 10
	399503	*LIF*	-1.64	Leukemia inhibiting factor
	397094	*IL1A*	-1.76	Interleukin 1 alpha
	397459	*ITGAM*	-1.80	Integrin alpha M
Endometrial Receptivity	100524951	*ACOD1*	8.09	Aconitate decarboxylase 1
	100134954	*MUC20*	2.82	Mucin 20, cell associated
	100125345	*STC1*	2.29	Stanniocalcin 1
	100626121	*MUC6*	1.95	Mucin 6, Oligomeric Mucus/Gel-Forming
	100125829	*MUC13*	1.68	Mucin 13, Cell Surface Associated
	100113360	*LPAR3*	1.64	Lysophosphatidic acid receptor 3
	100157640	*DKK1*	-1.66	Dickkopf-related protein 1
	100157103	*BMP2*	-1.72	Bone morphogenetic protein 2
Implantation-Related Uterine Remodeling and Proteolysis	100101475	*MMP12*	4.09	Matrix metalloproteinase 12
	397411	*MMP7*	2.57	Matrix metalloproteinase 7
	100519795	*KRT23*	2.4	Keratin 23
	100523811	*MMP8*	2.07	Matrix metalloproteinase 8
	397346	*MMP13*	1.58	Matrix metalloproteinase 13
	100518044	*ADAM23*	-1.55	A disintegrin and metalloprotease domain 23
	397006	*ADAM2*	-1.57	A disintegrin and metalloprotease domain 2
	100518181	*ADAMTS19*	-1.63	ADAM metallopeptidase with thrombospondin type 1 motif 19
	100156770	*ADAM7*	-1.64	A disintegrin and metalloprotease domain 7

Table 3. Differentially expressed endometrial miRNAs in high versus low iCrit gilts on PxD 13.

Gene ID	Swine miRNA Name	Swine miRBase ID[1]	FC
MI0023568;_1	ssc-miR-7135-5p	MIMAT0028145	1.92
MI0014773;_1	ssc-miR-338	MIMAT0015713	1.89
MI0013120;_2	ssc-miR-365-5p	MIMAT0017376	1.81
MI0031640;_1	ssc-miR-371-5p	MIMAT0037081	1.67
MI0022132;_1	ssc-miR-144	MIMAT0025364	1.64
MI0013169;_1	ssc-miR-129a-3p	MIMAT0013959	1.51

[1]Mature miRNA ID.

Table 4. Integrated target prediction analysis[1] for differentially expressed endometrial miRNAs and mRNA targets in high versus low iCrit gilts on PxD 13 identified by Ingenuity Pathway Analysis.

miRNA	Number of Targeted mRNAs[2]	Differentially Expressed Target mRNAs
miR-129a-3p	17	*LY6G6C, XKR4, KCNB1, MCF2, S100Z, TMEM257, AKAP5, SLC24A2, CPAMD8, PARD6B, SACS, MARCH1, BLOC1S6, PRKAR2A, KL, VNN2, KIAA1644*
miR-371-5p	16	*DUOXA2, HEPHL1, GABBR2, ANXA10, C7, HSD17B13, GDF11, ARNT2, MBL2, SCN4B, FAM131B, KL, RECK, CLOCK, INHBE, GPR173*
miR-365-5p	14	*CCND2, GREM1, FAM198A, CBY3, TGFBI, DQX1, BOLL, TREM2, RAB26, DMC1, KLK10, OMP, PLCH2, PRKAR2A*
miR-338	11	*DUOXA1, SCN1A, SLC26A7, FAM196B, DMRT2, ASTN2, TMEM196, FAM131B, SCAI, RAD54B, CHL1*
miR-144	9	*EYA1, CPS1, CCDC36, TMEM257, GALNT5, HIST2H4B, KIAA1024L, RAD54B, HEATR4*

[1]For cases where miRNA expression is decreased and mRNA expression is increased, or miRNA expression is increased and mRNA expression is decreased.

[2]Individual miRNAs can target multiple, overlapping populations of mRNAs.

Table 5. Top 10 enriched biological processes associated with endometrial miRNA-mRNA interactions in high versus low iCrit gilts on PxD 13 identified by DAVID functional annotation analysis.

Functional Terms of Overrepresented Biological Processes[a]	**Enrichment Score**[b]
Developmental maturation (5)	2.36
Pathway-restricted SMAD protein phosphorylation (3)	1.89
Regulation of pathway-restricted SMAD protein phosphorylation (3)	1.89
Regulation of thyroid hormone generation (2)	1.85
Positive regulation of metabolic process (16)	1.74
Cell development (12)	1.72
Double-strand break repair (4)	1.70
Negative regulation of cell migration (4)	1.66
Metanephros development (3)	1.59
Negative regulation of cell motility (4)	1.59

[a]Values within parentheses indicate the number of annotated mRNAs targeted by miRNAs that are involved with the corresponding functional term.

[b]Enrichment scores were determined by calculating geometric means of p-values involved in corresponding biological processes (in $-\log_{10}$ scale).

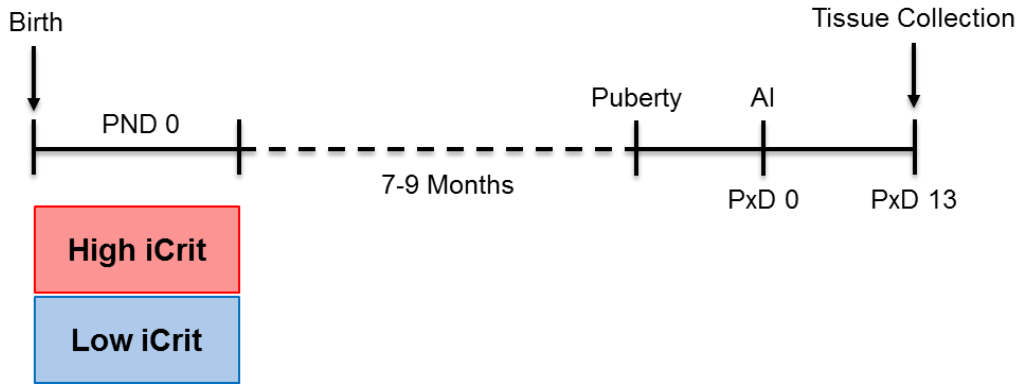

Figure 1. Experimental design. At birth (PND 0), jugular blood samples were collected from gilts, immunocrit (iCrit) ratios were measured, and used to assign gilts into high (n = 8) or low (n = 7) iCrit groups. At puberty (7-9 months of age), following one estrous cycle of normal duration, gilts were mated by artificial insemination (AI) using up to two doses of semen (pregnancy day = PxD 0). On PxD 13, gilts were euthanized and tissues were collected as described in Materials and Methods.

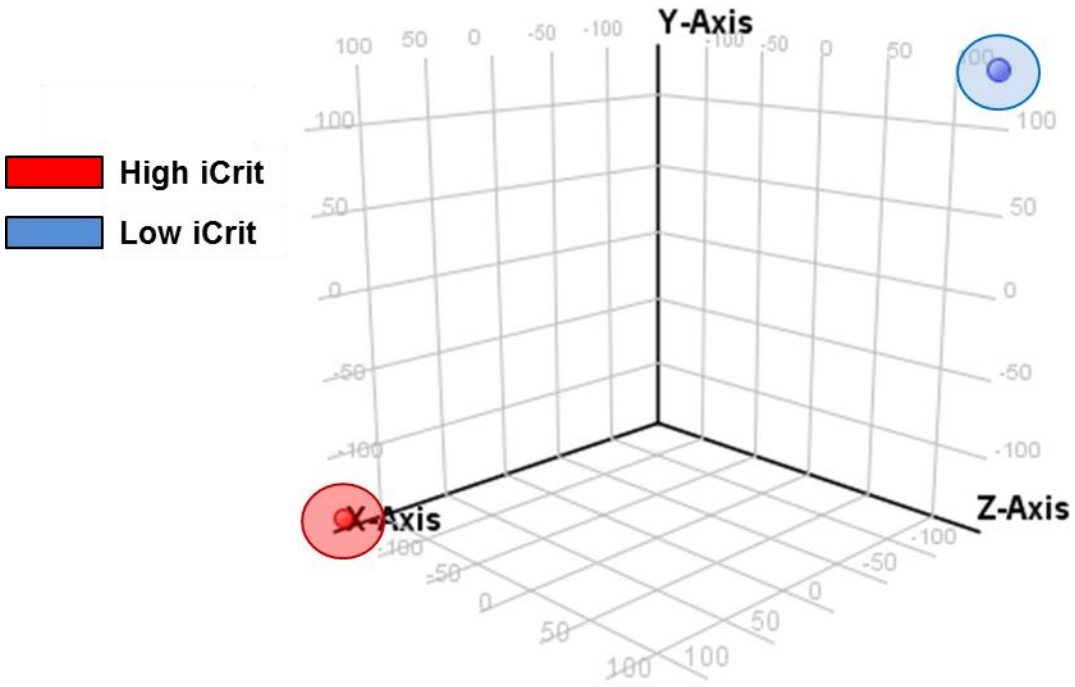

Figure 2. Principal component analysis (PCA) plot of mRNAseq data. PCA plot of pooled porcine endometrial samples on PxD 13 showing the clustering of expressed mRNAs for high iCrit (red) and low iCrit (blue) samples.

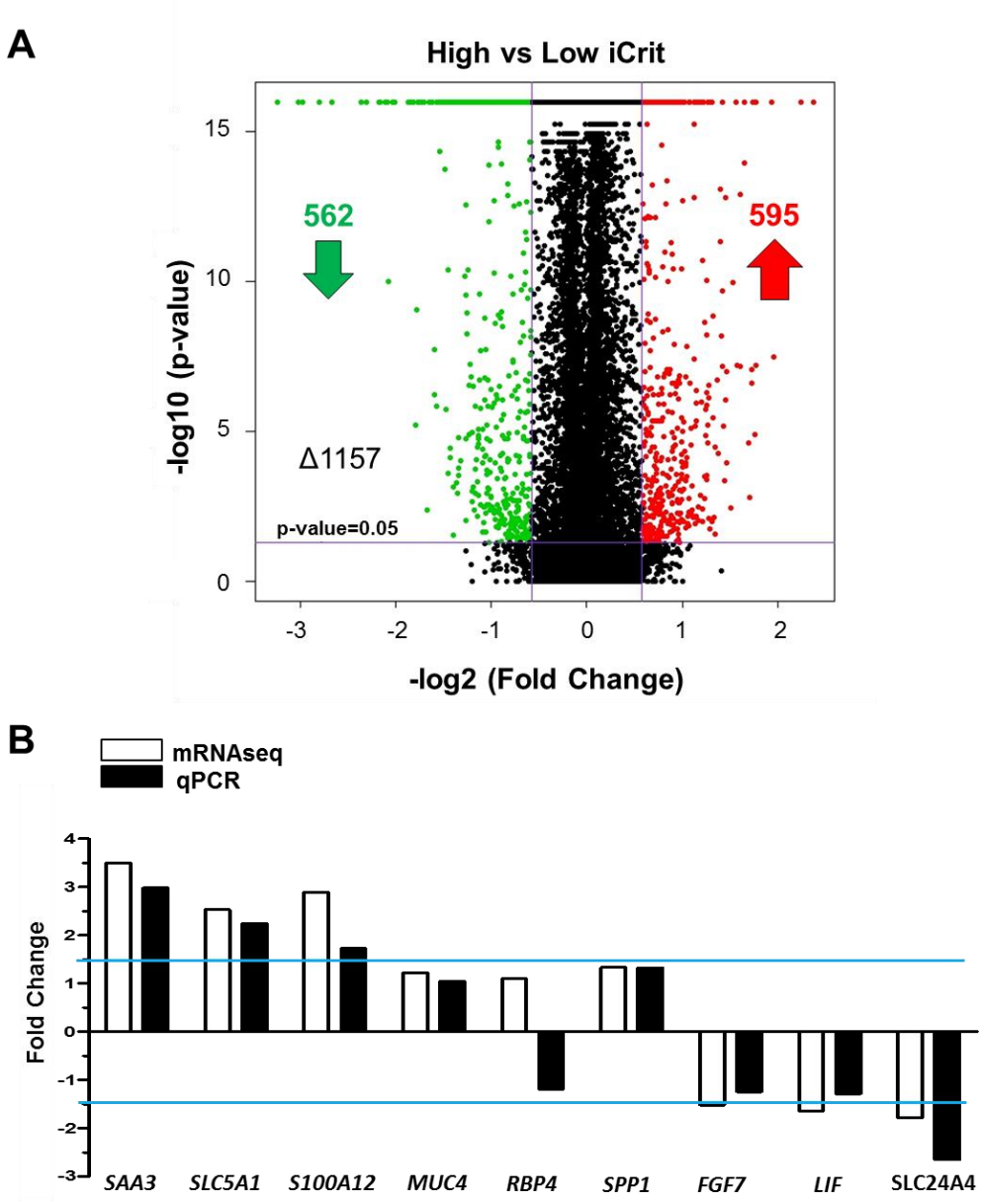

Figure 3. (A) Volcano plot demonstrating differences in endometrial expression of mRNAs on PxD 13 in high versus low iCrit gilts. Increased expression is indicated in red and decreased expression in green (fold-change ≥ 1.5). Black indicates mRNA expression that did not differ between groups. The horizontal line indicates $P = 0.05$. The total number of differentially expressed mRNAs is given (Δ value). The number of mRNAs for which expression was decreased (green) or increased (red) is shown. (B) Results of

qPCR validation for nine mRNAs identified by mRNAseq for high versus low iCrit groups. White bars indicate mRNAseq-based fold change; black bars indicate qPCR-based fold change. Horizontal lines indicate 1.5-fold change (+/-). A positive correlation between mRNAseq and qPCR results was identified ($r = 0.91$, $P < 0.001$).

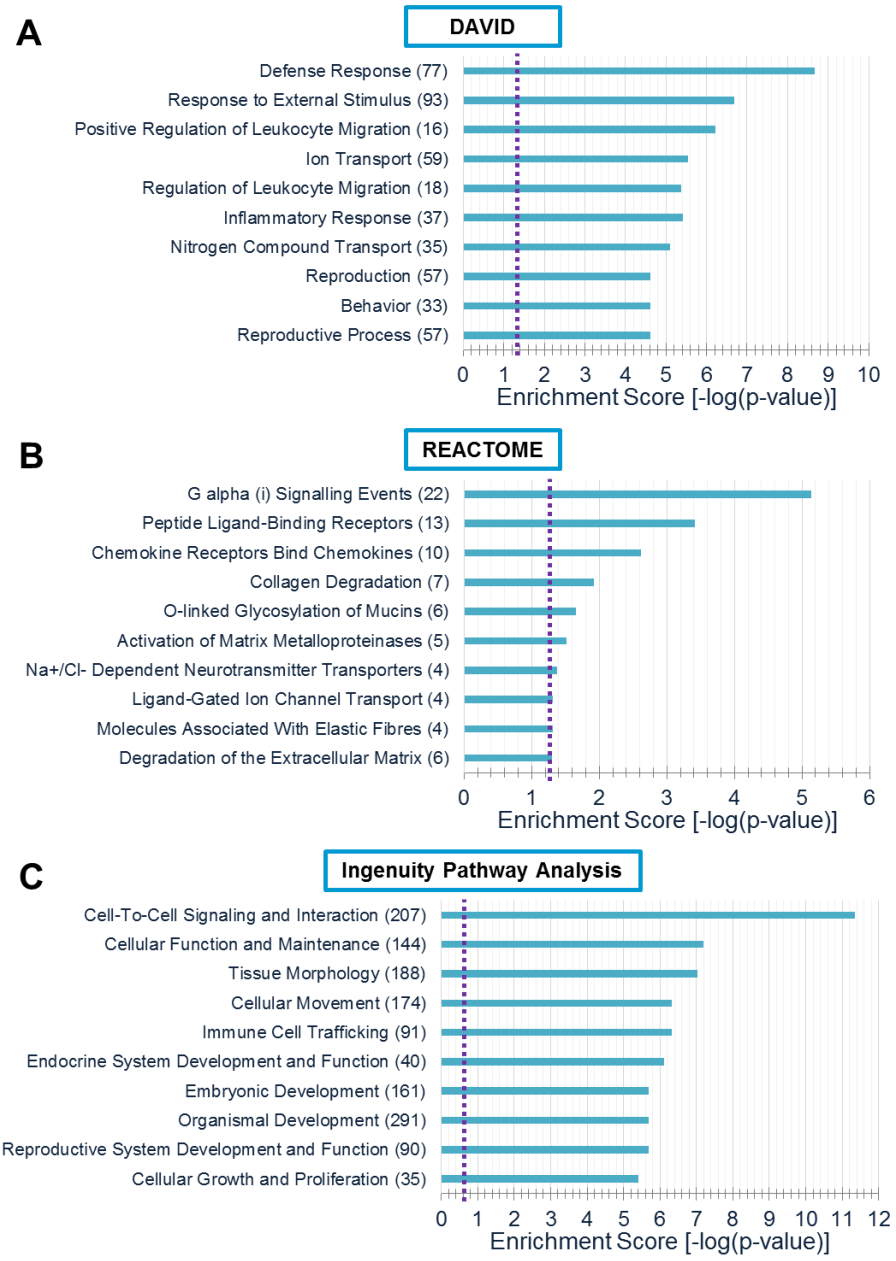

Figure 4. Selected functional annotation categories for differentially expressed endometrial mRNAs in high versus low iCrit gilts on PxD 13 as identified by: (A) DAVID, (B) REACTOME, and (C) IPA. Values within parentheses indicate the number of annotated mRNAs that are involved in the corresponding functional term. Enrichment scores were calculated by taking the geometric mean of the P-values associated with differentially expressed transcripts (in $-\log_{10}$ scale). Dashed vertical line: $P = 0.05$.

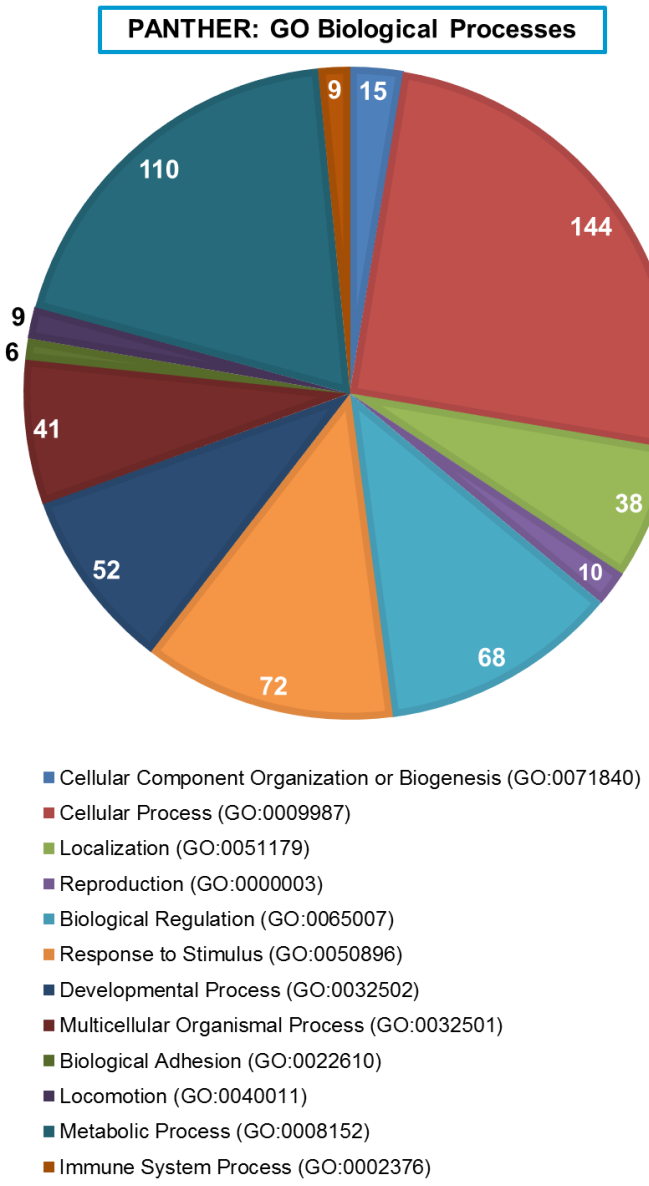

Figure 5. Gene ontology (GO) of biological processes for differentially expressed endometrial mRNAs in high versus low iCrit gilts on PxD 13 as classified by PANTHER. A total of 325 differentially expressed genes were identified by PANTHER and classified according to their gene ontology. The numbers on the pie chart represent the number of genes within the GO category. GO identification numbers are shown in parentheses. For orientation, the list begins with Cellular Component Organization or Biogenesis associated with 15 differentially expressed genes and proceeds clockwise.

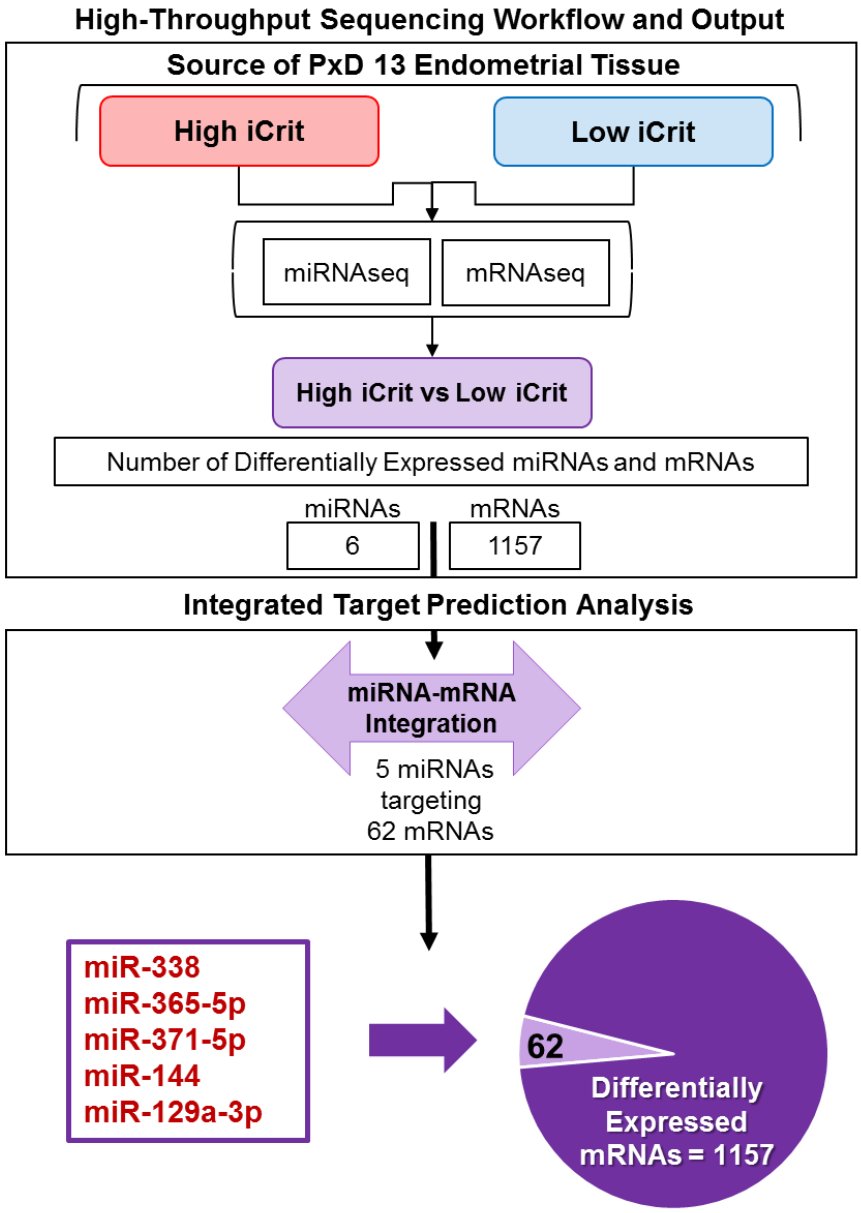

Figure 6. High-throughput sequencing workflow and output. Endometrial tissues were obtained from high and low iCrit gilts on PxD 13. Total endometrial RNA was isolated and both mRNAs and miRNAs were sequenced as described in Materials and Methods. Integrated target prediction analysis was conducted using IPA as described in Materials and Methods. The numbers and names of differentially expressed miRNAs targeting differentially expressed mRNAs are presented.

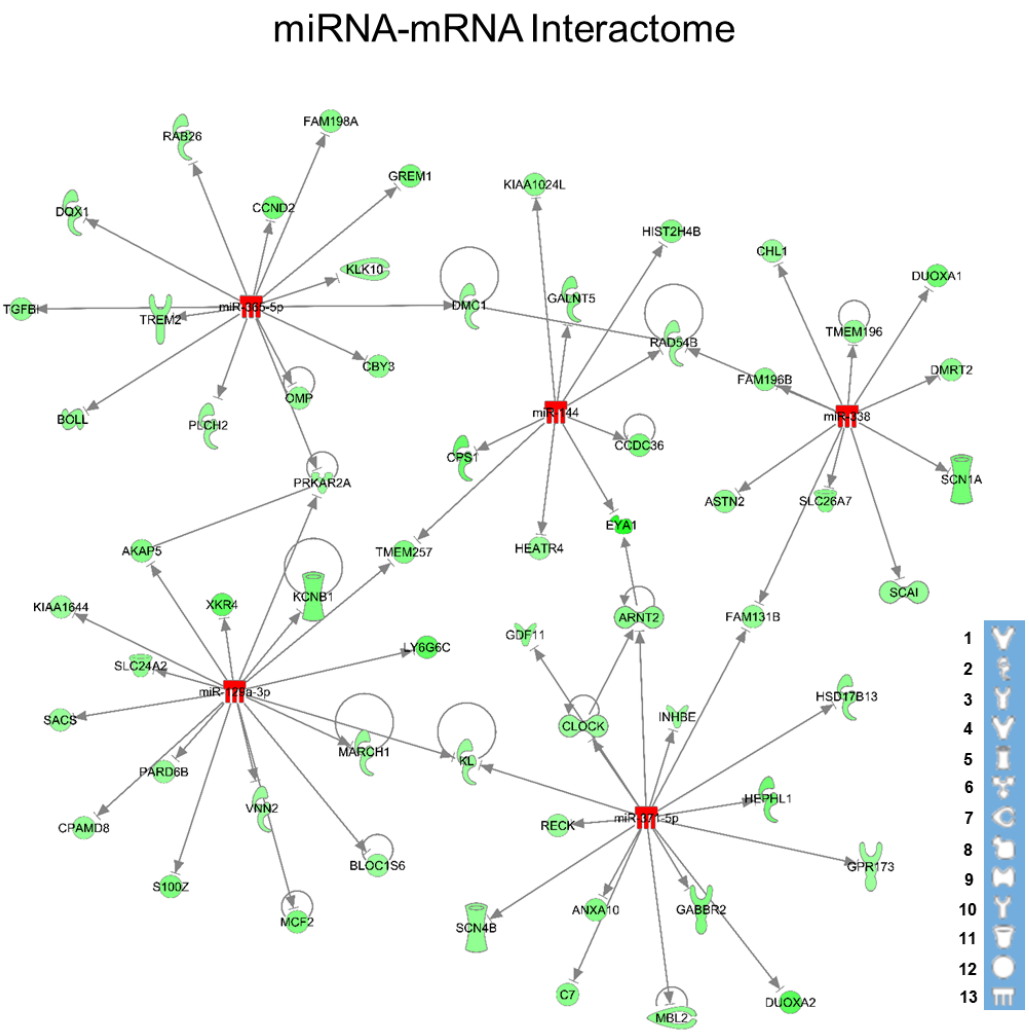

Figure 7. Integrated miRNA-mRNA interactome illustrating effects of high versus low iCrit on endometrial transcript interactions on PxD 13. Red denotes increased and green denotes decreased transcript expression ($P < 0.05$). Color intensity indicates degree of change. IPA legend key (bottom right): (1) cytokine/growth factor; (2) enzyme; (3) G-protein coupled receptor; (4) growth factor; (5) ion channel; (6) kinase; (7) peptidase; (8) phosphatase; (9) transcription regulator; (10) transmembrane receptor; (11) transporter; (12) other; and (13) mature miRNA. For a complete list of all predicted miRNA-mRNA interactions see Table 3.

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CHAPTER V

Dissertation Conclusions

CONCLUSIONS

As defined by the lactocrine hypothesis for maternal programming of uterine development (Bartol *et al.* 2008), disruption of lactocrine signaling from birth can alter the neonatal developmental program and trajectory of uterine tissues with long-term consequences for adult reproductive performance. Previous data indicate permanent impairment of reproductive performance in neonatally lactocrine-deficient female pigs (Bartol *et al.* 2013, Vallet *et al.* 2015, Bartol *et al.* 2017). Results presented here advance and extend studies designed to test the lactocrine hypothesis by identifying short- and long-term effects of colostrum consumption on uterine development in the neonate and the adult pig. Further, results provide the first molecular evidence of neonatal lactocrine programming effects on adult endometrium in the pig.

Nursing ensures delivery of maternally derived, MbFs to offspring, important for development of porcine uterine (Miller *et al.* 2013, Rahman *et al.* 2016, George *et al.* 2017, Ho *et al.* 2017), cervical (Camp *et al.* 2014), and testicular (Rahman *et al.* 2014) tissues between birth and PND 2. In addition to nursing, other methods of feeding colostrum, such as orogastric gavage or bottle-feeding, and associated maternal interactions have potential to affect neonatal development. For example, maternal licking and grooming behaviors in rodents are important for neuroendocrine development (Curley & Champagne 2016) and adult responses to stress, as well as memory and spatial learning (Liu *et al.* 1997, Caldji *et al.* 1998, Liu *et al.* 2000). Given that the first 12 h from birth may represent a critical period for lactocrine programming of porcine uterine function (Bagnell *et al.* 2017), establishment of a model system for study of known, potentially lactocrine-active factors found in porcine colostrum, such as IGF1 (Simmen *et*

al. 1990, Donovan *et al.* 1994, Donovan & Odle 1994), is important. Results of studies presented in Chapter II provide new information showing that lactocrine effects on the uterine organizational program are detectable by 12 h postnatally. Study 1 determined nursing from birth until 12 h supports: 1) normal patterns of endometrial cell proliferation; 2) uterine expression of cell cycle, morphoregulatory, and lactocrine-sensitive transcripts; and 3) uterine expression of MMP9 and TIMP1 proteins. Data from study 2 showed that a single feeding of colostrum at birth was sufficient to support aspects of uterine development at 12 h, regardless of method of feeding colostrum. Study 3 supports and extends results for the cervix (Camp *et al.* 2014) by showing positive effects of oral IGF1 on endometrial cell proliferation at 12 h from birth. While effects of IGF1 on endometrial cell proliferation were additive when administered in colostrum, supplementation of milk-replacer with IGF1 rescued the lactocrine-null phenotype at 12 h. Together, results reported in Chapter II suggest that lactocrine effects on postnatal uterine development are initiated with the first ingestion of colostrum, and establish the utility of this experimental model system for identification of lactocrine-active factors.

The first few days of neonatal life are organizationally important for the porcine uterus. In the pig, differentiation of uterine glandular epithelium from luminal epithelium, marked by ESR1 expression, is evident as early as 24 h postnatal (Bartol *et al.* 2013). Further, data for the pig showed that short-term imposition of a lactocrine-null state, by substitution of a porcine milk replacer for colostrum, altered global patterns of uterine gene expression by PND 2 (Miller *et al.* 2013, Rahman *et al.* 2016), and inhibited uterine endometrial gland development by PND 14 (Miller *et al.* 2013). Results presented in Chapter III extended those findings, and addressed a gap in knowledge regarding

potential involvement of miRNA-mRNA interactions in regulation of age- and lactocrine-sensitive events affecting the neonatal uterine transcriptome. Using mRNAseq data generated previously (Rahman *et al.* 2016) and extending those studies through application of microRNAseq, uterine miRNA expression profiles in nursed as compared to replacer-fed gilts between birth and PND 2 were defined. Further, integrated miRNA-mRNA target prediction analyses, performed *in silico,* revealed novel age- and lactocrine-sensitive interactions and biological processes associated with neonatal porcine uterine development. These observations implicate miRNAs in regulation of uterine gene expression post-transcriptionally between birth and PND 2. Results also revealed that dysregulation of lactocrine signaling between birth and PND 2 by milk replacer feeding affects neonatal uterine development epigenetically, as reflected by miRNA expression profiles. Additionally, results reported in Chapter III provide a rationale for future studies aimed at determining functional roles for age- and lactocrine-sensitive miRNA–mRNA interactions identified here for the porcine uterus at PND 2.

In a study following 799 female pigs over four parities, lactocrine deficiency from birth was associated consistently with reduced live litter size in adulthood. Lifetime effects of lactocrine deficiency from birth were estimated at 1.4 pigs/litter (Vallet *et al.* 2015). Results were interpreted to indicate that reproductive performance of adult, neonatally lactocrine-deficient female pigs is permanently impaired (Vallet *et al.* 2015, Bartol *et al*. 2017). Dysregulation of events required to support conceptus-endometrial interactions during the periattachment period is recognized to be a major contributing factors affecting reproductive performance, efficiency and overall uterine capacity in the pig (Bazer *et al.* 2011). Endometrial response to pregnancy and the presence of

conceptuses during this period, reflected by global patterns of gene expression, are significant during this period (Samborski *et al.* 2013a, Samborski *et al.* 2013b). Studies described in Chapter IV focused on the long-term impact of lactocrine deficiency from birth on the adult endometrial transcriptome in response to the challenge of pregnancy. Evidence of differential endometrial gene expression patterns between high and low iCrit gilts on pregnancy day 13 (PxD 13) provides strong support for the idea that impaired reproductive performance in lactocrine-deficient adult gilts is associated with dysregulation of endometrial gene expression during the periattachment period of early pregnancy.

Lactocrine deficiency from birth did not affect adult uterine horn length or wet weight, number of corpora lutea, number of conceptuses, or total recoverable uterine luminal fluid estradiol content. These results suggest that systemic and local signals affecting endometrial function in pregnancy were similar between high and low iCrit gilts on PxD 13. Considering that establishment and maintenance of pregnancy requires the endometrium to integrate maternal and conceptus signals (Waclawik *et al.* 2017), differential gene expression patterns observed between high and low iCrit groups likely reflect alterations in endometrial function. Collectively, results reported in Chapter IV provide compelling evidence for long-term effects of neonatal lactocrine programming on adult endometrial function in the pig.

Since the highest incidence of embryonic mortality in swine occurs before PxD 25 (Bazer *et al.* 2012b), it will be important to determine effects of neonatal lactocrine deficiency on other critically important periods of early pregnancy, such as completion of trophoblast attachment (PxD 20), and allantoic fluid expansion (PxD 20-30) (Bazer *et al.*

2012b). Intrauterine crowding is also associated with a high occurrence of fetal death, particularly during the second period of allantoic fluid accumulation and increase in placental surface area, which occur between PxD 40-70 (Bazer *et al.* 2012b). It is not known how lactocrine deficiency from birth affects events associated with these periods of pregnancy (Vallet *et al.* 2015). This will be important to elucidate.

In addition to functional changes in the endometrium, uterine secretions support developing conceptuses and facilitate implantation (Geisert *et al.* 2017, Waclawik *et al.* 2017). Uterine luminal fluid consists of uterine epithelial secretory products, as well as factors delivered to the uterine lumen from maternal plasma (Bazer *et al.* 2012a). Results reported in Chapter IV indicated lactocrine effects on in adult gilts on PxD 13 affecting endometrial expression of genes involved in epithelial cell transport mechanisms. Of note were members of the SLC gene family, products of which are required for movement of solutes across cellular compartments and into the uterine lumen (Vallet *et al.* 2014). Additionally, transcripts for a variety of secretory products were expressed differentially by endometrium from low versus high iCrit gilts, including those related to prostaglandin synthesis and secretion, immune response, and proteolysis. These data suggest lactocrine disruption of neonatal uterine development may affect patterns of adult endometrial secretory dynamics during early pregnancy. Studies now underway will determine effects of lactocrine deficiency from birth on the uterine luminal flushing proteome during early pregnancy, using samples from the same animals. Considering the complexity of post-transcriptional regulation, proteomic analyses will be important for understanding the functional consequences of birthday lactocrine deficiency on early pregnancy.

Beyond miRNAs, other epigenetic regulators of developmental programming include DNA methylation and post-translational histone modifications (Hoffman *et al.* 2017). Data presented here indicate lactocrine effects on aspects of the miRNA transcriptome and miRNA-mRNA interactome in the neonatal porcine uterus on PND 2, as well as in the adult endometrium on PxD 13. Whether effects of lactocrine deficiency on the neonatal and adult porcine uterus are mediated through other epigenetic mechanisms remains to be determined, and should be the focus of future studies. Further, identification of MbFs responsible for lactocrine-mediated effects on development and definition of their functional roles will be important. This information could be used to improve commercial milk-replacer formulas provided to porcine neonates, and could have translational value for human and other mammalian neonates. Considering that lactation and nursing are processes conserved among eutherian mammals, such studies will be important for understanding developmental origins of health and disease.

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