LACTOCRINE DEFICIENCY ALTERS POSTNATAL PORCINE UTERINE DEVELOPMENT

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ABSTRACT OF THE THESIS

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Nursing provides the mammalian neonate with nutrition and passive immunity and serves as a mechanism for delivery of milk-borne bioactive factors (MbFs) such as growth factors and hormones present in colostrum and milk to nursing offspring. The lactocrine hypothesis suggests that MbFs communicated from mother to nursing offspring have both short- and long-term effects on postnatal development of the female reproductive tract. Data for the pig show that experimental imposition of a lactocrine-null condition by milk replacer feeding from birth (postnatal day = PND 0) has negative effects on uterine endometrial development in the neonate. In addition, pigs determined to be lactocrine-deficient from birth, as reflected by a low serum immunoglobulin immunocrit (iCrit) ratio, showed long-term, negative effects on litter size and uterine capacity that persisted into adulthood. Effects of lactocrine deficiency on postnatal porcine uterine development at PND 14 have not been studied extensively. In addition,
FOXA2, a marker of uterine gland development, was identified in endometrial glandular epithelium in multiple species including mice, rats, humans, and in both the developing and adult ovine uterus. However, uterine FOXA2 expression and immunolocalization of FOXA2 have not been reported for the pig.

Therefore, the goal of this research was to learn more about the effects of lactocrine deficiency, indicated by low serum iCrit at birth, on maternal programming of porcine uterine development. Objectives of research described here were to: 1) identify effects of lactocrine deficiency from birth on endometrial morphology at PND 14; 2) determine if FOXA2 expression is detectable in neonatal porcine uterine tissues and whether neonatal porcine uterine FOXA2 expression is lactocrine-sensitive; and 3) evaluate effects of lactocrine deficiency from birth on patterns of FOXA2 distribution in the uterine endometrium on PND 14. Results showed that the endometrial area occupied by glandular epithelial cells (P < 0.05) and, to a lesser extent, endometrial thickness (P < 0.08) were reduced in low as compared to high iCrit gilts on PND 14. However, uterine gland penetration depth did not differ between the two groups. The FOXA2 protein was immunolocalized consistently and uniquely in nascent glandular epithelium in both low and high iCrit gilts on PND 14. Uterine FOXA2 expression was reduced (P<0.05) by approximately 3-fold in low iCrit gilts on PND 14. Results confirm that, under normal husbandry conditions, lactocrine deficiency from birth in nursing gilts alters patterns of neonatal uterine endometrial development and is associated with a decrease in uterine and glandular epithelial FOXA2 expression and inhibition of neonatal uterine gland genesis by PND 14.
DEDICATION

I dedicate this work to my parents, Satish and Anamika Paranjpe, my brother Neil Paranjpe, and to all of my extended family members. I would not have made it this far without all of you. Thank you for always being there for me and offering your unconditional love and support.
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CHAPTER I

Review of Literature
A. Purpose and scope of the literature review

The aim of this research was to test the lactocrine hypothesis for maternal programming of porcine uterine development. Based on data for the pig (Sus scrofa domesticus), the lactocrine hypothesis suggests that milk borne bioactive factors (MbFs) communicated from mother to nursing offspring as a specific consequence of nursing, have both short- and long-term effects on postnatal development of the reproductive tract (Bartol et al. 2008). Furthermore, the lactocrine hypothesis predicts that disruption of lactocrine signaling will alter the neonatal uterine endometrial developmental program with lasting consequences in adulthood. Goals of this research were to identify effects of lactocrine deficiency from birth (postnatal day = PND 0), as reflected by low serum immunoglobulin immunocrit (iCrit) ratio, on aspects of neonatal porcine uterine development at PND 14.

The purpose of this literature review is to provide the background and rationale for research described here. To start, an overview of literature pertaining to the reproductive biology of the female pig is presented. Evidence that there are maternal influences that contribute to prenatal and postnatal developmental programming is reviewed. The idea that colostrum/milk serves as a conduit for delivery of MbFs from mother to nursing offspring and studies that led to the concept of lactocrine signaling and the lactocrine hypothesis are described. Studies highlighting the immunoglobulin immunocrit assay as a tool to assess relative colostrum consumption in nursing pigs are described. In addition, events associated with development of the mammalian female reproductive tract dictating form and function are presented. Finally, evidence in support of the necessity of uterine glands for establishment and maintenance of pregnancy and
studies highlighting the role of FOXA2 and ESR1 in uterine gland development are discussed.

The research described here focused on the domestic pig as both an agriculturally important domestic animal species and as a translationally relevant, mammalian model for biomedical research. Given that lactation is a highly conserved process and the defining characteristic of all mammals, knowledge of acute and long-term effects of colostrum consumption on porcine uterine development could be used to advance animal agriculture as well as provide new information to improve human infant growth and development (Peaker 2002; Lefevre et al. 2010).

**B. Reproductive biology of the female pig**

The porcine ovary contains primordial, primary and secondary follicles that are incapable of ovulation (Christenson et al. 1985). In order to ovulate, large follicles need to produce adequate estradiol to initiate estrus and the surge of luteinizing hormone (LH) that causes ovulation (Soede et al. 1992; Soede et al. 2011). Growth of ovarian follicles depends on the release of gonadotropin-releasing hormone (GnRH) from the hypothalamus. Within the anterior pituitary, GnRH causes the release of follicle stimulating hormone (FSH) and LH from the gonadotrophs into the circulation. Increased LH pulses stimulate ovarian follicles to secrete increasing amounts of estradiol into the circulation (Esbenshade et al. 1990). The uterus continues to grow in response to the increasing concentrations of estradiol which ultimately results in an LH surge (Esbenshade et al. 1990). This LH surge leads to follicular rupture and ovulation.

Puberty in gilts generally occurs at 5-7 months of age. Age at puberty can be influenced by a variety of factors such as breed, nutrition, social environment, and
exposure to males (boars). The length of estrous cycle for gilts and sows ranges from 18 to 22 days, with the average length being approximately 21 days (Soede et al. 2011). The four stages of the estrous cycle include proestrus, estrus, metestrus, and diestrus. Gilts and sows ovulate from 20 to 30 ovarian follicles (Soede et al. 2011) and following fertilization the embryos are transported to the uterus where they will subsequently undergo attachment (Soede et al. 2011).

Endometrial receptivity is essential for a successful conceptus attachment, placentation and maintenance of pregnancy. During days eight to twelve of pregnancy preimplantation conceptuses use both peristaltic movements of the uterine wall and microvilli on their outer surface to move through the uterine horns until conceptuses are equally spaced throughout the uterus (Pope et al. 1982). In the pig, conceptus elongation typically starts on day 11. Trophoblast attachment to the uterine luminal epithelium occurs starting on pregnancy day (PxD) 13 and is completed by PxD 18 (Burghardt et al. 1997). From PxD 13 to PxD 18 uterine secretions support the developing conceptuses, and functional changes in the endometrium facilitate implantation (Geisert et al. 2017; Waclawik et al. 2017). As pregnancy progresses uterine secretions are essential for establishment of an embryotrophic intrauterine environment (Geisert et al. 2017; Waclawik et al. 2017). Parturition in the pig occurs between 114 to 117 days of gestation. Pigs typically give birth to 10-12 piglets per litter (Foisnet et al. 2010).

C. Maternal effects contribute to prenatal and postnatal developmental programming

Genetic and environmental factors affect the trajectory on developing tissues with lasting consequences (Bartol et al. 2013). The developmental programming of any
organism depends upon the establishment and maintenance of an optimal developmental trajectory during critical organizational periods (Burghgren 1999). When conditions required for normal development are altered during such periods, the developmental trajectory and ultimately the phenotype of any cell, or tissue is altered and a suboptimal phenotype results (Bartol et al. 2008).

Studies show a correlation between environmental conditions early in life and the risk of disease later in life (Barker et al. 1986). For example, maternal undernutrition during pregnancy was correlated with ischemic heart disease in offspring when they reached adulthood (Barker et al. 1989; Barker et al. 1993; Barker 1995). Together these studies were the basis for the ‘Barker Hypothesis’ now described as the ‘Developmental Origins of Health and Disease (DOHaD)’ (Barker et al. 1989). The DOHaD hypothesis states that if the developing fetus is exposed to a harsh uterine environment (such as one caused by poor nutrition or infection), the fetus will respond by developing adaptations that guarantee both immediate survival, and survival if a similar environment is encountered later in life (Hales et al. 2001; Gluckman et al. 2005; Gluckman et al. 2008). The foundation for the DOHaD hypothesis came from studies done during the Dutch Hunger Winter Famine of 1944-1945 during World War II. These studies showed that undernourished, pregnant women gave birth to underweight children that had increased risk for an array of disorders in adulthood that included schizophrenia (Susser et al. 1992), cardiovascular disease, and increased incidences of obesity (Roseboom et al. 2001; Roseboom et al. 2006). Together these studies demonstrated the importance of maternal effects in the prenatal period on offspring development and adult phenotype.
Prenatal exposure of human female fetuses to diethylstilbesterol (DES) resulted in increased incidences of clear cell adenocarcinoma of the vagina and cervix (Herbst et al. 1979), along with other complications of the female reproductive tract (Iguchi et al. 2015). These studies demonstrated that administration of steroid hormones during critical developmental periods can result in abnormal female reproductive tract programming. In mice (Cooke et al. 2012; Spencer et al. 2012) and sheep (Bartol et al. 1999; Gray et al. 2001a), neonatal progestin exposure throughout the time period associated with endometrial adenogenesis resulted in a uterine gland knockout phenotype characterized by lack of endometrial glands in adults. In rats, neonatal progesterone exposure disrupted endometrial gland genesis, and resulted in progesterone resistance and uterine gland dysfunction at the age of maturity (Dhakal et al. 2015). Studies in pigs showed that estrogenic disruption of female reproductive tract development in the neonatal period had both morphological and functional consequences in adulthood (Tarleton et al. 2003). Estradiol valerate exposure from birth to PND 13 reduced uterine weight and uterine horn volume in adult, cyclic and day 12 pregnant gilts compared with controls. Additionally, estrogen-induced disruption of uterine organizational events in the neonate altered the capacity of adult uterine tissues to respond normally to conditions of pregnancy evident by reduced uterine fluid protein and $^3$H-leucine incorporation into endometrial proteins.

D. Milk is a conduit for the delivery of MbFs from mother to nursing offspring

In all mammals the transition from intrauterine to extrauterine life involves a dependence on the mammary gland and milk as lactation is a defining characteristic of all mammals (Peaker 2002). In pigs, there are two phases of milk production or lactogenesis. The first phase of lactogenesis occurs between gestation days 90 and 105.
This phase is marked by the accumulation of milk components such as lactose and casein (Kensinger et al. 1986). The second phase of lactogenesis begins at parturition and defines the beginning of lactation. During this phase colostrum, or first milk, is the initial secretion produced by the porcine mammary gland from birth to 48 hours after parturition (Klobasa et al. 1987). The composition of colostrum changes during the transition to mature milk (Farmer et al. 2009). Compared to mature milk, colostrum contains high concentrations of proteins, including immunoglobulins, as well as reduced concentrations of fats and sugars (Klobasa et al. 1987; Langer 2009). In addition to providing the neonate with energy and nutrients, colostrum contains MbFs that are communicated from the mother to offspring prior to weaning (Donovan and Odle 1994; Grosvenor, Picciano, and Baumrucker 1993). More than 100 MbFs have been identified in colostrum, including peptide and steroid hormones, growth factors, cytokines (Burrin et al. 1992; Donovan and Odle 1994) and microRNAs (Chen et al. 2014).

Neonatal pigs are immunologically compromised at birth and depend on transmission of maternal immunoglobulins by way of nursing in order to survive. Since the pig has a diffuse and six-layered, epitheliochorial type of placentation, which lacks significant invasion of the uterine lining, large molecules, such as maternal immunoglobulins (150 kDa), are unable to pass into the fetal circulation in utero. Pigs acquire passive immunity by ingestion of immunoglobulin-rich colostrum (Furukawa et al. 2014). Immunoglobulin G is the most abundant immunoglobulin in pig colostrum (Poonsuk et al. 2018). Once these immunoglobulins are consumed by nursing pigs they cross the intestinal epithelium and enter the circulation to provide the nursing neonate with passive immunity (Coalson et al. 1973). Studies in neonatal calves showed that
MbFs can pass along the gut and act directly on the cells of the gastrointestinal tract to induce effects on other tissues indirectly (Blum 2006). For example, MbFs are reported to modulate the gut microbial population and influence gastrointestinal tract differentiation and growth.

E. Maternal programming of postnatal development involves lactocrine signaling

The term lactocrine was defined as the delivery of biologically active factors of maternal origin into the neonatal circulation as a consequence of nursing (Bartol et al. 2008). The lactocrine hypothesis predicts that disruption of lactocrine signaling will affect the developmental trajectory of uterine tissues and compromise reproductive performance in adults (Bartol et al. 2008). The lactocrine hypothesis has been studied in a variety of species. Studies to test the lactocrine hypothesis in mice showed that changes in milk tumor necrosis factor alpha (TNF) altered patterns of neonatal hippocampal development with long-term effects on spatial memory (Liu et al. 2014). Results were reproduced by the postpartum administration of an anti-TNF agent to suckling pups of TNF-deficient mothers. Administration of the anti-TNF agent restored both postnatal proliferation and spatial memory to normal levels. These results identified a TNF-dependent lactocrine pathway that programs neonatal hippocampal development and memory (Liu et al. 2014).

Maternal programming refers to maternal factors with the potential to affect the developmental program and trajectory of the embryonic, fetal, or perinatal tissues influencing overall phenotype of the neonate (Szyf et al. 2005; Bartol et al. 2008). Maternal effects on uterine development in their offspring begins during gestation in the prenatal period and does not end with parturition, but extends into the neonatal period via
lactation (Bartol et al. 2008). A study using a cohort of rhesus macaques demonstrated that mothers with fewer resources produced milk with increased cortisol concentrations. Elevated milk cortisol was associated with greater infant weight gain across time. These studies showed that mothers with fewer resources were programming their offspring through cortisol signaling, resulting in behaviorally cautious offspring that prioritize growth (Hinde et al. 2015). In other studies, newborn rats deprived of milk in early lactation had altered secretory function of mammotropes in adulthood (Nusser et al. 1997). These studies document the importance of nursing and the quality and quantity of colostrum/milk consumed on lactocrine programming of developing somatic tissues.

F. Serum Immunoglobulin-immunocrit (iCrit) assay: a tool with which to assess relative colostrum consumption in nursing pigs.

The quality and quantity of colostrum produced is variable among sows (Farmer et al. 2009). Factors affecting colostrum production include, but are not limited to, endocrine status of the sow, nutrition, and heat stress (Farmer et al. 2009). Disruption of lactocrine signaling in pigs can occur through natural mechanisms such as maternal mastitis, agalactia, or offspring competition for teat position (Wu et al. 2010; Kraeling et al. 2015; Vallet et al. 2015). Given the importance of colostrum intake on neonatal survival, development of strategies to understand both the amount and quality of colostrum delivered to nursing piglets is a priority. Studies by Vallet et al (2013) established a rapid method for measurement of the passive transfer of immunoglobulins from sow to piglet by measuring piglet serum immunoglobulins that provides an indirect measure of colostrum intake by piglets on their day of birth.
Effects of lactocrine deficiency in pigs, indicated by low serum iCrit at birth, were reported in a large scale study (Vallet et al. 2015). Immunoglobulin immunocrit measurements were collected on PND 0. Body weight measurements were taken from a subset of piglets to assess growth rates. Additionally, age at puberty was recorded from a subset of female piglets (Vallet et al. 2015). Results showed that gilts that were lactocrine deficient at birth, showed reduced growth rates, increased age at puberty (Vallet et al. 2015) and reduced ESR1-positive uterine gland development at PND 14 (Grey et al, 2017 SSR abstract). In addition, results involving data generated from almost 800 gilts over four parities showed that neonatally lactocrine-deficient gilts had reduced live litter size in adulthood that was unaffected by parity when compared with lactocrine-replete gilts. The difference in live litter size for females with high versus low serum iCrit was estimated at 1.4 piglets per litter (Vallet et al. 2015). This study validated results of an earlier study involving 381 gilts, for which PND 0 serum iCrit values were obtained and live litter sizes were recorded for the same pigs as adults (Bartol et al. 2013). This study also showed that low serum iCrit on PND 0, reflecting reduced colostrum consumption, was associated with reduced lifetime fecundity, as reflected by data for number of piglets born alive. Additionally, when serum iCrit ratio fell below 0.05 (relative units), live litter size was predicted to drop off substantially (Bartol et al. 2013). Together these studies demonstrated that lactocrine deficiency, indicated by low serum iCrit at birth, is predictive of reduced functional uterine capacity impaired reproductive performance in adult female pigs.
G. Events associated with the development of mammalian female reproductive tract
dictate form and function

The mammalian female reproductive tract develops from the Müllerian ducts, which differentiates into the oviducts, uterus, cervix, and anterior vagina (Massé et al. 2009). The female reproductive tract is essential for the continuation of mammalian species as it provides the site for fertilization of oocytes by spermatozoa, for implantation and subsequent development of the embryo, and the expulsive force required for delivery of the fetus (Kobayashi et al. 2003).

Uterine organogenesis begins prenatally and is completed postnatally. Temporospatial expression of patterning genes is necessary to support antero-posterior segmentation and radial patterning of the developing Müllerian duct. Antero-posterior segmentation and radial patterning is necessary for the Müllerian duct to develop into functional parts of the female reproductive tract including the uterine tubes, uterus, cervix and anterior vagina (Bartol et al. 2006). Genes required for female reproductive tract development include paired-box gene 2 (Pax2), Lim1 and Emx2, and members of both Wnt and Hedgehog (Hh) gene families (Kobayashi et al. 2003). Studies using mice demonstrated that Pax2-null mutant mice die soon after birth, have no kidneys and lack a reproductive tract (Torres et al. 1995), Lim1-null mutant mice lack oviducts, a uterus and the upper portion of the vagina (Kobayashi et al. 2004), and Emx2-null mutant mice lack reproductive tracts, gonads and kidneys (Miyamoto et al. 1997).

A subset of the Wnt gene family members are involved in the development of several reproductive tract organs (Miller et al. 1998). Studies show that Wnt4-mutant female mice lack a female reproductive tract (Jeays-Ward et al. 2003). In addition, in
*Wnt4*-null female mice at 18.5 days post coitum Müllerian ducts were absent and Wolffian ducts developed demonstrating the masculinization that occurs in *Wnt*-4 mutant females (Vainio *et al.* 1999). These results indicate that *Wnt4* is important for the initial step of Müllerian duct formation before sexual differentiation occurs as defined by the differentiation of the Müllerian duct into functional parts of the female reproductive tract including the uterine tubes, uterus, cervix and anterior vagina (Miller *et al.* 1998). Along the anterior–posterior axis of the Müllerian duct, *Hoxa9* is expressed in the oviduct, *Hoxa10* in the uterus, *Hoxa11* in the uterus and cervix, and *Hoxa13* in the cervix and upper vagina (Taylor *et al.* 1997; Warot *et al.* 1997). Mutations in *Hoxa10* cause an anterior homeotic transformation when one embryonic axial segment alters its identity to that of another. As a result the anterior part of the uterus transforms into the more anterior oviduct in the female (Benson *et al.* 1996). Together these results demonstrate that disruptions in gene expression patterns can prevent uterine development or female reproductive tract development entirely.

In pigs the vagina, cervix, and oviducts, but not the uterus, are fully developed at birth (Bartol *et al.* 1993). Postnatal uterine development in pigs includes differentiation and proliferation of uterine glands, stromal organization, development of endometrial folds, and growth of the myometrium (Spencer *et al.* 2019). Imposing a lactocrine-null condition in neonatal pigs, by feeding a milk replacer from birth, altered the neonatal porcine uterine transcriptome by postnatal day PND 2 (Rahman *et al.* 2016) and inhibited uterine gland formation by PND 14 (Miller *et al.* 2013). In these studies pigs were nursed or fed milk replacer for two days and then returned to nursing through PND 14 to see if the lactocrine-null effect could be rescued. Porcine uterine histology was similar in
nursed and replacer-fed gilts on PND 2 \cite{Miller2013}. However, by PND 14 endometrial development and gland formation were reduced in the replacer-fed gilts and expression of ESR1 in glandular epithelial cells declined \cite{Miller2013}. These results demonstrated that return to nursing after 48 hours of milk replacer feeding did not rescue the impaired uterine phenotype by PND 14.

Lactocrine-deficient pigs, indicated by low serum iCrit at birth, that reached adulthood displayed an altered endometrial transcriptome at pregnancy day (PxD) 13, reflected by differential expression of more than 1100 endometrial mRNAs in high- as compared to low-iCrit gilts \cite{George2018}. The differentially expressed genes had functions related to solute transport, endometrial receptivity and immune response \cite{George2018}. In other studies, a single feeding of colostrum at birth increased endometrial cell proliferation at 12 h in nursed gilts, as well as those that were bottle- or gavage-fed colostrum \cite{George2018}. Results indicated that lactocrine effects on postnatal uterine development are initiated with the first ingestion of colostrum within 24 h of birth. Together these results demonstrated common effects of neonatal uterine developmental disruption through experimental imposition of the lactocrine-null condition \cite{Miller2013} and lactocrine-deficiency from birth \cite{George2018}. In both conditions there is some form of organizational disruption as reflected by reduced endometrial thickness and uterine gland genesis.

**H. Uterine glands are necessary for establishment and maintenance of pregnancy**

The uterine wall consists of three tissue layers, including the: 1) endometrium or uterine mucosa, comprised of stroma and epithelium; 2) myometrium, which includes inner circular and outer longitudinal smooth muscle layers and; 3) perimetrium, or uterine
serosa (Cooke et al. 2013). Two distinct epithelial cell types in the adult endometrium include luminal epithelium (LE) and glandular epithelium (GE) (Bartol et al. 1993; Bartol et al. 1999).

At birth the porcine uterus consists of a simple columnar LE that is supported by undifferentiated mesenchyme and encircled by a rudimentary myometrium (Bartol et al. 1993; Spencer et al. 1993; Bartol et al. 1999). Endometrial adenogenesis is initiated after birth when GE develops into simple epithelial tubes that extend from the LE into underlying stroma (Cooke et al. 2013). By PND 7 there are distinct shallow and deep stromal zones. Additionally, uterine glands are present throughout the stroma. Uterine glands begin to branch within the stroma until they reach the myometrium, by PND 14 uterine glands extend approximately one-third of the distance from the LE to the myometrium. On PND 28, uterine glands have obvious branches and GE is present throughout the stroma. Endometrial folds are present by PND 28, and there are numerous endometrial glands by PND 56. By PND 120 the pig uterus is capable of supporting pregnancy (Bartol et al. 1999; Cooke et al. 2013).

The idea that uterine secretions are essential to pregnancy success evolved over many centuries (Cooke et al. 2013). Hippocrates (460–370 BC) argued that the fetus obtained nourishment by sucking on “uterine paps.” Studies to test the hypothesis that secretions from uterine glands are essential for maintaining a successful pregnancy showed that uterine gland–derived histotroph is crucial for pregnancy success (Daniel Jr et al. 1969; Roberts et al. 1987; Roberts et al. 1988). A study of proteins in pig uterine secretions revealed that histotroph proteins were of maternal origin and synthesized by GE (Bazer 1975). These proteins were absorbed via the placental areolae, transported
across the chorioallantoic membranes and taken up in allantoic fluid. Overall it was concluded that uterine protein secretions may affect both placental and embryonic/fetal development secondarily (Bazer 1975).

Other studies that demonstrated the importance of uterine glands for establishment and maintenance of pregnancy were reported in sheep. When administered to ewe lambs from birth to PND 13, norgestoment (a potent synthetic progestin) inhibited uterine gland development (Bartol et al. 1988). This original observation served as the foundation for the idea that prolonged exposure of neonatal ewes to progestins during the neonatal period associated with onset of uterine gland genesis could permanently inhibit uterine gland development and produce a ‘uterine gland knockout’ (UGKO) phenotype in adults, characterized by the permanent absence of uterine glands (Bartol et al., 1999). Adult UGKO ewes were infertile and exhibited recurrent pregnancy loss revealing an essential role for uterine glands and their secretions in conceptus survival and development during early pregnancy (Gray et al. 2001b; Gray et al. 2001a).

A progesterone-induced uterine gland knockout (PUGKO) in mice was used to investigate the biological role of uterine glands in blastocyst implantation and stromal cell decidualization (Filant et al. 2013b). Results showed that expression of implantation-related factors, including leukemia inhibitory factor (LIF) and prostaglandin-endoperoxide synthase 2 (PTGS2), were altered in PUGKO mice uteri. Additionally, administration of LIF failed to promote artificial decidualization in the uterus of PUGKO mice (Filant et al. 2013b). Studies done in mice demonstrated effects of progesterone treatment on uterine gland development (Cooke et al. 2012). Results showed that daily progesterone injections for a week during critical neonatal windows (PND 3–PND 9)
inhibited epithelial proliferation and permanently blocked uterine gland formation and adult fertility. This resulted in permanent loss of uterine glands and infertility in adulthood (Cooke et al. 2012). Together these studies in sheep and mice demonstrated that endometrial glands and their secretions are required for preimplantation conceptus survival and development.

I. FOXA2 is a regulator of uterine gland development

Forkhead box A2 (FOXA2) is a transcription factor that belongs to the FOXA family (Friedman et al. 2006), which includes FOXA1, FOXA2, and FOXA3. Organogenesis of multiple systems is controlled by FOXA family members (Kaestner 2010). Deletion of FOXA2 is embryonic lethal in mice since this transcription factor is required for development of endodermally-derived organs (Lee et al. 2005; Kaestner 2010), activation of the master gene of pancreas development Pdx1 (pancreas duodenum homeobox gene) (Gao et al. 2008), and for several phases of neuronal development. FOXA2 is the only FOXA family member expressed in uterine glands (Besnard et al. 2004). Studies showed that FOXA2 was localized in endometrial GE in neonatal and adult mice (Besnard et al. 2004; Filant et al. 2013a; Kelleher et al. 2017), pubertal and adult rats (Yamagami et al. 2014), adult humans (Kelleher et al. 2019) and in both the developing and adult ovine uterus (Spencer et al. 2019b). Additionally, FOXA2 mRNA levels increased substantially in the uteri of mice after PND 3 in association with the development of uterine glands (Dunlap et al. 2011).

Conditional deletion of FOXA2 in the neonatal mouse uterus resulted in reduced fertility (Jeong et al. 2010). At PxD 5.5, blastocyst implantation in FOXA2 mutant mice was disrupted and there was a severe impairment of the uterus in responding to artificial
induction of the decidual response (Jeong et al. 2010). In addition, uteri on PxD 5.5 had reduced number of endometrial glands which resulted in the reduction of LIF expression critical for blastocyst implantation. Overall, this study demonstrated that FOXA2 regulates endometrial gland development and that neonatal mice with a loss of endometrial glands cannot support implantation when they reach adulthood, due to the loss of LIF, which is necessary for fertility in the mouse.

In other studies, FOXA2 was conditionally deleted in both the adult and neonatal mouse uterus (Kelleher et al. 2017). Results showed that uteri of adult FOXA2-deleted mice were morphologically normal and contained uterine glands, while the uteri of neonatal FOXA2-deleted mice had no uterine glands. However, adult FOXA2-deleted mice were infertile due to defects in blastocyst implantation and stromal cell decidualization. Additionally, LIF was not expressed during early pregnancy in adult FOXA2-deleted mice. When adult mice were given injections of LIF, blastocyst implantation in the uteri of both gland-deficient and gland-containing adult FOXA2-deleted mice was initiated. Although pregnancy was rescued by LIF and was maintained to term in uterine gland-containing adult FOXA2-deleted mice, pregnancy failed by day 10 in neonatal FOXA2-deleted mice lacking uterine glands (Kelleher et al. 2017). Together these studies demonstrated the importance of uterine glands and their FOXA2-dependent secretions for adult uterine function and fertility.

**J. The transcription factors FOXA2 and ESR1 may cooperate in regulating neonatal endometrial development.**

Endometrial development involves differentiation of LE into GE and formation of uterine glands. A role for FOXA2 in epithelial budding was documented in multiple
organs including the pancreas (Besnard et al. 2004), liver (Lee et al. 2005) and prostate (Gao et al. 2005). These data, together with localization of FOXA2 exclusively in GE, support the idea that this transcription factor has a role in epithelial bud formation during uterine adenogenesis (Jeong et al. 2010). Likewise, the transcription factor ESR1, also expressed in nascent porcine GE, has a principal regulatory role in porcine uterine gland development. Administration of the antiestrogen ICI 182,780, a potent ESR1 antagonist, to neonatal gilts from birth inhibited endometrial adenogenesis and overall uterine growth at PND 14 (Tarleton et al. 1999).

FOXA1 and FOXA2 are termed ‘pioneer transcription factors’ because they can bind to compacted chromatin, decompact the chromatin and allow other transcription factors access condensed chromatin to influence transcription (Zaret et al. 2011). FOXA1/2 were reported to facilitate the binding of nuclear steroid receptor proteins, including ESR1 to target genes in human prostate or breast cancer cell lines (Gao et al. 2003; Carroll et al. 2005). In the liver, chromatin immunoprecipitation sequencing studies revealed enhanced coregulation of target genes by Foxa1/2 and ESR1 in female mice during hepatocarcinogenesis (Li et al. 2012). The same study found that enhanced coregulation of Foxa1/2 and ESR1 in response to carcinogen exposure plays a critical role in protecting females from hepatocellular carcinoma. In breast cancer cells, FOXA1 is important for ESR1 activity and estrogenic responses (Hurtado et al. 2011). These studies support the view that FOXA transcription factors cooperate with ESR1 and other transcription factors to regulate gene expression. Whether FOXA2 and ESR1, expressed in endometrial GE, play a role in coregulation of endometrial development is unknown and worthy of investigation.
K. Significance

Maternal influence on mammalian development begins during gestation and extends into the postnatal period by way of lactation. Nursing provides the neonate with nutrition and passive immunity and serves as a mechanism for delivery of MbFs such as growth factors and hormones present in colostrum and milk (Yan et al. 2006; Bagnell et al. 2009; Bartol et al. 2012). Studies supportive of the lactocrine hypothesis were reported for variety of species including rhesus macaques (Hinde et al. 2015), rodents (Nusser et al. 1997; Liu et al. 2014), and marsupials (Nicholas et al. 1997; Trott et al. 2003) and the pig (Vallet et al. 2015). These studies documented the importance of nursing and the quality and quantity of colostrum/milk consumed on lactocrine programming of developing somatic tissues. Data for the pig showed that disruption of lactocrine signaling by either experimental imposition of the lactocrine-null condition (Miller et al. 2013) or lactocrine-deficiency from birth (George et al. 2018) altered patterns of neonatal uterine development. In both conditions there is some form of organizational disruption as reflected by reduced endometrial thickness and uterine gland genesis.

Data presented in Chapter 2 support the view that lactocrine deficiency in nursing gilts, maintained under normal husbandry conditions, alters endometrial development by PND 14, resulting in reduced uterine FOXA2 expression and fewer FOXA2-positive glandular epithelial cells. This reduction in endometrial FOXA2 expression may contribute to alterations in neonatal lactocrine programing of porcine uterine tissues recognized to have lasting, negative effects on uterine capacity and fecundity in adulthood (Bartol et al. 2013; Vallet et al. 2015; George et al. 2018). Localization of
FOXA2 in nascent porcine endometrial GE cells at PND 14 reported here adds the pig to the list of mammalian species in which FOXA2 is expressed exclusively in uterine glandular epithelium (Spencer et al. 2019a). In addition, evidence for the neonatal pig indicates that two transcription factors, ESR1 (Tarleton et al. 1999; Masters et al. 2007; Miller et al. 2013) and now FOXA2, are markers of nascent endometrial glandular epithelium. Results reported and discussed in this thesis complement and extend those reported for gilts in response to imposition of a lactocrine-null state from birth on patterns of neonatal uterine wall development (Miller et al. 2013) and the neonatal uterine transcriptome (Rahman et al. 2016; George et al. 2017). In addition results presented here provide new information about how postnatal maternal lactocrine programming affects neonatal porcine uterine development, and introduce a new lactocrine sensitive marker of uterine gland development: FOXA2. Experiments described here advance and extend studies designed to test the lactocrine hypothesis for maternal programming of uterine development.

**L. Hypothesis and Objectives**

The hypotheses under consideration in the proposed research are as follows: 1) lactocrine deficiency from birth will affect patterns of porcine endometrial development by PND 14; and 2) lactocrine-sensitive porcine uterine FOXA2 expression will be detectable on PND 14 and serve as a marker of glandular epithelium. The overall goal of this research was to learn more about the effects of lactocrine deficiency, indicated by low serum iCrit at birth, on maternal programming of porcine uterine development. Objectives of the research were to:
1) Identify effects of lactocrine deficiency from birth on endometrial morphology at PND 14.

2) Determine if FOXA2 expression is detectable in neonatal porcine uterine tissues and whether neonatal porcine uterine FOXA2 expression is lactocrine-sensitive.

3) Evaluate effects of lactocrine deficiency from birth on patterns of FOXA2-distribution in the uterine endometrium on PND 14.
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CHAPTER II

Effects of lactocrine deficiency from birth on uterine gland development and FOXA2 immunolocalization in gilts at postnatal 14
ABSTRACT

Bioactive factors of maternal origin present in colostrum are delivered into the neonatal circulation via a lactocrine mechanism and affect patterns of uterine development in swine with lasting consequences. Imposition of a lactocrine-null condition by milk replacer feeding for two days from birth (postnatal day = PND 0) altered uterine gene expression patterns globally by PND 2 and inhibited uterine gland development by PND 14. Relative colostrum consumption can be assessed in nursing gilts by monitoring neonatal serum immunoglobulin immunocrit ratio (iCrit). Lactocrine deficiency, defined as low iCrit from birth, altered uterine development by PND 14 as reflected by reduced proliferation of endometrial glandular epithelial (GE) and stromal cells. Lactocrine deficiency from birth reduced lifetime fecundity in adults, indicated by reduced live litter size. Here, objectives were to determine effects of lactocrine deficiency from birth on porcine uterine endometrial development, forkhead homeobox A2 (FOXA2) expression and the distribution of FOXA2 protein in endometrium of neonatal gilts on PND 14. FOXA2, a transcription factor essential for the differentiation and development of uterine glands, marks uterine glandular epithelium in humans, mice and sheep, but has not been documented in the pig. Here, crossbred gilts were assigned to low (n = 12) or high (n = 10) iCrit groups based on iCrit ratio values determined on PND 0. Mean serum iCrit ratios were greater (P<0.01) in high (12.04 ± 0.27 relative units) versus low (1.92 ± 0.27 relative units) iCrit groups, indicating a difference in colostrum consumption between high and low groups of approximately 6-fold at birth. Uterine tissues were collected on PND 14. In a subset of high versus low iCrit gilts (6 litter-matched gilts per group) endometrial development was quantified
histomorphometrically and FOXA2 gene expression was measured. In addition, FOXA2 was localized in uterine GE cells immunohistochemically. Neither birth weights, PND 14 body weights, ovarian weights nor uterine wet weights differed between groups. However, both the number of GE/mm² (P < 0.05) and, to a lesser extent, endometrial thickness (P < 0.08) were reduced in low as compared to high iCrit gilts. Uterine gland penetration depth did not differ between the two groups. Uterine FOXA2 expression was detected in both low and high iCrit gilts and was reduced (P<0.05) approximately 3-fold on PND 14 in response to lactocrine deficiency from birth. Immunoreactive FOXA2 protein was localized consistently and uniquely in glandular epithelium of both low and high iCrit gilts on PND 14. The increased number of uterine GE cells/mm² in high iCrit gilts indicated an overall increase in endometrial FOXA2 expression. Results indicate that lactocrine deficiency from birth alters endometrial development, resulting in fewer endometrial glands and a general decrease in FOXA2 expression by PND 14. Reduction in endometrial FOXA2 expression may contribute to alterations in neonatal lactocrine programing of uterine tissues. Results reinforce the idea that, under normal husbandry conditions, lactocrine deficiency from birth in nursing gilts affects patterns of neonatal uterine endometrial development and is associated with inhibition of neonatal uterine gland genesis. Such changes in the neonatal uterine organizational program are ultimately associated with reduced fecundity in adults.
INTRODUCTION

Maternal influence on mammalian offspring development begins during gestation and extends into the postnatal period by way of lactation. Nursing provides the neonate with nutrition and passive immunity and serves as a mechanism for delivery of milk-borne bioactive factors (MbFs) such as growth factors and hormones present in colostrum and milk (Yan et al. 2006; Bagnell et al. 2009; Bartol et al. 2012). The term lactocrine was defined as delivery of MbFs of maternal origin into the neonatal circulation by consequence of nursing (Yan et al. 2006). This led to proposal of the lactocrine hypothesis for maternal programming of neonatal development (Bartol et al. 2008). The lactocrine hypothesis suggests that MbFs, communicated from mother to nursing offspring as a specific consequence of nursing, have both short- and long-term effects on postnatal somatic development, including effects on the uterine endometrial developmental program (Bartol et al. 2017).

Female reproductive tract development in pigs and other mammals, begins prenatally and continues postnatally (Cooke et al. 2013; Spencer et al. 2019a). Studies to test the lactocrine hypothesis showed that imposing a lactocrine-null condition, by feeding a porcine milk replacer from birth, altered the neonatal porcine uterine transcriptome by postnatal day (PND) 2 (Rahman et al. 2016; George et al. 2017) when almost 900 uterine genes were differentially expressed by uterine tissues obtained from nursed as compared to replacer-fed gilts. In addition, by PND 14 endometrial development and uterine gland formation were reduced in the gilts fed a commercial milk replacer for two days from birth and estrogen receptor (ESR1) immunostaining in glandular epithelial (GE) cells was reduced (Miller et al. 2013). These studies also
showed that return to nursing after PND 2 did not rescue the lactocrine-null phenotype by PND 14.

In addition to imposing a lactocrine-null state by milk replacer feeding, lactocrine deficiency can occur naturally due to conditions that limit availability of colostrum/milk (ex. maternal agalactia, competition for teat position) and/or affect milk quality (ex. mastitis) (Wu et al. 2010; Kraeling et al. 2015). A serum immunoglobulin immunocrit (iCrit) assay, developed (Vallet et al. 2013) to assess passive transfer of immunoglobulins from mother to nursing offspring, provided an practical method for indirect assessment of colostrum intake. Gilts with low serum iCrit at birth displayed reduced growth rates, delayed onset of puberty and reduced lactational performance (Vallet et al. 2015). Moreover, as predicted by the lactocrine hypothesis, low serum iCrit, indicating lactocrine deficiency on the day of birth, was associated with reduced live litter size and lifetime fecundity in adult, neonatally lactocrine deficient female pigs (Bartol et al. 2013; Vallet et al. 2015). Thus, both experimentally-induced and naturally occurring lactocrine deficiency from birth can affect the developmental trajectory of the neonatal porcine uterus.

The forkhead box A (FOXA) family of transcription factors includes FOXA1, FOXA2, and FOXA3 (Kaestner 2010). FOXA2 is expressed uniquely in uterine endometrial glands in mammalian species in which this has been examined (Besnard et al. 2004; Filant et al. 2013; Kelleher et al. 2017). FOXA2 was identified in endometrial glandular epithelium in mice (Besnard et al. 2004; Filant et al. 2013; Kelleher et al. 2017), rats (Yamagami et al. 2014), humans (Kelleher et al. 2019) and in both the developing and adult ovine uterus (Spencer et al. 2019b). However, uterine FOXA2
expression has not been reported for the pig. Therefore, objectives of this study were to determine: (1) effects of lactocrine deficiency from birth on endometrial morphology at PND 14; (2) if FOXA2 expression is detectable in neonatal porcine uterine tissues and if neonatal porcine uterine FOXA2 expression is lactocrine-sensitive; and (3) if lactocrine deficiency from birth affects patterns of FOXA2 protein distribution in the uterine endometrium on PND 14.
MATERIALS AND METHODS

Animals, Immunocrit Assay, and Tissue Processing

Gilts (*Sus scrofa domesticus*) were born and raised from a herd of crossbred maternal line (Landrace and Yorkshire genetics) pigs at the U.S. Department of Agriculture Meat Animal Research Center (USDA-MARC) in Clay Center, Nebraska. All work involving live animals was conducted at the USDA-MARC. Procedures involving animals were reviewed and approved by the USDA-MARC Institutional Animal Care and Use Committee and conducted in accordance with the Federation of Animal Science Societies (2010) Guide for the Care and Use of Agricultural Animals in Research.

All gilts were allowed to nurse *ad libitum* from birth (PND 0) to PND 14, when uterine tissues were collected. The experimental design is illustrated in Figure 1. Serum immunoglobulin immunocrit ratios were determined on PND 0 as described previously (Vallet *et al.* 2013). Briefly, this involved collection of jugular vein blood samples from each gilt within 24 h of birth. Clotted blood samples were centrifuged at 1000 × g for 10 min. Equal amounts (50 μl) of serum and 40% (w/v) ammonium sulfate in distilled water were mixed and the precipitated sample was loaded into a hematocrit centrifuge tube. Samples were centrifuged at 12,000 × g for 10 min. All samples were run in duplicate. The iCrit ratio was calculated by taking the length of the precipitate column divided by the length of the diluted serum column in each hematocrit tube. Gilts were assigned to low (n = 12) or high (n = 10) iCrit groups based on PND 0 iCrit percentages. Serum iCrit percentages were greater (P<0.01) in high (12.04 ± 0.27 relative units) versus low (1.92 ±
0.27 relative units) iCrit groups, indicating a difference in relative colostrum consumption between high and low groups of approximately 6-fold at birth.

On PND 14, gilts were euthanized and uterine tissues were collected and trimmed free of associated connective tissue. One uterine horn was snap-frozen in liquid nitrogen and stored at -80°C for gene expression analyses. The other uterine horn was fixed in Xpress Molecular Fixative (Sakura Finetek USA Inc; Torrance, CA) and, ultimately, embedded in Paraplast Plus (Fischer Scientific, Atlanta, GA, USA) for histomorphometry and immunohistochemistry (IHC). Uterine cross-sections (6 µm) were affixed to Superfrost Plus slides (VWR International, LLC, Radnor, PA, USA). Each slide had two nonsequential uterine sections/gilt and contained tissues from three high iCrit and three low iCrit animals (total of 12 uterine sections).

**Histomorphometric Analyses**

ImageJ (National Institutes of Health) was used for all histomorphometric analyses. Measurements were made on four nonsequential uterine sections per animal. A schematic to illustrate the protocol used for making histomorphometric measurements is presented in Figure 2. Endometrial thickness was measured as the distance from the base of the luminal epithelial (LE) cells to the inner circular layer of the myometrium. For each animal 24 endometrial thickness measurements were taken (four sections x six measurements per section, three each from the dorsal and ventral side of the lumen). To quantify GE per unit of endometrial area, all epithelial cells were counted for each uterine section. This measurement was reported as the average number of glandular epithelial cells/mm² as determined for four uterine sections from each animal. Gland penetration depth was measured from the mouth of a gland at the base of the LE to the bottom of
each gland where GE cells met stroma (ST). This measurement was repeated for all glands in four uterine sections per animal.

_Uterine RNA isolation and quantitative real-time polymerase chain reaction (qPCR)_

At Rutgers University total RNA was isolated from PND 14 uterine tissue (50 mg/uterine horn) for each sample using the miRNeasy Mini Kit (Qiagen Inc., Vallencia, CA, USA) following the manufacturer’s protocol. RNA quantity was measured using a NanoDrop ND-100 (Thermo Scientific; Waltham, MA, USA) and RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Applied Biosystems, Carlsbad, CA, USA). RNA integrity (RIN) values ranged from 2.1 to 6.4 indicating variable RNA quality and possible RNA degradation. After repeating the RNA isolation three times RIN numbers did not improve so a subset of uterine samples were shipped to Dr. Xu Wang at Auburn University.

At Auburn University, RNA was isolated from PND 14 uterine tissue (50 mg/uterine horn) using the Zymo Quick-DNA/RNA Miniprep Plus kit (Zymo Research, Irvine, 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). RIN numbers ranged from 4.8 to 6.5 confirming low RNA quality in uterine samples. This RNA was sent to Rutgers for evaluation of uterine FOXA2 expression.

Reverse transcription was performed using 100 ng of total RNA per sample, a Peltier Thermal Cycler -200 (Bio-Rad Laboratories, Inc.) and the Superscript III First-Strand Synthesis System (Life Technologies, Carlsbad, CA, USA). Individual sample libraries were diluted to a final concentration of 1ng/µl. Next, uterine expression of
FOXA2 in high vs low iCrit gilts at PND 14 was determined by quantitative real-time polymerase chain reaction (qPCR). Quantitative RT-PCR (10 ng/sample) was conducted using SYBR Green and universal thermal cycling parameters (40 cycles) as recommended by the manufacturer on a StepOne Plus System (Applied Biosystems/Life Technologies). Porcine primers for FOXA2 and cyclophilin A (PPIA) 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. Each cDNA sample was run in duplicate. Control qPCR reactions included substitution of water only, in place of primers and template, to ensure specific amplification in all assays. Standard curves for primer sets were run in triplicate and assessed to ensure that no amplicon–dependent amplification occurred. Data generated by qPCR were analyzed using the ΔΔCt method described by Applied Biosystems (ABI User Bulletin 2, 2001). With this method, Ct values of both sample and calibrator are normalized to an endogenous reference gene. In this case cyclophilin A was used as expression was unaffected by treatment. Amplification efficiencies for target genes were determined to be similar to those calculated for the reference gene, cyclophilin A (ABI User Bulletin 2, 2001).

Immunohistochemistry and Image Analysis:

In preliminary experiments, rabbit anti-human FOXA2 antibody (category #ab108422; Abcam, Cambridge, MA, USA), used for detection of FOXA2 in mice (Besnard et al 2004), sheep (Spencer et al 2019) and humans (Kelleher et al 2019), was evaluated for detection of FOXA2 in porcine uterine tissues. In these experiments, PND 21 pig uterine tissues and adult mouse uterine tissues (positive control) were incubated
with increasing dilutions (ie., 1:100, 1:500, 1:1000 v/v) of rabbit anti-human FOXA2 antibody. This enabled determination of the FOXA2 antibody concentration for optimum immunostaining with low background. Mouse and pig uterine sections were processed for immunochemistry as described below.

Immunohistochemistry was performed using a VectaStain Elite ABC Kit (Rabbit IgG) (Category number PK 6101; Vector Laboratories, Burlingame, CA, USA). Uterine sections were deparaffinized, rehydrated and subjected to heat-induced antigen retrieval in boiling sodium citrate buffer (pH 6). For evaluation of FOXA2 immunostaining, uterine sections were incubated overnight at 4°C with rabbit anti-human FOXA2 antibody (Abcam category #ab108422). Following overnight incubation, sections were washed in PBS and incubated with HRP-conjugated biotinylated secondary antibody for 1 h at room temperature. Endogenous peroxidase activity was blocked (3.0% hydrogen peroxide for 5 min) then sections were incubated with ABC reagent and developed using 0.05% diaminobenzidine substrate (DAB; Sigma-Aldrich) in 0.005M Tris and 0.01% hydrogen peroxide in water for 10 minutes. Negative controls were incubated with rabbit isotype control IgG (1.06 ug/ml; Invitrogen) in place of the primary antibody. In some experiments uterine sections were counterstained with Mayer’s hematoxylin (Thermo Scientific, TA-125-MH) for four minutes and then dehydrated. In other experiments to better view FOXA2 nuclear staining, hematoxylin was omitted. Images were taken at a final magnification of 10x or 40x using an Olympus FSX100 Digital Microscope. Images were converted to 8-bit grayscale in Adobe Photoshop and histomorphometric analyses conducted using ImageJ Software (National Institutes of Health). Nuclei were identified as positive for FOXA2 when staining intensity was within 25% of the highest values
generated by ImageJ (Masters et al. 2007). Grayscale images were converted to pseudocolor using ImageJ to better illustrate staining intensity patterns. Here, positively stained cells appear yellow-red on a dark blue-black background. Colors were assigned automatically based on relative staining intensity of grayscale digital images.

Statistics:

Data were subjected to analyses of variance using General Linear Models (SAS 2013; Cary, NC) and are presented as least squares means (LSM) ± standard errors of the mean (SEM).
RESULTS

*Effects of lactocrine deficiency from birth on uterine endometrial morphology at PND 14*

Neither birth weight (1.37 ± 0.08 vs 1.46 ± 0.07 kg), PND 14 body weight (4.63 ± 0.36 vs 4.46 ± 0.31 kg), ovarian weight (0.11 ± 0.01 vs 0.11 ± 0.01 g), nor uterine weight (0.99 ± 0.14 vs 0.89 ± 0.1 g) differed between high and low iCrit gilts. However, as shown in Figure 3, endometrial development was less advanced in low as compared to high iCrit gilts on PND 14. This was documented by reduced endometrial thickness (P < 0.08; Figure 3A) and endometrial glandularity (Figure 4). When uterine glands did develop in low iCrit gilts, mean uterine gland penetration depth (Figure 3B) did not differ (P < 0.22) between low (61.9 ± 2.2 µm) and high (80.1 ± 2.2 µm) iCrit groups.

*Effects of lactocrine deficiency on uterine FOXA2 expression on PND 14*

FOXA2 transcripts were detected consistently in uterine tissues from both low and high iCrit gilts. Overall, FOXA2 expression was reduced (P<0.05) approximately 3-fold in response to lactocrine deficiency from birth (Figure 5).

*Immunohistochemical localization of FOXA2 in porcine uterine tissues on PND 14.*

Immunohistochemical analyses confirmed that, as expected (Besnard *et al* 2004; Filant *et al* 2013; Yamagami *et al* 2014; Kelleher *et al* 2017; Kelleher *et al* 2019; Spencer *et al* 2019), FOXA2 was present in and confined to adult murine uterine GE (Figure 6B). A similar FOXA2 immunostaining pattern was observed in PND 21 porcine uterine tissue sections (Figure 6D). Incubation of mouse (Figure 6A) or pig (Figure 6C) uterine tissues with control IgG, in place of the FOXA2 primary antibody, resulted in low non-specific staining. High magnification of both murine (Figure 6B) and porcine (Figure 6E, 6F) endometrium showed nuclear localization of FOXA2 exclusively in GE cells.
Representative, pseudocolor images illustrating FOXA2 immunostaining in the endometrium of high and low iCrit gilts on PND 14 are presented in Figures 7A and 7B, respectively. FOXA2 immunostaining was confined to glandular epithelium in both high and low iCrit gilts. The average number of FOXA2-positive GE cells per unit endometrial area was more than 2-fold greater (P < 0.05) in high (302.9 ± 16.8 mm$^2$) as compared to low (139.2 ± 16.8 mm$^2$) iCrit gilts.
DISCUSSION

Lactocrine deficiency from birth, indicated by low serum iCrit on PND 0, reduces lifetime fecundity in pigs (Bartol et al. 2013; Vallet et al. 2015). Adult, neonatally lactocrine-deficient (low iCrit) female pigs are maintained under identical husbandry conditions as their lactocrine-sufficient (high iCrit) littermates. Both low and high iCrit gilts receive sufficient nutrition and passive immunity by nursing to survive into adulthood. However, lactocrine signaling in low iCrit gilts is insufficient to support an optimal, postnatal uterine developmental program (Vallet et al. 2015; George et al. 2018). Results presented here support the lactocrine hypothesis for maternal programming of porcine uterine development by establishing that lactocrine deficiency from birth has short-term effects on patterns of endometrial organization that are evident by PND 14. These observations complement and extend those reported for gilts in response to imposition of a lactocrine-null state from birth, by milk replacer feeding, on patterns of neonatal uterine wall development (Miller et al. 2013) and the neonatal uterine transcriptome (Rahman et al. 2016; George et al. 2017). Thus, lactocrine deficiency from birth, a condition that occurs naturally in nursing gilts under normal husbandry conditions, is likely to affect the neonatal uterine developmental program in a manner similar to that reported for gilts following experimental imposition of the lactocrine-null state (Miller et al. 2013; George et al. 2018).

Experimental disruption of neonatal porcine endometrial development was also reported for gilts given the type-II antiestrogen ICI 182, 780 (Tarleton et al. 1999). In those studies, endometrial thickness and gland genesis were reduced by PND 14 in response to antiestrogen treatment in a manner similar to that reported here in lactocrine-deficient gilts. In addition, present results confirm and extend previous findings for the
porcine uterus (Miller et al. 2013) involving imposition of a lactocrine-null state from birth by demonstrating analogous effects of reduced endometrial thickness and glandularity on porcine endometrial development on PND 14. Present observations indicating that lactocrine deficiency from birth did not affect body, ovarian, or uterine weights at PND 14 agree with findings in lactocrine-null gilts (Miller et al. 2013). Present data also confirm an earlier, preliminary report based on observations for lactocrine deficient gilts (Gray et al. 2017 SSR Abstract), in which endometrial thickness was also reduced in low iCrit gilts, while uterine gland penetration depth was unaffected. By contrast, results reported for lactocrine null-gilts fed milk replacer from two days from birth and returned to nursing until PND 14 in which endometrial adenogenesis, endometrial thickness and uterine gland penetration depth were all reduced on PND 14 (Miller et al. 2013). These observations suggest a greater degree of lactocrine deficiency in milk replacer-fed gilts.

Together these studies illustrate common effects of neonatal uterine developmental disruption associated with administration of ICI 182, 780 (Tarleton et al. 1999), experimental imposition of the lactocrine-null condition (Miller et al. 2013), and lactocrine-deficiency from birth (George et al. 2018). In all three conditions there is some form of organizational disruption as reflected by reduced endometrial thickness and uterine gland genesis. In the pig, glandular epithelium becomes ESR1 positive by 24 hours after birth, followed by endometrial stroma, which is ESR1-positive by 48 hours after birth (Bartol et al. 1993). Documented effects of ICI 182, 780 established that events associated with endometrial development and uterine gland genesis in the neonatal pig were, at least in part, ER-mediated (Tarleton et al. 1999).
Transient imposition of the lactocrine-null state on neonatal porcine female reproductive tract development (Camp et al. 2014; Ho et al. 2017; George et al. 2018) indicated that a lactocrine programing window is open within 24 h from birth. For example, in the neonatal cervix, a single feeding of colostrum at birth was sufficient to support normal cervical cell proliferation at 12 h postnatal. Effects of age at first nursing and duration of nursing from birth showed that colostrum consumption within 12 h of birth was both necessary and sufficient for neonatal uterine matrix metalloproteinease-9 (MMP9), tissue inhibitor of metalloproteinase, and cervical MMP9 expression (Ho et al. 2017). In the uterus, a single feeding of colostrum at birth increased endometrial cell proliferation at 12 h in nursed gilts, as well as those that were bottle- or gavage-fed colostrum (Ho et al. 2017). Results indicate that lactocrine effects on postnatal uterine development are initiated with the first ingestion of colostrum within 24 h of birth. While the duration of natural lactocrine deficiency from birth in low iCrit gilts evaluated here cannot be determined from the current study, differences in lactocrine signaling between high and low iCrit gilts on PND 0 alone may be sufficient to change the uterine endometrial organizational program, as reflected by observations documented here on PND 14. In addition, the observation that PND 14 body weights and uterine weights of low and high iCrit gilts were similar suggests that effects of lactocrine deficiency on uterine development were not due to a lower plane of nutrition in low iCrit gilts.

Localization of the transcription factor FOXA2 in porcine endometrial GE cells at PND 14 reported here adds the pig to the list of mammalian species in which FOXA2 is expressed exclusively in uterine glandular epithelium (Spencer et al. 2019a). Data for the neonatal mouse indicate that FOXA2 is essential for differentiation and development of
uterine glands (Jeong et al. 2010). Conditional deletion of Foxa2 in the murine uterus inhibited uterine gland genesis and resulted in infertility (Jeong et al. 2010; Dunlap et al. 2011). Development of uterine glands is necessary for establishment and maintenance of pregnancy (Kelleher et al. 2017; Spencer et al. 2019a). The conserved nature of FOXA2 expression and localization in GE cells across mammalian species suggests that this transcription factor is mechanistically essential for development and function of endometrial glands (Kelleher et al. 2019).

Evidence presented here indicating that porcine endometrial FOXA2 expression is lactocrine-sensitive suggests that lactocrine delivery of MbFs in colostrum affects FOXA2 expression in the neonatal porcine endometrium. Lactocrine-active MbFs include proteins, growth factors, and bioactive peptides, as well as exosomal microRNAs (Bagnell et al. 2017). While yet to be determined, effects of such MbFs on neonatal endometrial development could be direct, through actions on nascent glandular epithelium, or indirect through actions on endometrial stroma.

That epithelial – mesenchymal interactions are essential to the success of endometrial development is well established (Cunha 1976; Sharpe et al. 1988). Studies in ungulate species, including cattle (Bartol et al. 1995) and sheep (Bartol et al. 1999), and in other species such as mice (Cooke et al. 2012; Filant et al. 2012), showed that disruption of epithelial-mesenchymal interactions from birth results in altered endometrial phenotypes and impaired uterine function in adulthood (Cooke et al. 2013). Specifically, in mice (Cooke et al. 2012; Filant et al. 2012) and sheep (Bartol et al. 1999; Gray et al. 2001), neonatal progestin exposure throughout the time period associated with endometrial adenogenesis resulted in a uterine gland knockout phenotype characterized
by lack of endometrial glands in adults. In rats, neonatal progesterone exposure disrupted endometrial gland genesis, and resulted in progesterone resistance and uterine gland dysfunction at the age of maturity (Dhakal et al. 2015). Together these studies demonstrate that neonatal disruption of epithelial and stromal interactions alters uterine gland genesis and that these effects in the neonate can persist into adulthood.

Evidence for the neonatal pig indicate that two transcription factors, ESR1 (Tarleton et al. 1999; Masters et al. 2007; Miller et al. 2013) and FOXA2, are markers of nascent endometrial glandular epithelium. In the pig, it is not yet established mechanistically whether FOXA2 mediates the genesis of nascent endometrial glands in the neonate. Studies involving multiple tissues, including the uterus (Bazer et al. 2010; Cha et al. 2017) and others (Li et al. 2012; Zhao et al. 2015) suggested cooperative or co-regulatory roles for ESR1 and FOXA2 signaling in aspects of development. Current results illustrating the localization of FOXA2 in porcine endometrial GE cells, known to be ESR1-positive opens the door to the proposed idea of ESR1 and FOXA2 colocalizing in nascent GE where neonatal porcine uterine gland genesis is concerned.

Studies in rhesus macaques (Hinde et al. 2015), rodents (Nusser et al. 1997; Liu et al. 2014), and marsupials (Nicholas et al. 1997; Trott et al. 2003), as well as the pig (Vallet et al. 2015), documented the importance of nursing and the quality and quantity of colostrum/milk consumed on lactocrine programming of developing somatic tissues. Studies designed to determine effects of experimentally imposed lactocrine deficiency from birth by milk replacer feeding on porcine uterine development focused on the period from birth to PND 14 (Chen et al. 2011; Miller et al. 2013; Rahman et al. 2016; George et al. 2017; Ho et al. 2017). Naturally occurring lactocrine deficiency in pigs, indicated
by low serum iCrt within 24 h of birth, provides a physiological model for studying the impact of ingestion of colostrum on uterine development and function. Data presented here support the view that lactocrine deficiency in nursing gilts, maintained under normal husbandry conditions, alters endometrial development at PND 14, resulting in reduced uterine $FOXA2$ expression and fewer $FOXA2$-positive GE cells. This reduction in endometrial $FOXA2$ expression may contribute to alterations in neonatal lactocrine programing of porcine uterine tissues recognized to have lasting, negative effects on uterine capacity and fecundity in adulthood (Bartol et al. 2013; Vallet et al. 2015; George et al. 2018).

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Table 1. Primer sequences (forward and reverse) for porcine uterine transcripts used for qPCR

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Accession #</th>
<th>Forward and Reverse Primer Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOXA2</td>
<td>Forkhead Box A2</td>
<td>NC_010459</td>
<td>F: ACGATGTAAGGTCTGTTGTA AA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: AGCACGCGGAAACCATAA</td>
</tr>
<tr>
<td>PPIA</td>
<td>Porcine Cyclophilin A</td>
<td>AU_058466</td>
<td>F: TTATAAAGGTTCTGCTTTCA CAGAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: TGCCATTATGGCGTGTAAG</td>
</tr>
</tbody>
</table>
Figure 1. Experimental design. Within 24 h of birth (PND 0), jugular vein blood samples were collected from gilts, serum immunocrit (iCrit) ratios were measured, and these values were used to assign gilts to high (n=10) or low iCrit (n=12) groups. Uterine tissues were collected on PND 14.
Figure 2. Schematic of the PND 14 porcine uterus (cross section) illustrating the histomorphometric protocol used to measure endometrial thickness, glandular epithelial (GE) cell area (GE cells/mm²) and gland penetration depth. Red line shows how endometrial thickness was measured as the distance from the base of the luminal epithelial (LE) cells to the inner circular myometrium. Blue line shows how gland penetration depth was measured from the basal aspect of the LE to the depth of each gland where GE meets endometrial stroma.
Figure 3. Effects of lactocrine deficiency defined as low serum iCrit from birth on endometrial thickness (A) and gland penetration depth (B) in PND 14 gilts (black bars= high iCrit, white bars= low iCrit). Data are presented as LSM ± SEM, n=6 gilts/group). Plus sign indicates difference between iCrit groups (P<0.08).
Figure 4. Representative photomicrographs of FOXA2 and hematoxylin-stained uterine cross-sections from high (A) and low (B) iCrit gilts to illustrate effects of lactocrine deficiency on uterine glandularity. Magnification 2.5x. GE= glandular epithelium, LE= luminal epithelium, ST= stroma.
Figure 5. Effects of lactocrine deficiency on uterine *FOXA2* gene expression in gilts at PND 14. Black bar indicates high iCrit (n=5) and white bar indicates low (n=4) iCrit gilts. Data were normalized to cyclophilin gene expression and expressed as LSM ± SEM. Asterisk indicates difference between iCrit groups (P<0.05)
Figure 6. Representative photomicrographs of FOXA2 (1:500) immunostaining in adult mouse (A,B) and PND 21 pig (C-F) uterine endometrial tissues. All of the sections were developed using DAB substrate without hematoxylin counterstaining. A) mouse, IgG control, 40 x; B) mouse, FOXA2, 40 x; C) pig, IgG control, 10x; D) pig, FOXA2, 10x; Red circle indicates inset for higher magnification in E; E) pig, FOXA2, 40x; F) pig, FOXA2, 40x, pseudocolor imaging in which FOXA2-stained GE cell nuclei appear green-blue on a black background.
Figure 7. Effects of lactocrine deficiency on FOXA2 immunostaining in uterine GE cells in high (A) and low (B) iCrit gilts at PND 14. Uterine tissues were developed using DAB substrate and counterstained with hematoxylin. Images were pseudocolored to illustrate FOXA2 labeling patterns in GE cells. Representative photomicrographs of FOXA2-stained and hematoxylin labeled GE cell nuclei appear yellow-orange on a black background. ST, stroma; Myo, myometrium. Quantification of FOXA2 labeling of GE cells, (C). Black bar indicates high iCrit (n=6) and white bar indicates low (n=6) iCrit gilts. Data are presented as LSM ± SEM. Asterisk indicates difference between iCrit groups (P<0.05).
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CHAPTER III

THESIS CONCLUSIONS
CONCLUSIONS

Maternal influence on neonatal development begins during gestation and extends postnatally via signals communicated to nursing offspring in colostrum/milk. Colostrum, or first milk, is the initial secretion produced by the porcine mammary gland from birth to 48 hours after parturition (Klobasa et al. 1987). The importance of colostrum/milk in providing nutrition and passive immunity for developing offspring is clear (Bartol et al. 2008). However, the role of colostrum as a conduit for delivery of maternally-derived signals by way of lactocrine signals affecting the programming and trajectory of neonatal development is not well understood. Studies in lactocrine-null pigs, deprived of colostrum by feeding a commercial milk replacer from birth, focused on short-term effects on neonatal uterine (Chen et al. 2011; Miller et al. 2013; Ho et al. 2017) and cervical (Frankshun et al. 2012; Camp et al. 2014) development. In addition, studies involving nursing pigs deemed lactocrine-deficient pigs from birth as reflected by low serum iCrt, revealed long-term, negative effects of disrupted lactocrine signaling on uterine capacity and fecundity in adult female pigs (Bartol et al. 2013; Vallet et al. 2015). Still, the impact of lactocrine deficiency from birth on the neonatal uterine developmental program is not well understood. The overall goal of this research was to learn more about the effects of lactocrine deficiency on maternal programming of porcine uterine development in the neonatal pig.

Objectives of this research were to: 1) identify effects of lactocrine deficiency from birth on endometrial morphology at PND 14; 2) determine if FOXA2 expression was detectable in neonatal porcine uterine tissues and whether neonatal porcine uterine FOXA2 expression was lactocrine-sensitive; and 3) evaluate effects of lactocrine deficiency from birth on patterns of FOXA2 distribution in the uterine endometrium on
PND 14. Results showed that lactocrine deficiency from birth retarded endometrial development in low- as compared to high-iCrit gilts on PND 14. This was documented by reduced endometrial thickness and endometrial glandularity. These uterine morphological results were similar to those reported in lactocrine null-gilts fed milk replacer from two days from birth and returned to nursing until PND 14 (Miller et al. 2013).

The pioneer transcription factor, FOXA2, was immunolocalized in nascent porcine uterine GE (Chapter 2, Figure ??). This adds the pig to the list of mammalian species in which FOXA2 is expressed exclusively in neonatal uterine GE (Spencer et al. 2019). Further, uterine FOXA2 expression was determined to be lactocrine-sensitive, decreasing approximately 3-fold in uterine tissues of lactocrine deficient gilts at PND 14. Results presented here provide new information about how lactocrine deficiency from birth affects neonatal porcine uterine development and introduces FOXA2 as a new, lactocrine-sensitive marker of nascent porcine uterine GE and endometrial development.

The goal of future studies should be focused on determining whether epigenetic reprogramming in the porcine uterus plays a role in lactocrine-related transcriptional regulation. Previous studies showed that lactocrine deficiency from birth altered the endometrial miRNA-mRNA interactome in high- versus low-iCrit gilts in adulthood at PxD 13 (George et al. 2018). In addition to miRNAs, other epigenetic modifications include DNA methylation and histone modification, which alter DNA accessibility and chromatin structure, thereby regulating patterns of gene expression (Handy et al. 2011).

Studies to determine whether lactocrine deficiency from birth alters epigenetic marks including histone modifications in the adult porcine endometrium of early pregnancy at PxD 13 were part of my original thesis plans and initiated prior to the start
of the COVID-19 pandemic when research activity at Rutgers was abruptly shut down. The hypothesis to be tested was that lactocrine deficiency from birth will result in histone modifications that regulate porcine endometrial gene expression at the time of implantation. Differential recruitment of histone methyltransferases (H3K4me3, H3K9me3, and H3K27me3) in endometrial tissues was expected between high vs. low iCryt gilts at PxD 13. Plans were to test the previously mentioned hypothesis using chromatin immunoprecipitation (ChIP). Using ChIP, chromatin fragments of high and low iCryt endometrium samples at PxD 13 (n=5) per group were sheared to ~150bp an optimal shearing length for ChIP studies. Next steps in this study should focus on immunoprecipitating these chromatin fragments with antibodies directed against histone methyltransferases mentioned previously. Next ChIP qPCR can be done using FOXA2 primers to determine whether differential recruitment of histone methyltransferases due to lactocrine deficiency results in differential binding and expression of FOXA2 in the endometrium of high vs. low iCryt gilts at PxD 13. Studies to test whether lactocrine deficiency from birth alters other epigenetic marks in both the neonatal and adult porcine endometrium could provide valuable information about mechanisms involved in lactocrine mediated developmental programming.

Additionally, studies using chromatin immunoprecipitation sequencing (ChIP-seq) can be used to determine whether FOXA2 cooperates with ESR1 to coregulate neonatal porcine endometrial development. Previous studies using ChIP-seq showed that FOXA1/2 facilitates the binding of nuclear steroid receptor proteins including ESR1 to target genes in human prostate and breast cancer cells (Gao et al. 2003; Carroll et al. 2005). In the liver, ChIP-seq studies indicated enhanced coregulation of target genes by
Foxa1/2 and ESR1 in female mice during hepatocarcinogenesis (Li et al. 2012). New experiments to investigate the epigenetic regulation of maternal lactocrine programming will enhance understanding of maternal programming of neonatal and adult uterine development by way of nursing and the role of epigenetics in both short and long-term lactocrine effects.
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