Neonatal Exposure to Estrogen and Estrogen Receptor Agonists and Antagonists: Effects on the Adult Endometrial Proteome and Morphoregulatory Gene Expression in the Neonatal Uterus

by

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Abstract

Development of the porcine uterus begins prenatally but is completed postnatally. In mammals, the uterus provides an embryotrophic environment for conceptus development and integrates maternal and conceptus signals needed for maintenance of pregnancy. The first two weeks of postnatal life are an important period for developmental programming of the endometrium. Disruption of this program by exposure of neonates to estradiol valerate (EV; 50ug/kg BW/day) for two weeks from birth affects adult uterine morphology, endometrial function and, ultimately, decreases embryo survival. Several morphoregulatory genes including Wnt4, Wnt5a, Wnt7a, Hoxa10 and Hoxa11 have been implicated in the development of the porcine female reproductive tract. Studies were conducted to determine if Indian Hedgehog (Ihh), Muscle Segment Homeobox 1 and 2 (Msx1, Msx2) were present in the developing porcine endometrium as well as if the expression patterns were altered by exposure to hormones. The adult endometrial proteome from day 12 pregnant pigs was also studied to determine effects of neonatal estrogen exposure.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

APC—Adenomatous polyposis coli

BPA—Bisphenol A

BrdU—5-bromo-2’-deoxyuridine

CO—Corn Oil

Cox2—Cycloxygenase 2

DES—Diethylstilbestrol

Dhh—Desert hedgehog

EGFR—Epidermal growth factor

ER—Estrogen receptor α

αERKO—Estrogen receptor α knock out

βERKO—Estrogen receptor β knock out

αβERKO—Estrogen receptor α and β knock out

FRT—Female reproductive tract

FSH—Follicle stimulating hormone

GE—Glandular epithelium

GPCR—G-protein coupled receptor

GSK3β—Glycogen synthase kinase 3β

^{3}H-Thd—Methyl-^{3}H thymidine

Hh—Hedgehog

Ihh—Indian hedgehog

KGF—Keratinocyte growth factor

LE—Luminal epithelium

LH—Luteinizing hormone

LI—Labeling index

LGR-7—Leucine rich G-protein coupled receptor 7

LRP6—Low-density lipoprotein receptor-related protein 6

MRP—Maternal recognition of pregnancy

Msh/Msx—Muscle segment homeobox gene

NF-κB—Nuclear factor κB

PCNA—Proliferating cell nuclear antigen

$PGF_{2\alpha}$—Prostaglandin $F_{2\alpha}$

PR—Progesterone receptor

Ptc—Patched

RANK—Receptor activator of nuclear factor κB

RANKL—Receptor activator of nuclear factor κB ligand

RBP—Retinol binding protein

RT-PCR—Real time-polymerase chain reaction

SERM—Selective estrogen receptor modulator

SFRP2—Secreted frizzled related protein 2

Shh—Sonic hedgehog

Smo--Smoothened

UGKO—Uterine gland knock out

UHOX—Unilaterally ovariohysterectomized

VEGF—Vascular endothelial growth factor

INTRODUCTION

Reproduction is a normal and essential part of life for both humans and animals. Moreover, reproductive efficiency of females is an important trait in domestic animals upon which society depends for food and fiber, not to mention companionship and recreation. Consequently, it is imperative to study the development of the female reproductive tract (FRT) in order to understand factors affecting reproductive health and performance. The FRT consists of the ovaries, oviducts, uterus, cervix and vagina. This review will focus on the uterus. Emphasis will be placed on aspects of uterine development, function, and consequences of uterine developmental disruption in the domestic pig, a classic animal model that is also economically important.

In the domestic pig (*Sus scrofa domesticus*), the uterus is functionally and morphologically immature at birth (postnatal day = 0; i.e. PND 0). During the first two weeks of life, the porcine uterine mucosa, or endometrium, undergoes a remodeling process where glandular epithelium (GE), which differentiates from luminal epithelium (LE), begins to proliferate and form coiled tubular glands that penetrate deep into the endometrial stroma. Uterine gland formation is associated with the appearance of nuclear estrogen receptor-α (ER) expression during the first two weeks of life in stromal cells as well as in GE, but ER expression is not regularly apparent in cells of the LE [1]. Uterine ER expression is detectable by PND 2 [2]. Data for the pig indicate that the success of

estrogen-sensitive, ER–dependent organizational events associated with development of the uterine wall during the early neonatal period dictate the extent to which adult tissues are able to function optimally in support of reproduction [3, 4].

Functions of the adult uterus include: (1) regulation of cyclicity; (2) sperm transport and maturation; (3) integration of conceptus-initiated signals responsible for maternal recognition of pregnancy; and (4) provision of a suitable environment for conceptus development. In the non-pregnant pig, ovarian cyclicity requires that the uterine endometrium produce prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) for luteolysis and continuation of the ovarian cycle [5]. During estrus, myometrial contractions aid in the transport of sperm through the FRT into the oviducts where fertilization occurs [6]. Before fertilization is possible, sperm must undergo capacitation (maturation) in the FRT for several hours prior to ovulation [7]. After fertilization in the oviduct, developing embryos move into the uterine lumen. Porcine conceptuses begin to secrete estrogen as the signal for the maternal recognition of pregnancy by day 12 of gestation, causing the uterus to begin preparing for implantation and conceptus support [8]. Correct uterine morphogenesis (structural development) and cytodifferentiation (functional development) are necessary to insure that the adult uterus can support these processes.

Structural and functional development of the porcine uterus is a complex, interdependent series of processes. During development uterine cell populations organize, differentiate and proliferate. Maturation, growth and development of the porcine uterus appear to be ovary-independent processes between PND 0 and PND 60,

although the neonatal uterus is both steroid and peptide hormone sensitive during this period [1, 9]. Effects of steroid and peptide hormones are mediated by stromal-epithelial interactions that are central to the success of uterine morphogenesis and cellular differentiation [10]. Highly conserved morphoregulatory genes expressed by the neonatal porcine endometrium, including members of both *Wnt* [3, 11] and *abdominal-B HoxA* [3, 12] families, are required for developmental programming and patterning of the FRT [3]. The actions of their products are facilitated by stromal-epithelial cell interactions.

Morphoregulatory gene expression patterns can be disrupted by transient exposure of neonates to estrogen or factors that affect the state of ER activation. Estrogen-induced disruption of the porcine uterine developmental program during the first two weeks of life not only alters the morphology of the neonatal uterus [13], but can also have long-term consequences in the adult. These effects include an inhibited uterine growth response to local conceptus signals, reduced uterine luminal protein content and endometrial protein biosynthetic activity. Not surprisingly, the affected uterus displays altered endometrial mRNA expression patterns for several primary endometrial proteins including keratinocyte growth factor, retinol binding protein and uteroferrin [14]. Effects of this kind are known to lead to increased embryo mortality and decreased reproductive efficiency in adults.

LITERATURE REVIEW

Uterine biology has intrigued scientists since the 4th century BC as evidenced by the writings of Hippocrates and Aristotle describing the basic external structure of the uterus of domestic animals [15]. Structure, function and development of the uterus of many species, including humans, have been studied extensively since that time. Nevertheless, many aspects of uterine biology remain incompletely understood. Following is a review of some important aspects of uterine development and function with emphasis placed on porcine uterine biology.

1. THE UTERUS

The uterus, an anatomical component of the FRT, is a mesodermally derived organ which develops from the paramesonephric or Müllerian ducts. The paramesonephric ducts are paired structures [16] that develop from invaginations of coelomic epithelium on the lateral sides of the urogenital ridges [5]. These ducts give rise to all segments of the FRT including the oviducts, uterus, cervix and anterior vagina [3]. Anterior-posterior patterning creates histological boundaries between these anatomically distinct segments of the FRT [3]. The ovaries originate as bipotential structures from the genital ridges medial to the embryonic kidney [17].

During embryonic development female mammals acquire one of three anatomically distinct types of uteri (simplex, bicornuate or duplex), the nature of which depends upon whether fusion of the paramesonephric ducts is complete, partial or incomplete [5, 18]. Fusion of the paramesonephric ducts is almost complete in a simplex uterus, typical of humans, resulting in a single uterine body. At the other end of this spectrum is the duplex uterus found commonly in rodents. In the duplex uterus paramesonephric fusion can be entirely incomplete, leaving the uterine horns as separate tubular compartments which can extend through the cervical canals and even, in the case of the opossum, resulting in separate vaginal canals [5, 18]. The porcine uterus is of the intermediate, long bicornuate type [16]. Histologically, all mammalian uteri are composed of an outer perimetrial layer of connective tissue cells, outer longitudinal and inner circular smooth muscle layers that constitute the myometrium, and a secretory mucosa called the endometrium. Basic morphology and histology of the uterus are discussed in greater detail below.

1.1 BASIC MORPHOLOGY

The bicornuate porcine uterus forms when the hollow interiors of the paramesonephric ducts merge forming a common uterine lumen. This partial fusion results in a short uterine body connected to two long tubular uterine horns or cornua [4, 16]. Each adult uterine horn is 150-200 cm in length [19]. Growth of the porcine uterus during the first 60 days of life proceeds in an ovary-independent manner [1]. Uterine horn lengths and weights for ovariectomized gilts were comparable to those observed for intact gilts from birth to day 60 [4, 20]. Ovary-independent uterine growth and

development was also observed for the ewe [21, 22], mouse [23, 24] and rat [25]. Moreover, ovarian support was not required for histological development of the porcine endometrial wall between birth and postnatal day (PND) 120 [1].

1.2 UTERINE HISTOLOGY AND HISTOGENESIS

In the pig, histological development of the uterus begins prenatally but is completed postnatally [3]. Significant structural development of the porcine uterine wall takes place during the first eight weeks of neonatal life [4]. Both the myometrial and endometrial layers of the uterus are derived from undifferentiated mesenchyme of the paramesonephric ducts and are established during radial patterning of the FRT. The myometrium consists of both inner circular and outer longitudinal smooth muscle layers that develop from the intermediate layer of ductal mesenchyme and the sub-perimetrial mesenchyme respectively [4]. Basic uterine histology is illustrated for the PND 14 neonatal porcine uterus in Figure 1.

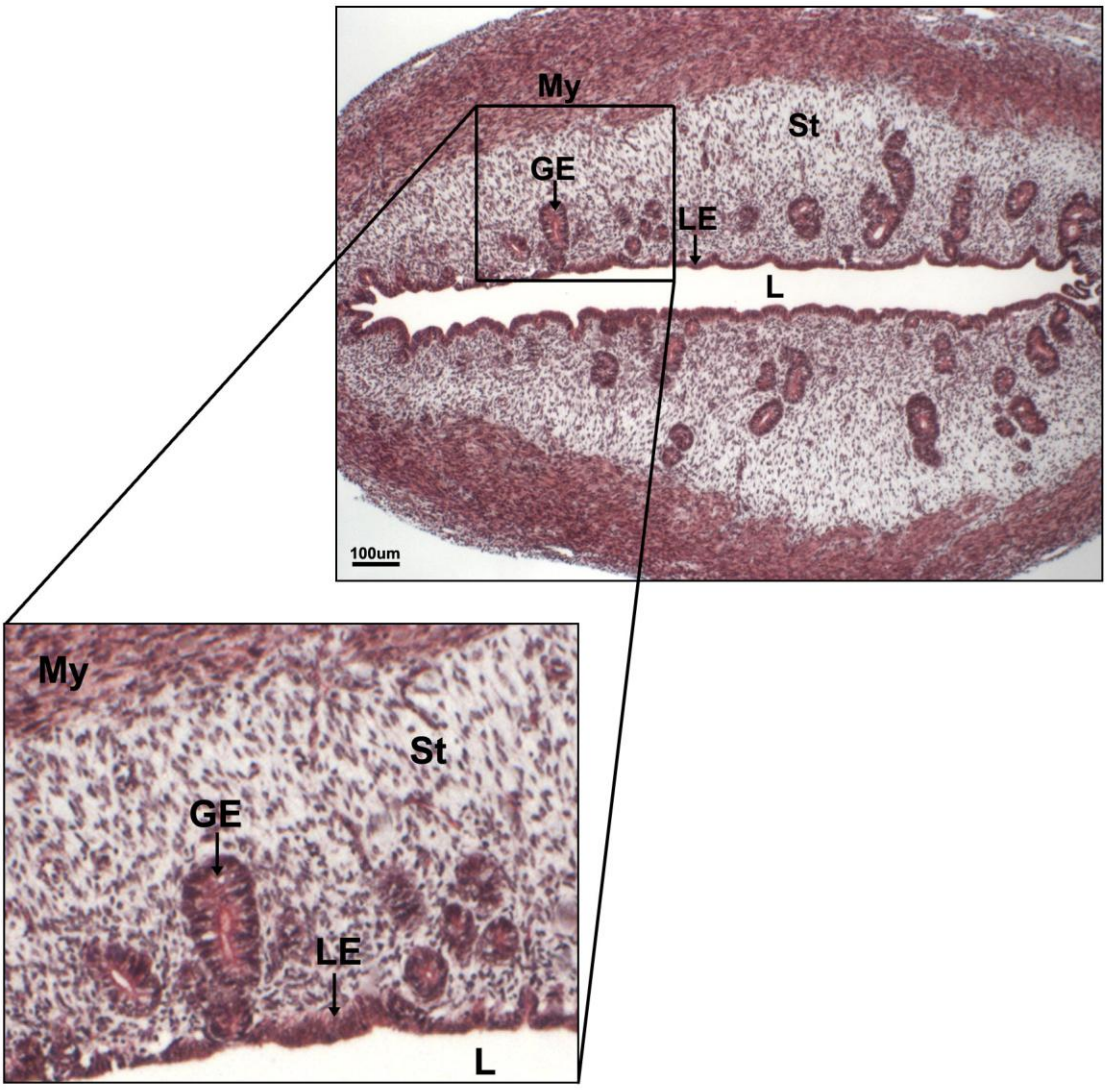


Figure 1. Histology of the porcine uterus in cross-section typical of PND 14 (top). Inset (bottom) provides detail of the endometrium and the endometrial-myometrial interface. LE: luminal epithelium, GE: glandular epithelium, St: stroma, My: myometrium, L: lumen. Trichrome stain.

Histological elements of the uterine wall not identified in Figure 1 (above) include blood vessels, nerves, lymphatic ducts and immune cells.

In the porcine endometrium, mesenchymal cells become stratified and form a dense layer of stromal cells under the epithelium but maintain a loosely organized

network of fibroblasts in the stroma adjacent to the myometrium. During histogenesis of the uterus, GE differentiates from LE and begins to proliferate and form coiled tubular glands that penetrate deep into the endometrial stroma.

2. UTERINE FUNCTION

Primary functions of the adult porcine uterus include regulation of cyclicity, sperm transport and maturation and both recognition and maintenance of pregnancy. Elements of each of these processes are described below.

2.1 REGULATION OF CYCLICITY AND SPERM TRANSPORT

Pigs are non-seasonal breeders and spontaneous ovulators with onset of puberty occurring between six and nine months of age [26]. Once puberty is reached, recurring estrous cycles are observed approximately every 21 days [18, 19, 26]. The four main stages of the estrous cycle include proestrus, estrus, metestrus, and diestrus. Proestrus, lasting two to five days, is characterized by recruitment of ovarian follicles for ovulation as a result of the actions of gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) [18, 27]. In the transition from proestrus to estrus, recruited follicles increase estrogen production. Estrogen, the dominant steroid hormone in estrus, drives a surge of LH secretion that induces ovulation [18]. Under the influence of estrogen, the uterus undergoes several changes, including increased fluid content and increased tone of the myometrium, both important for sperm transport. During estrus, which lasts about 50 hours, the female is receptive to a male and will allow copulation [18]. At the time of mating in the pig, sperm are deposited in the cervix and body of the

uterus and are transported toward the oviductal ampullary-isthmic junction by anti-peristaltic uterine contractions [28, 29]. Capacitation occurs as sperm are transported through the uterus and oviducts to the site of fertilization. This gives sperm the ability to fertilize ova. The first two stages of the estrous cycle are dedicated to follicular recruitment, selection, maturation, ovulation and, ultimately, fertilization of the ova [18, 30]. Together, proestrus and estrus are considered to be the 'follicular phase'.

Metestrus and diestrus, which encompass the luteal phase of the estrous cycle, are characterized by development of the corpora lutea (luteinization) and their regression (luteolysis) if pregnancy is not established [27]. Metestrus is defined as the time between ovulation and formation of functional corpora lutea, which occurs over two to five days [18]. During this stage of the estrous cycle ovarian estrogen production declines rapidly and progesterone production begins to increase. The longest stage of the estrous cycle, lasting ten to fourteen days, is diestrus. During diestrus progesterone is the predominant steroid hormone secreted by functional corpora lutea [18, 27]. This hormone plays a role in creating a receptive endometrium. Uterine epithelium undergoes structural and functional changes required to prepare the endometrium to interact with and support developing conceptuses effectively in direct response to ovarian steroids [31]. Endometrial response to progesterone insures the increase in endometrial glandular secretions found in the uterine lumen during early pregnancy [31, 32].

If pregnancy is not established, the uterine endometrium secretes prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) into the vasculature where it targets corpora lutea. Without endometrial secretion of $PGF_{2\alpha}$, corpora lutea can remain functional for months [18]. The presence of $PGF_{2\alpha}$ causes the corpora lutea to degenerate and progesterone secretion to diminish

through the process of luteolysis [33]. When luteolysis is complete, the estrous cycle starts once again, giving females the opportunity to become pregnant. In the pig, the estrous cycle continues until pregnancy is established or an anestrous condition occurs. Under normal conditions, anestrus in pigs is most often due to pregnancy and lactation [18].

2.2 PREGNANCY

Establishment and maintenance of pregnancy are complex processes involving close interactions between the conceptus(es) and the uterine endometrium. In eutherian mammals, a significant function of the adult uterus includes maintaining and providing a suitable environment for conceptus growth and development during pregnancy [5]. In the pig, before pregnancy is established, four-celled embryos develop from fertilized ova (zygotes) and travel from the ampullary-isthmic junction into the uterine horn on day 3 or 4 of gestation [19]. Porcine embryos do not continue to develop past the blastocyst stage if they remain in the ampullary-isthmic junction [34-36] . Once in the uterine horn, the four-celled embryo attains morula status before developing into an early blastocyst on day five [37]. In the pig, a polytocous animal, blastocysts hatch from their individual zonae pellucidae and, ultimately, become distributed throughout both uterine horns between days 10 and 12 of gestation. Similar to embryos in a cow or ewe, pig embryos develop from a spherical shape to a tubular shape and finally a 100-200 mm filamentous thread, through cellular remodeling, to cover the surface area of the uterine mucosa [38-40]. Pig conceptuses continue to elongate through cellular hyperplasia, reaching a length of 800-1,000 mm by day 15 [41]. This rapid elongation process in the pig allows

conceptuses to interact with the uterine endometrium along most of its length and begin the process of establishing pregnancy.

Maternal recognition of pregnancy (MRP) is defined as those conceptus-induced events that prevent luteolysis and insure a luteal source of progesterone [42, 43]. In the pig, MRP occurs between days 11 and 12 of gestation [44]. As embryos elongate into a filamentous state and spread out along the uterine lumen they secrete estrogen as the primary signal for MRP [8]. For this signal to be effective, embryos must be present in both uterine horns [45, 46]. Conceptus-secreted estrogen affects the receptive endometrium. First, there is an estrogen-induced shift from an endocrine release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) to that of an exocrine secretion. Normally, $PGF_{2\alpha}$ is secreted from the myometrial side of the endometrium into the uterine vasculature (endocrine) of cyclic gilts, but $PGF_{2\alpha}$ is secreted into the uterine lumen (exocrine) between days 10 and 12 of gestation. Because $PGF_{2\alpha}$ is secreted into and contained within the uterine lumen instead of into the capillaries of the uterus, luteolysis does not occur. This estrogen-induced shift is also associated with a transient increase in the intraluminal calcium concentration [44]. Estrogen also induces upregulation of endometrial prolactin receptors that stimulate calcium cycling across the uterine epithelium [44]. Calcium is then taken up by the endometrium or conceptus within twelve hours after uterine intraluminal calcium concentrations reach maximal values [44]. Second, conceptus-secreted estrogen causes secretory vesicles (present in the glandular epithelium from days 10.5-12 of gestation) to release biochemical factors into the uterine lumen. These secretions are thought to be necessary for conceptus survival.

Porcine corpora lutea are highly vascularized ovarian structures that secrete progesterone throughout gestation as required to maintain pregnancy. Luteal progesterone acts on the porcine endometrium by binding to and activating the progesterone receptor (PR) in endometrial LE, GE and stroma, as well as in the myometrium. However, by day 10 of gestation, progesterone downregulates its own receptor in LE and GE [47]. After day 11 or 12 of the estrous cycle or pregnancy, PR expression is undetectable in LE and detectable only in deep GE [48]. Since progesterone inhibits cell cycle progression, and accordingly, cellular proliferation and differentiation [49, 50], downregulation of PR is a highly conserved predecessor of GE differentiation and function during pregnancy. PR downregulation in the uterine epithelium, is critical to opening a window for implantation in the pig [48], mouse [51], sheep [52] and human [53].

The molecular mechanisms associated with negative autoregulation of the PR in uterine epithelium are unknown. Foremost among competing hypotheses, is a model reviewed by Geisert et al [54], that correlates expression of nuclear factor kappa B (NF-κB) with PR downregulation. Interestingly, progesterone has also been implicated in negatively regulating the expression of NF-κB. Progesterone binds PR, and inhibits NF-κB activation by one of the following mechanisms: (1) blocking NF-κB from binding to its DNA response element on progesterone receptor DNA; (2) inhibiting production of factors that activate NF-κB; or (3) increasing the expression of factors that inhibit activation of NF-κB [55]. Interestingly, NF-κB is also implicated in downregulating PR expression through activation of receptor activator of nuclear factor κB ligand (RANKL) and its cognate receptor RANK. Induced by both progesterone and prolactin, RANKL,

plays a role in mammary gland development, and progesterone induction of RANKL may require estrogen priming [56]. Coincidentally, RANK is expressed in the endometrium during early pregnancy in the pig [47]. After PR expression is downregulated and NF-κB is activated in the uterine epithelium, factors such as keratinocyte growth factor (KGF) and cyclooxygenase-2 (COX-2), which are necessary for implantation, growth, and development of the conceptus, are expressed in the uterine epithelium.

Although PR expression is downregulated in the pregnant uterus, progesterone is a requirement for maintenance of pregnancy and uterine secretory activity during normal conceptus growth and development. Unlike the epithelial components of the uterus, PR expression is maintained in uterine stroma and myometrium during pregnancy. Activated stromal PRs induce paracrine regulation *via*endometrial glands that penetrate the uterine stroma. The glands secrete biochemical factors into the uterine lumen. These factors, first termed "histotroph" by Grosser [34, 57], serve as important functional elements of an embryotrophic intrauterine environment required for developing conceptuses. Conceptus-endometrial interactions thought to be important for establishment and maintenance of pregnancy, and potential roles for uterine histotroph during the peri-attachment period have been extensively reviewed [46, 58].

Porcine progesterone-induced uterine factors include uteroferrin, retinol binding protein (RBP) and lysozyme. Although induced by progesterone, uteroferrin is secreted by glandular epithelium devoid of PR. Uteroferrin is a purple acid phosphatase glycoprotein which binds iron so that it can be transported into the fetal circulation [59, 60]. Retinol binding protein is also induced by progesterone, yet secreted by the PR-negative uterine epithelium. Retinol binding protein transports vitamin A in the form of

retinol [46], needed by the conceptus for normal organ development, cell division and placental growth [46, 61]. Progesterone induced secretion of lysozyme arises from an unknown cellular source. Lysozyme is a minor component of porcine uterine secretions thought to play an anti-bacterial role in pregnancy [58].

As the uterus transitions to a receptive state, the porcine conceptus interacts with the uterine endometrium through apposition and attachment to form a non-invasive epitheliochorial placenta. Alterations in the quality of uterine histotroph may affect uterine capacity for conceptus support and contribute to early embryonic death. These concepts are reviewed below.

2.2.1 UTERINE CAPACITY

In the pig, establishment of pregnancy does not guarantee that all embryos will survive to term. Many embryos are lost before the 25th day of pregnancy [62, 63], but the cause of such early embryonic losses remains uncertain. One possible cause of early embryonic death is lack of uterine capacity, which is the number of conceptuses that the uterus can support at a defined stage of gestation [64]. Many factors are hypothesized to play a role in defining porcine uterine capacity including uterine space, stage of gestation, and related biochemical and developmental factors [64].

Researchers thought that uterine crowding played a role in early embryonic death during the first 25 days of gestation in pigs. Methods employed to test this hypothesis in pigs included superovulation, superinduction [65] and ligation of oviducts [66], as well as using the unilaterally ovariohysterectomized (UHOX) model [67]. Superovulation involves treating animals with gonadotropins to stimulate the maturation and release of

abnormally high numbers of oocytes [18]. Superinduction refers to the transfer of additional fertilized ova to animals that have been mated and their estrous cycle synchronized with the donor animal [65]. Establishing the UHOX model involves surgical removal of one uterine horn and the ipsilateral ovary. The compensatory behavior of the remaining ovary produces about the same number of ovulations as that of both ovaries in an intact gilt [67].

Using the method of superinduction, Bazer et al. [64] showed that increasing the total number of potential embryos to 16, 22 or 28 in gilts did not increase litter size by day 90 of gestation. Fenton and coworkers also used superinduction to determine the stage of gestation at which uterine capacity limits porcine litter size [68]. Three trials involving superinduction of 2.5 day and 7 day embryos revealed two stages limiting embryo survival during the first 25 days of gestation. The first, between day 7 and 25 of gestation and the second near day 25, when a smaller percentage of embryos was lost. Fenton et al. [67] ruled out uterine crowding as a reason using the UHOX model. When pregnant UHOX gilts were compared to intact controls at slaughter on day 25 of gestation, there was no significant difference in the number of viable embryos. However, when gilts were slaughtered at 105 days of gestation, *fewer* fetuses were found in the uterine horn of the UHOX gilts when compared with intact controls. Data were interpreted to indicate that uterine crowding does not play a significant role in embryonic loss during the first 25 days of gestation, but it does affect embryo loss between days 25 and 105 of gestation [67]. Pope et al. [65] supported this finding by transferring 12 or 24 fertilized ova into the oviducts of non-mated gilts and slaughtering animals on day 25 of

gestation. Litter size was increased in animals that received 24 fertilized ova, indicating that uterine crowding does not limit litter size before day 25 of gestation [65].

Even though uterine crowding was not the cause of early embryonic death, some researchers believed that limited uterine space did have an effect on litter size after day 30 of gestation [64, 66, 67]. Webel et al. [66] tested this hypothesis using three different pig models. The first group of animals was considered "roomy" and had one oviduct ligated before ovulation. The second group of animals was considered "normal". In the "normal" group, some animals were subjected to sham surgery and some animals were superovulated but one oviduct was ligated prior to superinduction. The "normal" group served as a control. The third group was considered "crowded" and consisted of UHOX animals. Animals from all three groups were slaughtered at three different time points: (1) 25-30 days, (2) 31-40 days and (3) 41-112 days. It was determined that increasing uterine space does not affect litter size when compared with control animals at all stages of gestation, but when there is only half the normal amount of uterine space, litter size is reduced after day 30 of gestation. Thus, while embryonic losses are significant before day 30 of gestation in swine, such losses are not due to limited uterine space. It is only after day 30, when early embryonic losses have already occurred, that uterine space may have an effect on litter size [66]. Interestingly, this is not the case for feral Ossabaw swine. Hagen et al. [69] determined that litter size in feral Ossabaw swine is reduced in the UHOX animal model by day 45 of gestation instead of day 30. It appears that the uterine horn compensates for UHOX treatment at day 30 by increasing in length, but uterine horn length does not change after day 30. Chen et al. [70] determined that fetuses in the pig need at least 25 cm of initial uterine length to survive and develop fully. Early

embryonic loss was still observed when initial uterine space was greater than 25 cm by day 17 of gestation, but litter size was reduced between 29 and 35 days of gestation when initial uterine space was limited to 25 cm or less [70]. If intrauterine space does not play a role in defining uterine capacity until after day 30 of gestation and the majority of early embryonic losses occur before day 25 of gestation, other factors must be responsible. These factors have yet to be defined.

Biochemical limitations associated with the intrauterine environment during early pregnancy may contribute to early embryonic death in the pig [64, 71, 72]. Under the influence of progesterone, there is an increase in uterine luminal fluid protein content between days 12 and 15 of the estrous cycle, whereas there is a dramatic decrease in the protein content of uterine flushings after day 15 of the estrous cycle. The latter time period corresponds with luteal regression [72]. Rampacek et al. [73] evaluated the plasma progestin levels of superinducted gilts on days 13 and 20 of gestation. Animals were slaughtered and litter size was determined on day 25 of gestation. A significant positive correlation was found between progestin levels on day 13 of gestation and litter size on day 25 of gestation which supports the hypothesis that embryos may compete for a limited biochemical factor or protein produced during a short time period early in gestation [71]. Murray et al. [72] concluded that a consistent set of proteins was produced between days 12 and 15 of the estrous cycle—a time period influenced by progesterone. Rampacek et al. [73] theorized that one or more of the proteins evaluated by Murray et al. [72] between days 12 and 15 of the estrous cycle may be the limiting factor needed for normal growth and development that embryos compete for during the

early stage of gestation. These proteins have been analyzed using two dimensional electrophoresis (2DE) [74, 75] as reviewed by Roberts et al. [58].

Another factor likely to affect uterine capacity is the presence of the conceptus and its effect on uterine protein secretion. Knight et al. [76] showed that low concentrations of estradiol, the signal for MRP, can work with progesterone to increase endometrial protein secretion [76]. Basha et al. [75] determined that the presence of conceptuses did not change the endometrial protein secretion of pregnant gilts qualitatively or quantitatively on day 60 of pregnancy. To determine if increased endometrial protein secretion affected uterine capacity after day 30 of gestation when uterine crowding plays a role in early embryonic death, Vallet et al. [77] inserted estrone implants into UHOX gilts on day 30 of gestation. Estrone implants were placed subcutaneously in the neck and released 5 mg of estrone a day. Animals were then slaughtered on day 45 of pregnancy and litter size was evaluated. Estrone treatment between days 30 and 45 of gestation did not affect the uterine capacity of UHOX gilts [77].

Vallet and coworkers also tried to affect uterine capacity positively by altering endometrial gland development in the neonatal porcine uterus [78], which, in turn, would theoretically increase protein secretion in the pregnant gilt. Starting at birth, animals received a weekly injection of retinyl palmitate for twelve weeks because it was shown that retinyl palmitate increases uterine gland development when administered during the first 14 days of life [79]. During this treatment period, animals were unilaterally hysterectomized and ovariectomized on days 28, 84, and 112 days of age or were left intact as controls before being mated at their second or third observed estrous cycle.

Animals were slaughtered between days 44 and 47 of gestation and litter size was determined. After treatment with retinyl palmitate during the first twelve weeks of life, uterine horn length increased in adult UHOX gilts, but litter size was not affected by day 45 of gestation [78]. Data were interpreted to indicate that an increase in uterine protein secretion during early pregnancy does not positively affect uterine capacity in the pig.

The ability of the uterus to support a limited number of conceptuses to term can be affected by nutrients ingested by the pregnant gilt. Nutrient intake affects development of the placenta which, in turn, alters the development of the embryo. The effect of maternal nutrition on fetal and placental development in sheep has been reviewed by Redmer et al. [80]. Many studies [81-85] have been conducted to understand how maternal nutrition affects fetal growth and development as well as uterine capacity in the pig. Both underfeeding [83, 84] and overfeeding [81, 82] have an effect on fetal growth and development. A reduction in fetal weight and survival of female embryos at day 30 of gestation was observed when feed intake was restricted in lactating primiparous sows before mating [85]. Also, restriction of feed intake after day 80 of gestation impaired fetal growth [83]. To compensate for the effects on fetal growth in underfed pregnant gilts, researchers increased the amount of feed given to pregnant pigs. Similar to results for underfeeding pregnant gilts, fetal weight decreased and embryo and fetal mortality increased in response to overfeeding gilts during pregnancy [81, 82, 86, 87]. Recently, maternal nutrient intake was used to affect uterine capacity positively. Mateo et al [88] fed pregnant gilts diets supplemented with L-arginine (treatment group) and L-alanine (control group) starting at day 30 of gestation. Animals supplemented with L-arginine had 23% more piglets born alive when compared with the

control group. Thus, nutrients and nutrient intake can affect conceptus development and uterine capacity.

3. DEVELOPMENTAL BIOLOGY OF THE UTERUS

Histogenesis and functional programming of the porcine uterus during the first 60 days of post-/neonatal life is an ovary- and possibly steroid hormone-independent process [4, 32]. At birth, estrogen receptor-α (ER) is not detectable in the porcine uterus [1]. However, ER expression is detectable by PND 2 [89] and increases thereafter to PND 15 in uterine stroma and GE where ER expression is associated with both the differentiation and proliferation of nascent endometrial glands [1]. Although uterine growth and development do not require ovarian support, the uterus is steroid and peptide hormone sensitive. It was hypothesized that steroid hormone-induced disruption of porcine uterine development during the first two weeks of life would have lasting consequences for adult uterine function [4, 13, 90]. Administration of estrogen to neonatal pigs between PND 0 and PND 13 disrupted organizational events characteristic of uterine development during this period and altered functions of the adult uterus including the capacity for conceptus support and survival. Therefore, it was determined that the first two weeks of neonatal life constitute a critical period for uterine endometrial development in the pig [4, 14].

In domestic ungulates including the pig, development of the uterine wall during the early neonatal period is marked by the appearance and proliferation of endometrial glands (uterine adenogenesis) [13, 32]. This process, reviewed below, is essential for establishment of a properly functional uterine mucosa, involves stromal-epithelial

interactions that are supported by organizationally critical, hormone-sensitive, uterine morphoregulatory gene expression patterns.

3.1 GLAND GENESIS

Endometrial glands are a necessary part of a functional uterus. Their presence and functionality can define uterine capacity [32]. During pregnancy, uterine glands are responsible for the production of histotroph consisting of proteins and other substances required to nourish developing conceptuses, as reviewed above (section 2.2) [32].

In the pig, as well as in sheep [21, 22], mice [23, 24] and rats [25], uterine glands are absent at birth and develop rapidly during early postnatal life [13, 32]. At birth, small indentations present along the corrugated, simple columnar LE are assumed to be the anlagen of coiled, tubular glands that will ultimately be part of the mature endometrium. Nascent glands are evident by PND 3 and have a simple tubular shape extending into the shallow stroma by PND 6 [90, 91]. Simple, tubular glands become coiled and penetrate deeper into the stroma in relation to the myometrium by PND 14. By PND 28, simple, tubular, coiled glands as well as a few branched glands are evident throughout the stroma and include some that extend to the myometrium [90]. By day 56, complex, branched glands are present throughout the stroma and endometrial folds are apparent in the uterine wall [90]. Gland genesis is complete and the uterus achieves histological and functional maturity by PND 120 [92].

During the first few weeks of postnatal life, patterns of epithelial cell proliferation, especially GE, undergo dramatic changes [90, 91]. Several methods have been employed to identify cells in the “S” phase of the cell cycle, indicating that cells are

synthesizing DNA and, ultimately, proliferating. Some of these methods include metabolically labeling cells with [methyl-^{3}H] thymidine (^{3}H-Thd) [90] or 5-bromo-2'-deoxyuridine (BrdU) [13]. Following metabolic labeling, cells actively synthesizing DNA are identified by autoradiography (^{3}H-Thd) or immunohistochemistry (BrdU). Another method for identifying cells that are actively proliferating involves basic immunohistochemistry using commercially available antibodies for proliferating cell nuclear antigen (PCNA) and image analysis. This technique can be used with tissues that are appropriately preserved and archived without the *a priori* need for metabolic labeling [91, 93].

Spencer and coworkers used ^{3}H-Thd to study cell proliferation in the neonatal porcine uterus [90]. All neonatal uterine tissue incorporated ^{3}H-Thd in vitro as evidenced by autoradiography. Labeling index (LI) for LE was greatest on PND 0, but LI for LE was consistently lower than that observed for GE on PND 7 and 14. Labeling index for GE was maximal on PND 7 and 14 indicating that a large proportion of GE cells were actively synthesizing DNA and proliferating. Tarleton et al [13] used BrdU to label actively proliferating cells in the neonatal porcine uterus. Results were consistent with data obtained using ^{3}H-Thd. The BrdU LI for LE was less than that for GE, and LI indicating proliferation was highest for GE on PND7 and PND 14. Results obtained using PCNA immunostaining in porcine uterine tissues obtained between birth and PND 15 were consistent with observations involving both ^{3}H-Thd and BrdU labeling [13, 90, 94]. At birth, when GE is absent, both LE and stroma exhibited positive PCNA labeling indices indicative of cell proliferation [90, 91]. After PND 0, with the appearance of GE, PCNA LI was consistently higher in GE than in LE [13, 90, 91]. Epithelial PCNA

labeling indices were high on PND 3, remained high at PND 6, and declined sharply by PND 9 through PND 12 in both LE and GE [91]. Positive epithelial PCNA LI at PND 3 indicates that the developing porcine uterus transitions from an 'infantile' state to a more active 'proliferative' state earlier than reported initially by Spencer et al. [90]. The fact that PCNA LI on PND 15, increased for GE but not for LE suggested development of independent mechanisms regulating proliferation in these cell populations [91]. Development of glands in the neonatal porcine uterus requires that LE cells differentiate and that nascent GE proliferate.

Not only are endometrial glands absent at birth, but ER is undetectable in all uterine cell types at birth [95]. Using Western blot analysis, ER protein was detected in neonatal porcine uterine tissues on PND 2 [2]. By PND 7, ER mRNA expression was observed by *in situ* hybridization in both nascent glands and endometrial stroma but was absent in LE even at PND 14 [13]. Regular, weak nuclear staining observed for ER protein by PND 30 increased in intensity to moderate levels by PND 120 [1]. Temporo-spatial patterns of ER expression in the porcine uterus between birth and PND 15 indicate that ER is a marker of GE differentiation [1, 13].

To test the hypothesis that ER activation is required for normal gland genesis, Tarleton et al. [13] administered ICI 182,780 (ICI), a steroidal anti-estrogen and inhibitor of ER function, during two different time periods in neonatal porcine uterine development. This pharmacological approach was taken because gene knockout (i.e. loss-of-function) technologies did not exist for the pig at the time and remain impractical in most instances. Gilts that received ICI during the infantile period (PND 0-PND 6) had fewer well developed glands than did gilts treated with corn oil (CO). The most

pronounced negative effect on uterine gland development was observed on PND 14 when ICI was administered from birth through PND 13. This effect was less pronounced when animals were treated with ICI only during the proliferative phase (PND 7- PND 13) [13]. Evidence of early ER expression in nascent GE and stroma, taken together with data showing that gland genesis could be inhibited with ICI, indicated that differentiation and development of porcine uterine glands are marked by ER expression in GE and mediated by ER activation.

3.2 ENDOCRINOLOGICAL CONSIDERATIONS OF UTERINE DEVELOPMENT

Regulation of growth, differentiation and function of the uterus are just a few events affected by hormone action in the uterus. A hormone is defined generally as a substance produced by one or more ductless or endocrine glands that is secreted into and transported by the blood to exert a specific effect upon a target organ, which bears receptors for the hormone [18]. Both steroid and protein/peptide hormones and their cognate receptors and related signal transduction systems can contribute to events affecting uterine development and function. The impact of two hormones - estrogen, a steroid hormone, and relaxin, a peptide hormone - and their related signaling systems will be reviewed here, with emphasis placed on their effects on uterine growth and development.

Estrogen receptor-α was once thought to be the only type of estrogen receptor responsible for mediating the hormonal effects of estrogen. However, a second estrogen receptor, ERβ, encoded by the ESR2 gene, was identified in the rat [96], mouse [97], and

human [98]. The predominant estrogen receptor in the uterus is now known to be ERα (ER), encoded by the ESR1 gene. A better understanding of the functions of estrogen in the FRT was obtained when three mouse models were created to knockout expression of ER (αERKO) [99], ERβ (βERKO) [100], and both ER and ERβ (αβERKO) [101, 102]. It was established that estrogen is required for normal uterine growth, evidenced by the hypoplastic uterus of the αERKO and αβERKO mice [102, 103]. Because the uterine compartments—epithelium, stroma and myometrium—are hypoplastic, uteri in these loss-of-function animal models is half the weight of those in control mice. Both αERKO and αβERKO uteri are unresponsive to estrogen. Uterine wet weight and estrogen-responsive gene expression did not increase after estrogen administration in either the αERKO or αβERKO uterus. Both αERKO and αβERKO mice are infertile. In contrast to these observations for the αERKO and αβERKO mice, βERKO mice have a normal uterus that responds to estrogen and can support pregnancy [103]. Therefore, ER is required for correct uterine development and function.

Until recently, steroid actions were thought to be mediated through a classic and unique genomic signal transduction pathway that has been studied extensively [104-109]. Genomic actions of steroid hormones are characterized by the comparatively long period of time (hours or even days) required for induction of the full, complement of hormone-specific biological effects, as well as sensitivity of signaling pathways to inhibitors of transcription and translation, particularly actinomycyin D and cyclohexamide [110, 111]. Generally, the classic steroid hormone signaling pathway involves binding of the steroid hormone to an intracellular receptor and subsequent interactions between the hormone-receptor complex with relevant nuclear steroid response elements upstream of target

genes that affect patterns of gene expression and protein synthesis. For example, estrogen, a lipophilic molecule, can easily pass through the lipid bilayer of target cell membranes by simple diffusion, although there is some speculation about active transport of steroid hormones [112]. In most cases, estrogen binds to plasma proteins for transport to target tissues, a phenomenon that can also increase steroid half life in circulation. Once the estrogen and plasma protein complex comes into contact with the target cell, the plasma proteins and estrogen dissociate allowing the hormone to penetrate the lipid bilayer of the cell where, upon estrogen receptor binding, the receptor undergoes a conformational change that causes the receptor to condense into a smaller molecule with a higher affinity for DNA [18].

In addition to the now classic genomic mechanism of action determined for steroid hormones [105, 113], more recent data indicate that steroids can act through nongenomic mechanisms [110, 111, 114]. Nongenomic actions of steroids are characterized by rapid effects—occurring within seconds to minutes—and are comparatively insensitive to inhibitors of transcription and protein synthesis, although they can respond to steroid receptor antagonists. Unlike classical genomic actions of steroid hormones that regulate the rate of target gene expression directly, nongenomic actions of steroid hormones involve second messenger cascades that mediate steroid-induced actions on cellular function indirectly. Defining nongenomic mechanisms of steroid hormone action, particularly with respect to the type of receptor involved, has become a controversial topic in the field of endocrinology [108, 115-117]. A major question is whether classic steroid receptors or receptors completely unrelated to the

nuclear steroid receptor superfamily are involved in mediation of nongenomic steroid effects in target cells.

Most steroid hormones, including estrogen, are known to exhibit nongenomic actions in target cells or tissues. For example, a large and rapid calcium influx was observed in cultured rat endometrial cells after treatment with 17β-estradiol [118]. It is thought that nongenomic actions of estrogen are mediated through a membrane bound receptor that is closely related to the nuclear ERα. Membrane estrogen receptors have been found using antibodies against several ERα–specific epitopes [119]. Available data indicate that membrane-associated ERs are localized within caveolar rafts as well as other parts of the plasma membrane of target cells [120]. For example, in human sperm, a rapid increase in calcium concentration due to treatment with 17β-estradiol was also seen following treatment of cells with a BSA-17β-estradiol conjugate. The conjugate cannot pass through the plasma membrane and induce genomic effects. Thus, data were interpreted to indicate that a membrane receptor was responsible for the influx of calcium [121]. Recently, evidence was presented identifying a membrane estrogen receptor using SKBR3 breast cancer cells [122]. Detected in several tissues including the heart, ovary, and prostate, the orphan G-protein coupled receptor (GPCR) -like protein, GPR30, had a similar structural sequence to receptors for chemokines and other molecules, suggesting its role as a peptide receptor [122, 123]. Interestingly, SKBR3 cells do not express nuclear ER or ERβ, but do specifically bind estrogen to the plasma membrane [122] suggesting that GPR30 is involved in estrogen signaling as a membrane estrogen receptor.

A proposed mechanism of nongenomic estrogen action involves the membrane-bound estrogen receptor associating with calveolin-1, a scaffolding protein, as well as with G-proteins and other signaling molecules [107]. Once the G-protein has been activated by the active membrane-associated estrogen receptor, phospholipase C, protein kinase C, nitric oxide synthase, phosphatidylinositol 3-kinase and extracellular regulating kinase could be stimulated to activate several signal transduction cascades [107]. For example, in endothelial cells the membrane ER associates with the $G_{\alpha i}$ protein to activate endothelial nitric oxide-synthase. Similar to nuclear ERs, stimulation of nitric oxide synthase was inhibited completely with the ER antagonists ICI and tamoxifen [107]. The extent to which effects elicited by estrogen in the FRT involve membrane-associated ER signaling remains to be determined.

Throughout the course of studying estrogen receptor structure and function as well as the role of estrogen receptor signaling in genomic and nongenomic steroid actions, it has been observed that ligand-activated receptors such as the epidermal growth factor receptor (EGFR) can activate ER and its target genes in an estrogen-independent manner [124]. This is referred to as crosstalk. Crosstalk has also been described for other steroid hormones and their receptor systems [107, 125, 126]. Often the action of crosstalk has an inhibitory effect on cell function, but the effect can be stimulatory as well. For example, crosstalk has been seen between epidermal growth factor and ER in a breast cancer cell line [124]. Serine and tyrosine residues were phosphorylated on ER after treatment with epidermal growth factor. This led to a direct interaction of ER and the EGFR. The formation of this complex stimulated cell proliferation and prevented apoptosis independently of the estrogen response element, which is typically required to

induce transcriptional activity via ER [124]. This has also been shown in αERKO mice with regard to uterine growth [127]. Mice expressing a functional ER displayed increased uterine DNA synthesis and expression of progesterone receptor mRNA when treated with epidermal growth factor. In contrast, αERKO mice did not display any of these effects. Results supported the hypothesis of crosstalk between epidermal growth factor and estrogen receptor systems in the murine uterus [127]. With evidence of crosstalk between estrogen receptor and peptide growth factor signaling systems in the uterus, it is apparent that some developmental events involving estrogen receptor activation may not require estrogen, since other factors can activate the estrogen receptor system.

In contrast to estrogen, relaxin is a peptide hormone that acts through a G-protein coupled receptor (GPCR) first identified as leucine-rich G-protein coupled receptor-7 (LGR7) and now called RXFP1 [128]. Once RXFP1 is activated by relaxin, the adenylate cyclase and protein kinase A-dependent pathway is activated in target cells [129-134]. Activation of this signal transduction pathway, allows relaxin to act on its target cells within the body including both cardiovascular and central nervous systems [135-138].

Although relaxin has been shown to have several functions within the body, it has, until recently, been mainly considered as a hormone of pregnancy. Secreted primarily from the corpus luteum during pregnancy in women, baboons, monkeys, rats, and pigs, relaxin bioactivity was first shown, during the 1920s, to facilitate the process of parturition by widening the birth canal [139, 140]. Since that time relaxin has been

detected in neonatal pigs [141] non-pregnant humans [140] and to be secreted by cultured granulosa cells from preovulatory porcine follicles [142].

In some species, relaxin may be another key player in uterine programming and development. Recently it was reported that RXFP1 is present in the neonatal porcine uterus at birth [141]. Also, relaxin has been identified in the colostrum and milk of dogs [143], humans [144], and pigs [141]. The highest concentrations of relaxin in milk are observed within the first 24-36 hours after parturition in sows, prior to gut closure in nursing piglets. Relaxin is detectable by radioimmunoassay in the peripheral plasma of newborn pigs only if they are allowed to nurse and cannot be detected in neonates fed relaxin-free milk replacer [141]. Thus, it appears that relaxin is transmitted from dam to offspring as a specific consequence of nursing via a lactocrine mechanism [141]. Consequently, maternally derived relaxin is available to affect uterine programming during a critical period of estrogen-sensitive development shortly after birth in the pig.

Both relaxin and estrogen are uterotrophic. In addition to stimulating uterine growth, effects of both hormones include stimulation of uterine insulin-like growth factor-I production [145], gap junction development [146], vascular endothelial growth factor expression [147] and uterine vascularization [148]. Because these two hormones enhance many of the same effects, it has been hypothesized that ER and RXFP1 use the mechanism of crosstalk to induce uterotrophic effects. The potent estrogen receptor antagonist, ICI has been used in a number of studies to resolve the question of whether crosstalk between these two receptor systems is a mechanistic possibility.

Pillai et al. [149] examined the effect of uterine edema induced by relaxin treatment in ovariectomized Sprague-Dawley rats. Treatment with both relaxin and

estrogen increased uterine wet weight within six hours. Pretreatment with ICI completely inhibited the uterotrophic effects of both relaxin and estrogen. From these data it was hypothesized that once relaxin activated its receptor, a signaling cascade would activate the nuclear ER to turn on target genes that regulate uterine edema [149]. A similar situation was documented in the neonatal porcine uterus [94]. Relaxin administered to neonatal gilts from PND 0- PND 2, beginning before onset of uterine ER expression, increased endometrial LE height, as well as both ER and VEGF expression in uterine tissues obtained on PND 2 [9, 89, 150]. Interestingly, effects of relaxin on induction of uterine VEGF expression were attenuated by pretreatment of neonatal gilts with ICI. Further, relaxin administered to gilts for two days from PND 12 to PND 14, after the onset of ER expression in the neonatal porcine uterus, increased both LE height and uterine weight [9, 150]. These uterotrophic effects were also inhibited by pre-treatment with ICI. Collectively, these observations can be interpreted to suggest that a maternally dependent, lactocrine-driven mechanism functions to deliver relaxin into the neonatal circulation at birth where it can act via the cognate relaxin receptor, RXFP1, in the uterine stroma to induce and/or support ER expression that is essential for normal uterine growth and development. Moreover, data indicate relaxin may act via cross-talk involving interactions between RXFP1 and ER signaling systems to support critical uterine programming events shortly after birth in the pig. [9].

3.3 CELL-CELL/STROMAL-EPITHELIAL INTERACTIONS

Development of uterine structure and function requires interactions between epithelium and mesenchyme or stroma [151]. Stroma plays a large role, with both permissive and directive influences, in determining the fate of adjacent epithelium [152]. Permissive actions of the stroma were observed when epithelium of the urogenital sinus—a prostatic inductor—was combined with stroma from either embryonic urogenital sinus, seminal vesicle, neonatal vagina or embryonic bladder and prostatic morphogenesis still occurred [152-155]. In these examples, the stroma supported differentiation of urogenital sinus epithelium into its normal prostatic phenotype. Conversely, stroma from the Müllerian duct-derived FRT has a more directive influence on adjacent epithelium. When stratified squamous epithelium from the murine vagina is combined with species-homologous uterine stroma, vaginal epithelium differentiates into simple columnar uterine epithelium [156]. Similarly, vaginal stroma combined with uterine epithelium causes the uterine epithelium to develop into stratified squamous vaginal epithelial cells [156].

Events in epithelial differentiation of the FRT are dependent on factors released by the stroma which move to and act upon adjacent epithelium to induce gene expression that is cell-type specific [157]. These stromal factors move in the direction of the inductive effects. For example, there is evidence that vaginal stroma directly beneath the vaginal epithelium is actively exocytosing material at the same time vaginal epithelium is actively endocytosing molecules from the intercellular space [152]. These cell determining factors passed from the stroma to the epithelium appear to be minimally diffusible. The directive characteristic of the stroma towards the epithelium allows the

developing FRT to differentiate as required for the formation of individual, structurally and functionally unique organs that comprise the FRT.

Epithelium also plays a role in stromal organization and function. In order for stromal cells to proliferate in response to steroid hormones, uterine epithelial cells must be present. Bigsby et al. [158, 159] combined 10-15 day old epithelium from developmentally determined vagina or uterus with two day old uterine stroma in order to study the effects of estrogen and progesterone on stromal cell proliferation in mice. Tissue recombinants were grown in athymic murine female hosts for five weeks when animals were ovariectomized. Hosts were then treated for one day with either corn oil (control) or estradiol and some were treated with progesterone for two days before following up with a combination of progesterone and estradiol. Twenty hours after the last treatment, mice were injected with [^{3}H]-thymidine and the tissue recombinants were removed an hour later. Uterine stromal proliferation was observed only when it was combined with uterine epithelium and treated with a combination of progesterone and estradiol. Therefore, it appears that the function and organization of uterine stroma depends on the presence of and interaction with uterine epithelium in response to steroid hormones [158, 159].

Just as the instructive effects of uterine stroma on developing epithelium occur during a limited time period, epithelial responses to instructive signals of stromal origin are age dependent. Using heterotypic recombination experiments in mice, Cunha et al. [156] demonstrated that the instructive properties of the uterine stroma are present in newborn mice and continue to have an effect on the epithelium until seven days after birth. In contrast, vaginal stroma continues to have inductive characteristics until 150

days of age. Cytodifferentiation of both uterine and vaginal epithelium is determined by postnatal day 10 [156].

Interactions between the stroma and epithelium mediate hormonal effects on the developing uterus. For example, the uterine epithelium depends upon the adjacent ER-positive stroma to proliferate in response to estradiol. At birth, murine uterine epithelium is devoid of ER. This is in contrast to the stroma, which is ER positive [160-162]. Data for the neonatal mouse showed that even though the uterine LE does not contain estrogen receptors, luminal epithelial cells proliferate in response to treatment with estradiol [160, 163]. It was proposed [163] that this response is mediated through the ER-positive stroma. Using the murine αERKO model and tissue recombination experiments proved to be a useful technique in analyzing the possible hormone effects mediated by the stroma [163]. When epithelium from the αERKO mouse was combined with stroma from a wild-type mouse, the ER-negative epithelium responded mitogenically to estradiol exposure when compared with the wild-type control as evidenced by an increase in ^{3}H-thymidine labeling index [163].

Like steroid hormones, actions of the peptide hormone relaxin may be mediated by the stroma in the pig [141]. The cognate receptor for relaxin, RXFP1, is localized to the uterine stroma and RXFP1 mRNA can be detected as early as birth in the pig by both RT-PCR and *in situ* hybridization [141]. After administration of relaxin for only two days from birth, luminal epithelial height increased when compared with vehicle-treated controls [2]. In a recent study, RXFP1 knockout mice were used to show that relaxin does indeed work through the stroma to affect epithelial cell behaviors [164]. When stroma from RXFP1-null mice was combined with epithelium from wild type mice and

the steroid-primed nude mice carrying these tissue recombinants were treated with relaxin, no effects on proliferation or apoptosis in either stromal or epithelial compartments of the cervical or vaginal tissue recombinants were observed. In contrast, proliferation of stromal and epithelial cells increased and apoptosis decreased in both vaginal and cervical tissue recombinants following relaxin treatment when wild type stroma was used. Stromal mediators of such relaxin-induced effects have yet to be identified.

3.4 MORPHOREGULATORY GENES

From studies involving a murine model and targeted mutagenesis, it is evident that expression of genes encoding the transcription factors *Pax2*, *Lim1* and *Emx2*, as well as *Wnt4* are required for FRT development. Mice not expressing *Pax2* die soon after birth and lack kidneys as well as a FRT [30, 165]. Similarly, loss of function of the *Lim1* gene led to the absence of the FRT [166]. Expression of *Emx2* is required for development of the reproductive tract, gonads and kidneys in mice [30]. *Wnt4* null female mice do not develop a FRT, but do develop a normal male reproductive tract [167]. Other morphoregulatory genes are required for correct patterning of the developing FRT, including the uterus. This list includes *Wnt4*, *Wnt5a*, *Wnt7a*, *Hoxa10*, *Hoxa11*, *Indian Hedgehog* (*IHH*), *Msx1* and *Msx2*. Characteristics of these gene products and the signal transduction pathways through which they are thought to act are reviewed below.

3.4.1 WNT GENES

The vertebrate *Wnt* gene family contains nineteen members that encode secreted glycoproteins. Related to *wingless*, a segment polarity gene in *Drosophila melangoster* (fruit fly), several vertebrate *Wnt* gene products including *Wnt4*, *Wnt5a* and *Wnt7a* are implicated in development as well as patterning of the FRT. Within the developing FRT, *Wnt* gene products are thought to be involved in mediation of cell proliferation, cell-cell communication, specification of cell fate, and regulation of cell death [168-170]. *Wnt* gene expression has been studied extensively in the FRT of the mouse and was recently detected in the uterus of the neonatal pig [3, 171].

The creation of *Wnt* gene mutations in rodents allowed researchers to study and understand the signal transduction pathways of *Wnt* genes. These gene products are thought to act through both canonical and non-canonical signaling pathways [172]. Canonical *Wnt* signaling involving the *frizzled* family of receptors shown in Figure 2A and 2B will be reviewed here.

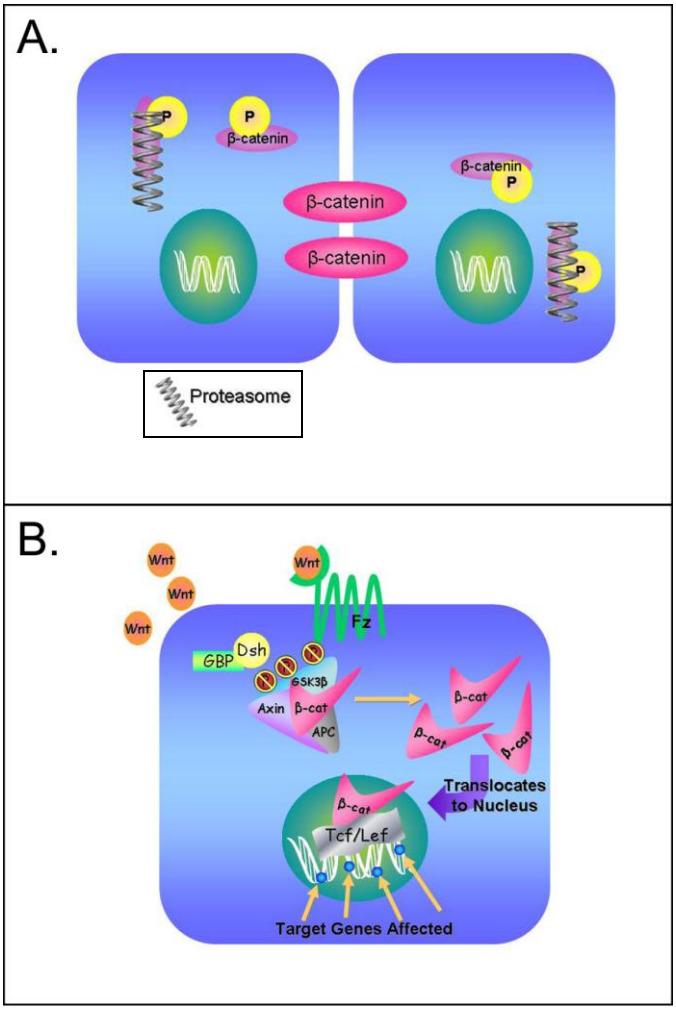


Figure 2. Wnt signaling via the *frizzled*-mediated canonical pathway. (A) In the absence of Wnt, β-catenin that is not incorporated into the junctions at the cell border is phosphorylated and marked for proteasomal degradation. Blue boxes represent cells. (B) When Wnt binds to its receptor (Frizzled = Fz), actions of the destruction complex molecules are inhibited leaving β-catenin unphosphorylated. The destruction complex molecules include GSK-3β, Axin, and APC. Dephosphorylated β-catenin then accumulates in the cytoplasm where it can translocate to the nucleus and effect downstream signaling events. Figure adapted from [173].

Frizzled receptors are seven transmembrane G protein coupled receptors with an extracellular cysteine-rich domain for *Wnt* binding. In the absence of *Wnt* molecules, a complex composed of glycogen synthase kinase 3β (GSK3β), axin, adenomatous polyposis coli (APC) and β-catenin forms. Through serine-threonine kinase activity,

GSK3β phosphorylates β-catenin targeting it for proteasomal degradation by ubiquitination. When *Wnt* molecules bind to frizzled and the co-receptor, low density-lipoprotein-related protein (LRP6), β-catenin escapes phosphorylation and is able to translocate to the nucleus. Once in the nucleus, β-catenin activates the lymphoid enhancer factor (Lef)/ T-cell factor (Tcf) family of transcription factors by forming a complex with them to induce transcription of *Wnt* target genes. Another player in *Wnt* signaling is the secreted frizzled related protein-2 (SFRP2) that acts as an antagonist to *Wnt* gene signaling. SFRP2 inhibits *Wnt* signaling by competing with *Wnt* molecules to bind and form a nonfunctional complex with frizzled. Several of these signal transduction elements have been identified in both the murine and porcine uterus.

Three *Wnt* genes and their products regulate FRT/uterine patterning and development. In the developing mouse endometrium, both *Wnt4* and *Wnt5a* mRNA expression were detected in the stroma at birth. In contrast, *Wnt7a* expression was limited to the uterine LE at birth [174]. Similar to the murine model, *Wnt4*, *Wnt5a* and *Wnt7a* are expressed in the developing endometrium of the porcine uterus [3]. At birth, mRNA was detected for these three *Wnt* genes in the uterine stroma, but signal was more pronounced for *Wnt4* and *Wnt5a*. Signal intensity increased for *Wnt4* and *Wnt5a* in the stroma on PND 14. Although a weak signal for *Wnt7a* mRNA was detected in the stroma at birth, expression increased and was detected clearly in LE by PND 14.

Gene knockout technology was used in mice to gain a better understanding of *Wnt* gene function in the developing murine uterus [175-177]. While both *Wnt4* [167] and *Wnt5a* mutants die at birth, Mericksay and coworkers [178] were able to graft mutant *Wnt5a* FRT tissues into adult hosts. After development in the host, it was observed that

the uterus fails entirely to form endometrial glands in the absence of *Wnt5a* [178]. In *Wnt7a* mutants, cell compartments of the developing uterus are altered. For example, thickness of the stromal compartment is reduced, endometrial glands are absent, the myometrium is unorganized and the epithelium becomes stratified at puberty instead of maintaining a columnar phenotype [179].

3.4.2 HOMEOBOX GENES

Highly conserved homeobox genes are developmental control genes the products of which regulate aspects of morphogenesis and cell differentiation [180]. Homeobox genes encode DNA-binding proteins that have a 60-amino acid homeodomain involved in sequence-specific DNA binding of much larger homeodomain-containing or homeobox proteins. First discovered in *Drospophila melangoster*, homeobox genes encode proteins that are transcriptional regulators that can activate or repress target genes. Vertebrate homeobox genes are divided into two groups—nonclustered and clustered. The nonclustered homeobox genes are scattered throughout the genome and fall into a number of sub-groups based on sequence similarities [180]. Clustered homeobox genes, also known as Hox genes, play an important role in morphogenesis and structural patterning during development of many systems including the FRT. In mammals there are at least 39 homeobox genes arranged in four clusters (A, B, C and D). Each cluster is localized to a different chromosome. On each chromosome the cluster of Hox genes is arranged linearly from 3' to 5' in the order in which they are expressed along the anterior-posterior body axis during development [181]. Vertebrate Hox genes expressed in the FRT are

related to the *Abdominal-B* gene family in *Drosophila* and are referred to as the Hoxa gene cluster [182].

During embryonic development, four of the *Hoxa* genes, including *Hoxa9*, *Hoxa10*, *Hoxa11* and *Hoxa13* are expressed uniformly along the anterior-posterior axis of the murine Müllerian duct. However, with antero-posterior differentiation of FRT tissues, expression patterns for these genes become segment-specific such that *Hoxa9* can be detected in the oviduct, *Hoxa10* and *Hoxa11* in the uterus, and *Hoxa11* and *Hoxa13* in the cervix and anterior vagina [182]. The developing porcine endometrium also expresses both *Hoxa10* and *Hoxa11* [3]. Expression of both of these homeobox genes, which is predominantly if not exclusively stromal, is observed as early as birth and, particulary *Hoxa10*, increases by PND 14.

Although the *Hoxa* gene family is required for segment identity along the FRT, overlapping expression domains may be required for some functional redundancy [183]. In the FRT, redundancy of *Hoxa* gene expression appears to function in preventing complete developmental disruption of a portion of the FRT if one of the *Hoxa* genes is mutated or absent. For example, targeted deletion of *Hoxa10* or *Hoxa11* altered the oviduct-uterine boundary and partially transformed the anterior portion of the murine uterus into a structure that was histologically similar to the oviduct [184, 185]. Thus, redundant uterine expression of *Hoxa10* and *Hoxa11* may cause a partial instead of complete transformation of the uterus into oviduct when either of these genes is deleted or mutated. Redundant *Hoxa* gene expression does not appear to induce partial function of the adult uterus when expression of *Hoxa* genes is altered. Instead, it has been shown that mice displaying decreased expression of *Hoxa10* and *Hoxa11* do not have problems

ovulating or with fertilization, but do have difficulty with implantation and are sterile [186, 187].

3.4.3 HEDGEDHOG GENES

Hedgehog (*Hh*) genes and their products, the Hh proteins, play key roles in the orchestration of many critical developmental processes. First identified as a single invertebrate gene from studies of *Drosophila melangoster*, three *Hh* genes are now known to be expressed in vertebrates. These include *Sonic hedgehog* (*Shh*), *Indian hedgehog* (*Ihh*) and *Desert hedgehog* (*Dhh*). Mutation of the *Hh* gene caused a duplication of denticles on the cuticles along the anterior portion of the *Drosophila* larval body. These spiky growths reminded researchers of spiny processes on a hedgehog's body and thus the gene was given the name *Hh* [188]. Acting locally through the 'Patched' (Ptc) receptor system, Hh proteins can signal target cells to proliferate, differentiate, and may also affect cell stability and survival [189]. Normal development and organization of many tissues, organs and organ systems, including the respiratory, gastrointestinal, nervous, cardiovascular and musculoskeletal systems, as well as skin, eyes and the reproductive tract, depend upon the success of Hh signaling events [189, 190].

The Hh signaling system, illustrated in Figure 3, will be reviewed briefly here.

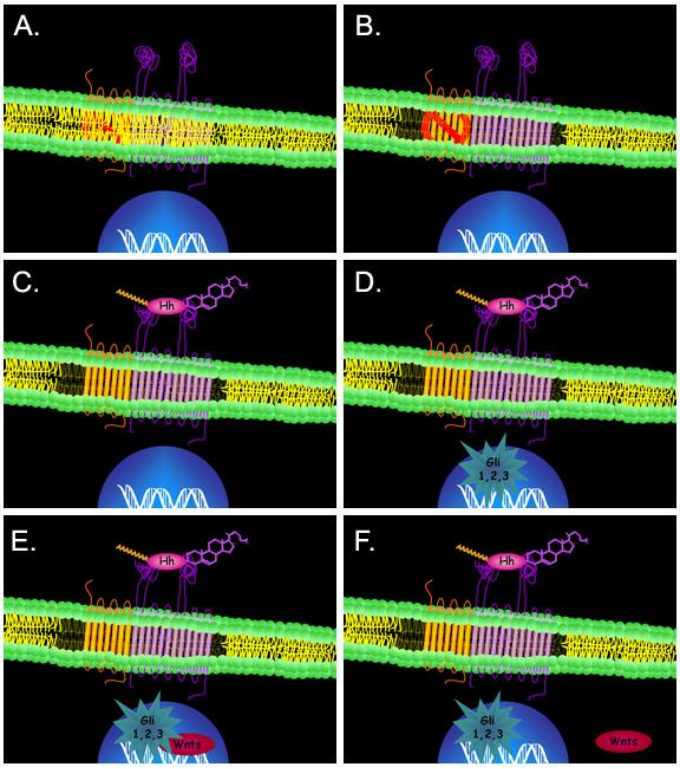


Figure 3. Simplified Model of Hedgehog Signal Transduction. (A&B) In the absence of the Hh protein, its twelve-transmembrane receptor, patched (purple), has an inhibitory effect on the G-protein-coupled seven-transmembrane receptor, smoothened (orange). The lipid bilayer of a cell is represented by the green and yellow. (C) The Hh protein binds to patched eliminating inhibition from smoothened. (D-F) This activates the pathway and allows the family of Gli transcription factors to enter the nucleus and stimulate gene expression as indicated here by induction of Wnt. Adapted from [191].

The Hh pathway involves two transmembrane proteins including patched (Ptc)—a 12-pass transmembrane protein and smoothened (Smo)—a 7-pass transmembrane protein. The Hh proteins must first undergo activation via unique post-translational modifications which include autocatalytic cleavage, sterolation and acylation before being secreted by signaling cells. The double lipid-modified form of the protein is tethered to the cell membrane and can induce short range signaling. However, Hh proteins can be further modified in order to release the Hh proteins that are tethered by cholesterol to the plasma membrane to induce long range signaling. In the absence of Hh

protein, Ptc inhibits Smo activity and ultimately inhibits downstream Hh signaling. Signaling events downstream of Smo are unclear, but ultimately involve inhibition of Gli, a family of zinc finger transcription factors, from activating Hh target genes. When the multimeric Hh protein binds Ptc, Smo is released to upregulate downstream target genes. Gli transcription factors are released from this inhibitory complex and translocate to the nucleus where they are able to activate Hh target genes such as Wnt genes. Hedgehog targets many genes involved in cell adhesion [192] and apoptosis [193], as well as Wnt signaling antagonists [193].

Indian hedgehog (*Ihh*) has been implicated in bone development, gastrointestinal tract development, embryonic vasculogenesis and may play a role in preparing the uterus for implantation in the mouse [194]. For example, mice that lack the *Ihh* gene have severe skeletal defects and die at birth [195]. In several epithelial-mesenchymal organs and under the influence of hormones, *Ihh* plays a role in regulating proliferation and differentiation of different cell types during development as well as during pregnancy in adults. Identificaton of *Ihh* and *Ptc* in neonatal and adult porcine uterine tissues would provide baseline data to suggest that *Ihh* is an element of the uterine developmental program and could be involved in support of uterine function.

3.4.4 MUSCLE SEGMENT HOMEOBOX GENES

Vertebrate *Msx* (muscle segment homeobox-like) genes are related to the *Drosophila melangoster* muscle segment homeobox (*msh*) gene. Msx genes are part of a highly conserved family of genes that have been identified in animals ranging from mammals to coelenterates [196]. Msx genes have a highly conserved homeobox motif

that encodes proteins with a DNA binding domain that act as transcription factors, but they share a limited sequence identity outside of the homeobox [197]. In the mouse, three Msx genes have been identified —Msx1, Msx2 and Msx3— with Msx1 and Msx2 having been cloned and characterized in detail [197]. Evidence of both Msx1 and Msx2 expression during embryogenesis and organogenesis suggests that these two genes play a role in development [168, 198-200].

Msx1 and *Msx2* may also play a role in porcine uterine development. Similar to other Hox genes, expression of *Msx1* and *Msx2* is localized to areas of epithelial-mesenchymal interactions as exemplified by the mammary gland [198]. Expression of *Msx1* and *Msx2* was detected in developing mammary glands of ovariectomized, virgin female mice as well as in the mammary gland of mice during early pregnancy [198]. In murine mammary glands, *Msx2* expression appears to be regulated by ovarian steroids. Expression levels of *Msx2* in the periductal stroma of the mammary gland are low in ovariectomized mice, but increased to levels found in intact mice in response to estrogen [198]. Transcript levels of *Msx1*in the mammary gland epithelium did not change when mice were ovariectomized. To determine if *Msx2* expression requires epithelium, mammary gland-free fat pads with the epithelium surgically removed were studied in prepubertal mice [201]. As adults, mice used for this study had inguinal glands consisting only of mammary adipose stroma and no mammary epithelial component [198]. *Msx2* expression was not found in the epithelium-free tissue by *in situ* hybridization, suggesting a fundamental inductive role for epithelium in Msx2 expression. Expression of *Msx1* was also identified in the epithelium of the adult mouse uterus—another epithelial-mesenchymal organ [168]. Because *Msx1* and *Msx2* are

expressed in epithelial-mesenchymal organs and are regulated by steroid hormones, it is possible that Msx genes play a role in neonatal porcine uterine development.

4. CONSEQUENCES OF DEVELOPMENTAL DISRUPTION

Developing conceptuses are continuously exposed to maternal factors *in utero*. After birth, nutritional, environmental, and endocrinological factors continue to influence development. Critical periods of development can be defined as periods of pre- and perinatal life during which disruption of the course of development – the developmental program - can have lasting consequences as reflected by alterations in adult phenotype. Aberrant exposure to environmental, nutritional or endocrinological factors during these critical developmental time periods can alter the structure and function of many organs and tissues including those of the FRT. The process through which lasting or lifelong alterations in structure and function of a tissue or organ occur as a consequence of the action of natural or manmade bioactive factors during critical developmental periods is called programming [202, 203].

During pregnancy, conceptuses are exposed to some circulating endogenous estrogens, but it is clear that exogenous estrogen and estrogen-like factors influence the organizational program and alter the developmental trajectory of many organs and tissues including those of the FRT. Not only was fetal development of the murine FRT affected by *in utero* exposure to diethylstilbestrol (DES)—a potent synthetic estrogen [204, 205], but long term effects in mice exposed to DES *in utero* were also evidenced by either subfertility or infertility of both males and females [206]. In pregnant women, DES was once prescribed to prevent miscarriages and other complications, but it has been shown

that women who were exposed to DES *in utero* have reproductive tract abnormalities as adults [207]. Reproductive tract anomalies of this kind not only affect fertility, but are associated with increased probability of vaginal adenocarcinoma [206, 207]. Other selective estrogen receptor modulators (SERMs), including chemicals such as bisphenol A (BPA) and naturally occurring phytoestrogens such as genistein, are also recognized to affect development and function of FRT tissues [208, 209]. Natural and sythethic SERMs can induce estrogen receptor-mediated gene expression at the wrong place and time during critical developmental periods, leading to changes in the organizational program of FRT tissues. Uterine programming events affected by administration of exogenous estrogen during perinatal life in laboratory animals and domestic ungulates have been studied extensively [14, 210-214].

Uterine gland genesis can be altered in response to exogenous hormones such as progesterone or estrogen. It was hypothesized that removal of the fetus from a progesterone-dominated prenatal environment at birth was a cue for adenogenesis in the ovine uterus [214, 215]. In a test of this hypothesis, it was shown that implanting neonatal ewes with a synthetic progestin called norgestomet from PND 0-PND 13 inhibited gland genesis [21] resulting in a uterine gland knockout (UGKO) phenotype. Glands, although histologically abnormal and not well developed, developed by PND 26 when the progestin implant was removed at PND 13. This ovine UGKO model has provided a better understanding of uterine function and development [32, 214]. In contrast to the ovine UGKO model, estrogen had a different effect on gland genesis in the porcine uterus. In a study by Tarleton and coworkers [13], estrogen was administered during the first two weeks of neonatal life, a critical developmental time period. This

treatment advanced uterine gland development in gilts evaluated on PND 14 when compared to those treated with vehicle alone. By PND 14, uterine glands had penetrated the endometrial stroma and extended all the way to the myometrium in animals exposed to estrogen [13]. When neonates were treated with estrogen during the proliferative phase of endometrial development (PND 6 – PND 13), penetration of GE throughout the stroma was advanced and BrdU labeling index was increased [13]. Similar results for uterine cell proliferation were observed in studies by Masters et al. [94], where neonatal pigs were treated with estrogen on PND 12 and 13, and uteri were obtained on PND 14. Estrogen advanced development of uterine glands and increased the PCNA labeling index when compared with control animals.

Morphoregulatory gene expression can also be altered in response to exogenous estrogen. In the FRT, it is normal for *Wnt* gene expression to be mediated by circulating hormones. For instance, in the murine female reproductive tract, *Wnt4*, *Wnt5a*, and *Wnt7a* expression patterns change in association with increasing and decreasing ovarian hormone concentrations such as estrogen and progesterone throughout the estrous cycle [171]. Prenatal exposure of mice to exogenous estrogens such as DES resulted in a uterine phenotype similar to that observed for the *Wnt7a*-null mouse (reviewed in Section 3.4.1) [175]. A thickened layer of smooth muscle, stratified epithelium and reduced endometrial stroma and glands were found in the uterus of both the DES-exposed and *Wnt7a*–null mice [179]. *Wnt7a* expression, observed normally in uterine LE was reduced in perinatally DES-exposed mice [179]. In the mouse, exogenous estrogens act to down regulate *Wnt* gene expression and induce a uterine phenotype in adults which resembles that of *Wnt4, Wnt5a, and Wnt7a*-null mutants (reviewed in Section 3.4.1) [167, 178, 179,

211, 216]. Similarly, estrogen administered for two weeks from birth in the pig decreased apparent expression of *Wnt4*, *Wnt5a* and *Wnt7a* in the developing porcine endometrium as evaluated using *in situ* hybridization [3, 11]. Collectively, data can be interpreted to suggest that *Wnt* gene expression patterns are associated with and may affect the developmental program governing morphogenesis and cytodifferentiation of FRT tissues in the neonatal pig.

Similar to results observed for mice with loss of function mutations for specific Hoxa genes, mice exposed to DES *in utero* displayed altered expression of *Hoxa* genes and shifted expression boundaries for these genes along the anteroposterior axis of the FRT at two weeks of age [217]. *Hoxa9*, normally expressed in the oviduct, was detected clearly in the uterus, but expression levels were very low in the oviduct. *Hoxa10* expression shifted to the caudal end of the uterus and was not detected in the glands or stroma in the upper part of the uterus. Expression of *Hoxa11* decreased dramatically in the uterus. Similar to data for the mouse, treatment of neonatal pigs with estrogen for two weeks from birth downregulated the expression of *Hoxa10*, but did not consistently have an affect on *Hoxa11* expression in the porcine uterus.

In pigs, estrogen exposure from birth not only alters patterns of uterine development and morphoregulatory gene expression in the neonate but can have lasting effects on uterine function in adults [14]. Tarleton et al. administered estrogen to a group of neonatal pigs and corn oil vehicle (CO) to another group for two weeks from birth. On postnatal day 170, estrus detection began and nine gilts from each group were assigned to remain cyclic or to be bred at the second observed estrus. On day 12 postestrus or mating, animals in all groups were hysterectomized and uteri were analyzed for treatment

effects. Neonatal estrogen treatment was antiuterotrophic in both cyclic and pregnant adult gilts as evidenced by reduced uterine wet weight and uterine horn volume when compared with control cyclic and pregnant gilts. Reduced luminal fluid protein and endometrial keratinocyte growth factor (KGF) mRNA levels in neonatally estrogen-exposed pregnant gilts indicated an alteration in adult uterine function [14]. In a similar study, neonatal estrogen administration from birth to PND 13 reduced the number of viable conceptuses on gestational day 45 in pregnant gilts [4]. These data have been interpreted to indicate that transient exposure to estrogen from birth can have long term consequences that alter uterine capacity as a result of altered developmental programming in the pig.

It is clear that long term effects on FRT/uterine function can be induced when the organizational program is altered during critical periods by aberrant exposure to specific environmental, nutritional or endocrinological factors. Research is needed to determine what these factors are and how they effect changes in the developmental program with lasting consequences for adult uterine function. Purposely disrupting development of the uterus can also be used as a tool to understand the mechanisms associated with optimal development and function.

5. SUMMARY/IMPLICATIONS

In light of observations discussed in this literature review, three studies were proposed to further the understanding of developmental events associated with porcine uterine development and the consequences of hormone-induced disruption of the normal uterine developmental program. Objectives of the first study were to determine if: (A)

IHH mRNA and protein, as well as the cognate Hh receptor Patched (Ptc) protein were present in neonatal and adult porcine uterine tissues; and (B) estrogen administration from birth through PND 13 affected patterns of IHH mRNA and/or IHH and Ptc protein expression in the neonatal uterus. Objectives of the second study were to determine if (A) Msx1 and Msx2 mRNA and/or protein were expressed in the neonatal porcine uterus; and (B) treatment with estrogen and estrogen agonists and antagonists on PND 12 and 13 would affect these expression patterns on PND 14. Finally, objectives of the third study were to determine effects of neonatal estrogen exposure from birth through PND 13 on adult uterine function during the peri-attachment period of early pregnancy as reflected by potential changes in the porcine endometrial proteome on gestational day 12.

CHAPTER I

Coordinate Expression of Indian Hedgehog and the Hedgehog Receptor Patched in Neonatal and Adult Porcine Uterine Tissues

Secreted vertebrate hedgehog proteins act via the patched (Ptc) receptor to affect developmentally critical cell behaviors including proliferation, survival and differentiation. Indian hedgehog protein (Ihh) may play a role in uterine patterning during perinatal life and has been implicated as a mediator of steroid hormone-sensitive cell interactions in adult endometrium. Here, objectives were to evaluate Ihh expression and to document Ihh and Ptc distribution in uterine tissues obtained on postnatal days 0 (PND 0=birth) 7 and 14, a period associated with onset of uterine gland genesis in the pig, and in adult porcine uterine tissues from diestrus and pregnancy. A 221 bp porcine cDNA (NCBI: CK172438) that displayed 94% sequence identity with the human Ihh transcript was generated by RT-PCR using total uterine RNA from PND 14. This cDNA template was used to generate [^{35}S]-UTP-labeled sense (negative control) and antisense Ihh cRNA probes for in situ hybridization (ISH). Immunohistochemical (IHC) localization of Ihh and Ptc proteins in uterine tissues was accomplished using Ihh (SC-1196) and Ptc1 (SC-6149) antibodies (Santa Cruz Biotechnology, CA). Uterine tissues obtained on PND 0, 7 and 14 ($N \geq 4$ gilts/day), and adult tissues from day 8-12 postestrus and pregnancyday 45, were processed together to evaluate expression and

distribution of Ihh mRNA and both Ihh and Ptc proteins in situ. On PND 0, when glandular epithelium (GE) is absent, Ihh mRNA signal was pronounced in luminal epithelium (LE) and developing myometrium (My) and weak in uterine stroma (St). On PND 7 and 14, Ihh mRNA signal was strong in LE and nascent GE and persisted in St and My, as seen on PND 0. In adult tissues, Ihh mRNA signal was evident in LE, stronger in deep GE, and strongest in My. Neonatal Ihh immunostaining, negligible at birth, mirrored mRNA signals by PND 14. In adult tissues, Ihh protein was detected in deep GE, St and My. Neonatal Ptc staining was observed in LE, GE and St. Adult Ptc staining was seen in all cell compartments, especially in deep endometrial zones. Spatially coordinated expression of Ihh and Ptc associated with morphogenetically active endometrial zones in neonates and adults suggests a role for Ihh-Ptc signaling in regulation of neonatal uterine morphogenesis and adult endometrial patterning and function. [USDA-NRICGP 2003-35203-13572]

Introduction

Vertebrate hedgehog (Hh) genes, including Sonic hedgehog (Shh), Desert hedgehog (Dhh) and Indian hedgehog (Ihh) are homologues of the Hh gene discovered in *Drosophila* [218]. Hedgehog genes encode secreted glycoproteins that act via the patched (Ptc) receptor system to affect developmentally critical cell behaviors including differentiation, proliferation and survival [189]. Indian hedgehog may play a role in uterine patterning during perinatal life and was recently implicated as a mediator of steroid hormone-sensitive cell interactions in adult endometrium [194, 218-223].

In the pig (*Sus scrofa*), development of the uterine wall between birth [postnatal day (PND) 0] and PND 14 involves differentiation of glandular epithelium (GE) from luminal epithelium (LE) and proliferation of nascent GE through endometrial stroma as myometrial smooth muscle layers become organized [4]. Differentiation of GE from LE between PND 0 and PND 15 is marked by onset of Wnt7a expression in LE and ERα expression in nascent GE [1, 11-13].

The normal porcine uterine organizational program can be disrupted by daily administration of estradiol valerate (EV) to gilts from PND 0-13 [4, 14, 224]. This transient exposure to estrogen from birth alters temporospatial patterns of morphoregulatory gene expression in the neonatal uterus, affects adult endometrial functionality and, ultimately, reduces the capacity of the adult uterus to support conceptus development. Thus, the period from PND 0-13 constitutes a critical period for estrogen-sensitive uterine development and endometrial programming in the pig.

Secreted hedgehog proteins may be upstream effectors of *Wnt* expression [218] and could contribute to events associated with uterine patterning and tissue programming [194, 218, 220, 221, 225]. The extent to which elements of a HH-Ptc signaling system are present and may be involved in perinatal uterine tissue programming and/or regulation of endometrial function is unknown and unexplored in ungulate species.

Objectives of this study were to determine if IHH is expressed by the neonatal porcine uterus.Also, to evaluate IHH expression and document IHH and Ptc distribution in porcine uterine tissues obtained from: (1) neonatal gilts between PND 0 and 14; and (2) neonatal gilts on PND 7 and 14 following daily EV exposure from birth; as well as

characterize endometrial IHH expression in adult gilts during diestrus and pregnancy (day 45).

Materials and Methods

Tissue collection and processing

All uterine tissues were obtained from female white crossbred pigs (gilts) at hysterectomy following procedures approved by the Auburn University Institutional Animal Care and Use Committee. The experimental design used to generate normal and estrogen-exposed neonatal uterine tissues is shown in Figure 6. At birth [postnatal day (PND) = 0], gilts were assigned randomly to serve as either vehicle (corn oil) –treated controls or to receive estradiol-17β valerate (EV; 50μg/kg bw/day, i.m.) from PND 0-13. Uterine tissues were obtained from gilts representative of all categories on PND 0, 7 and 14 (N=4-6 gilts/day). Tissues were also obtained from adult cyclic gilts during diestrus (days 8 and 12, estrus = day 0) and from pregnant gilts on gestational day 45 (3-5/day) [4, 14]. Pregnancies were confirmed by evidence of the presence of conceptus tissues *in utero* at the time of hysterectomy.

At the time of collection approximately one half of each neonatal uterus was frozen immediately in liquid nitrogen and stored at -80º C until needed for extraction of total RNA. Representative pieces of each uterus were fixed immediately in 4% (w/v) buffered neutral formalin and processed for *in situ* hybridization (ISH) and immunohistochemical (IHC) procedures as described elsewhere [1, 13].

RT-PCR amplification and cloning of porcine uterine IHH cDNA

Forward (cgg ctt tga ctg ggt gta tta) and reverse (gaa aat gag cac atc gct ga) primers, designed using Vector NTI software and sequence data generated by others from porcine cartilage (NCBI: AJ536288), were obtained from Qiagen (Alameda, CA). An IHH cDNA was generated from total RNA extracted with TRIzol (Invitrogen; Carlsbad, CA) from porcine uterine tissues obtained on PND 14. Procedures were as described for the Qiagen OneStep RT-PCR kit. The product obtained in this step was re-amplified using HotStarTaq (Qiagen) to generate a single 221 bp amplicon (see below). This porcine IHH (pIHH) cDNA was cloned into a pDrive vector (Qiagen PCR Cloning Kit) and submitted to the Auburn University Genomics and Sequencing Laboratory. The resulting sequence, confirmed in both directions, was submitted to the NCBI dbEST database.

In Situ Hybridization (ISH) for IHH mRNA

For ISH, antisense and sense (negative control) [α-35S]UTP-labeled cRNA probes were generated by *in vitro* transcription (IVT) using a MAXIscript kit (Ambion; Austin, TX), SP6 or T7 RNA polymerases, and appropriately linearized pIHH cDNA-containing plasmid vectors as templates. Template for generation of antisense cRNA was generated by linearizing the pDrive vector with KpnI. Sense transcripts were generated from XhoI linearized vector. Individual slides containing representative cross sections from each day and treatment, as well as both radiolabeled antisense and sense probes, were prepared. Tissues from all groups were processed together. Auto-radiography was performed using NTB-2 emulsion (Eastman Kodak, Rochester, NY) with 4-wk exposures at 4ºC as in [1, 13].

Immunohistochemical localization of IHH and Patched

Uterine tissues from neonatal and adult pigs were sectioned at 5-7 µm and placed onto SuperFrost plus slides (Fisher Scientifc). After deparaffinization in HemoDe and rehydration through graded ethanol baths, sections were subjected to heat-induced epitope retrieval (HIER; Zymed Laboratories, Inc., San Francisco, CA). Briefly, slides were placed into a Pyrex® beaker containing boiling 10mM citrate buffer, pH 6.0, for 20 min. The beaker was then removed from heat and cooled at room temperature for 20 min. Sections were then rinsed in PBS and non-specific binding was blocked by incubating sections with diluted rabbit serum. Primary antibodies for IHH (c-terminus, SC-1196) and Ptc (n-terminus, SC-6149) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and used at a concentration of 1 µg/ml. Incubations were performed overnight at 4oC. Procedures were as outlined for the Vector ABC Elite Kit. Sections were incubated in DAB substrate and counterstained with hematoxylin. Tissues from all categories were processed together.

Microscopy

Sections were examined by bright field microscopy for analysis of the immunocytochemical tests on an Olympus BHS microscopy with a 1.30 n.a. condenser, and lenses ranging from 4X to 20X. In situ hybridization samples were examined by darkfield illumination using an Olympus oil immersion ultracondenser providing 1.2-1.4 n.a.; slides were examined with lenses ranging from 4-20X magnification for most samples; high magnification required use of an Olympus planapochromatic 100X UV objective with a built – in iris to limit the numerical aperture as needed for the darkfield condition. Images were collected with either a Dage-MTI VE1000 video camera, with

Image-Pro software (Media Cybernetics Corporation, Bethesda, MD), using digital background subtraction, or with a SIT 68 camera (also Dage-MTI, Roeske City, IN). Later microscopical analyses used the same microscopical optics coupled to a MicroPublisher 3.3 digital CCD camera (Cooled model, 3.3 megapixel, QImaging Corporation, Vancouver, BC).

Results

PCR cloning and characterization of IHH

A 221 base pair amplicon was generated by RT-PCR with primers designed for Ihh from total RNA extracted from neonatal uterine tissues obtained on PND 14 and adult endometrial tissue obtained on day 8 post-estrus.

Ihh cDNA generated from RNA extracted from PND14 uterine tissue was cloned and sequenced and found to share 94% and 89% identity with corresponding human and mouse Ihh sequences. The porcine Ihh cDNA was registered with the NCBI dbEST database and assigned the GenBank Accession no. CK172438.

In situ expression of Ihh in neonatal and adult uterine tissue

Effects of age and treatment with EV from PND 0 on temporospatial patterns of Ihh expression in neonatal porcine uterine tissues detected by in situ hybridization is shown in Figure 1. At birth, expression (white grains) above background was observed in luminal epithelium (LE), stroma (St), and myometrium. Expression was evident in all uterine cell types including glandular epithelium (GE) on PND 7 and 14. Treatment with EV from birth dramatically decreased Ihh expression by PND 14 in all cell types. Expression patterns of Ihh in the adult porcine uterus on day 12 post-estrus (Figure 2)

differ from those in the neonatal uterus. Expression was observed consistently in GE and minimal or absent expression in LE. Low levels of expression were also observed in the stroma.

Immunohistochemical localization of Ihh and Ptc expression in neonatal and adult uterine tissue

Immunohistochemical localization of Ihh and Ptc proteins in neonatal porcine endometrium on PND 14 is shown in Figure 3. Signal (brown) for both Ihh and Ptc protein were observed in LE, GE, and St. Right panels show background staining when goat IgG is substituted for caprine-derived primary antibodies for Ihh and Ptc. Figure 4 depicts immunohistochemical localization of Ihh and Ptc proteins in adult porcine endometrium from a day 8 cyclic gilt. Signal for both Ihh and Ptc proteins is minimal or absent in LE, weak in St, and pronounced in GE.

Discussion

First discovered in *Drosophila*, Hh genes encode secreted glycoproteins that play a role in morphogenesis, growth, and patterning during development. There are three vertebrate Hh genes including Sonic hedgehog (Shh), Desert hedgehog (Dhh), and Indian hedgehog (Ihh) that works through the Patched1 (Ptc) receptor system. Of the three vertebrate Hh genes, Ihh has been implicated in uterine development. The Hh proteins induce developmental events by creating a concentration gradient with short and long range signaling which allows the protein to work in both an autocrine and/or paracrine manner [226]. Autocrine and paracrine signaling is important in uterine development mediated by stromal-epithelial interactions.

Similar to the mouse, porcine uterine development begins prenatally but is completed postnatally. During the first two weeks of life, a critical period for development, the uterus undergoes an extensive remodeling process where GE differentiates from LE. The GE begins to proliferate and form coiled branched, tubular glands that penetrate into the stroma towards the myometrium. Differentiation of GE is marked by the appearance of nuclear estrogen receptor-α (ER) expression, and LE differentiation is marked by Wnt7a expression [3, 13].

In the murine uterus, Ihh mRNA and protein were identified in the LE and GE, and both the mRNA and protein of the Hh receptor, Ptc, was detected in the LE, GE, and stroma [194, 221]. The location of Ihh and Ptc suggests autocrine and paracrine signaling within the uterus. Stromal expression of Ptc as well as stromal proliferation induced by progesterone may be a consequence of the paracrine actions of Ihh expressed by the epithelium [221, 227]. Stromal cell proliferation and mediation of steroid hormones are important in uterine development.

Expression of Ihh and Ptc were observed in the neonatal and adult porcine uterus. Starting at birth, expression for Ihh mRNA was observed in the LE, stroma and myometrium of the neonatal porcine uterus. By PND 7 and 14, Ihh mRNA was also expressed in GE as well as the LE, stroma and myometrium. Treatment with EV for two weeks from birth significantly decreased the mRNA expression of Ihh in the neonatal uterus on PND 14. Effects were pronounced in the LE. Immunohistochemical localization of Ihh mirrored the expression pattern observed with *in situ hybridization*. Protein localization for Ptc, the receptor for Ihh, was also observed in the LE, GE and stroma of PND 14 uterine tissue. Expression of Ihh and Ptc was also detected in the adult

uterus. Unlike the neonate, Ihh mRNA was evident in the stroma, pronounced in the GE, and was minimal or absent in LE. Localization of Ihh and Ptc protein in the adult uterus is weak in the stroma, minimal or absent in LE and pronounced in GE.

In the *Drosophila* embryo, Hh induced local regulation of *wingless* (*wg*), a segment polarity gene, expression in cells adjacent to Hh-expressing cells [228, 229]. Related to *wg,* several vertebrate Wnt gene products including Wnt4, Wnt5a and Wnt7a are implicated in development as well as patterning of the FRT. These three Wnt genes are expressed in the neonatal porcine endometrium such that Wnt7a is expressed in the LE and Wnt4 and Wnt5a are expressed in the stroma beginning at PND 7 and increasing by PND 14 [3]. Like Ihh expression, after transient exposure to EV for two weeks from birth, Wnt7a expression is significantly decreased in the LE by PND 14 [3]. Therefore, it is possible that Ihh regulates Wnt gene expression in the neonatal porcine endometrium.

Expression of Ihh and Ptc in the neonatal porcine endometrium during a critical time period for development suggests a potential role for Ihh in neonatal uterine development. Patterns of IHH expression documented here can be interpreted to suggest that Ihh and the Hh-Ptc signaling system are components of the organizational network of factors that regulate porcine uterine development, endometrial patterning and function.

Figure 1.

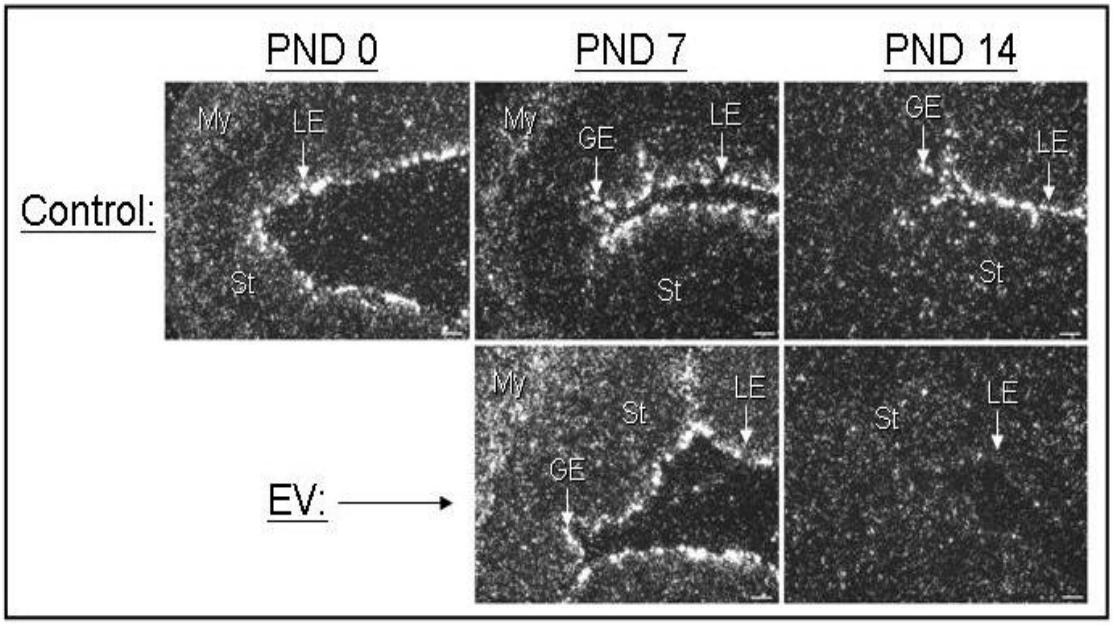


Figure 2.

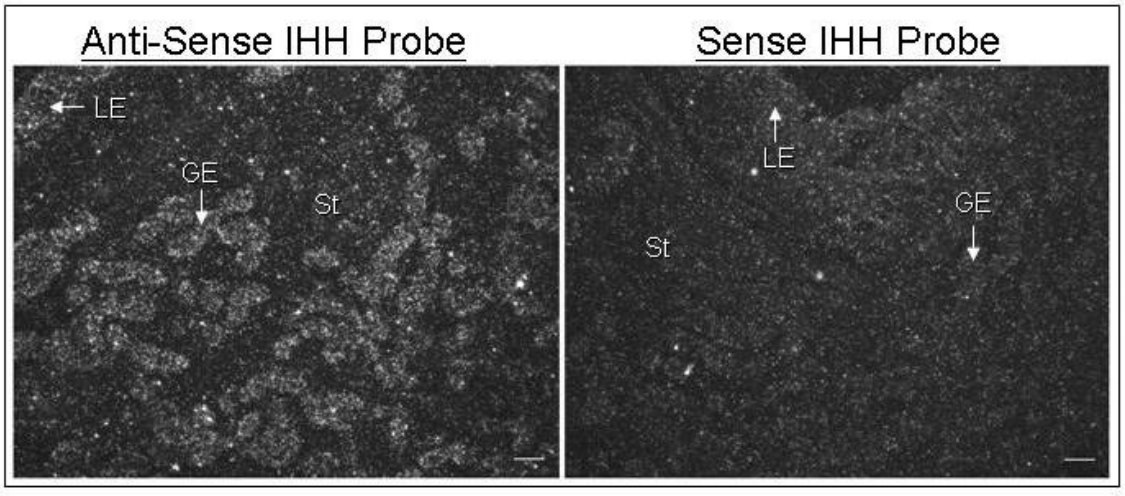


Figure 3.

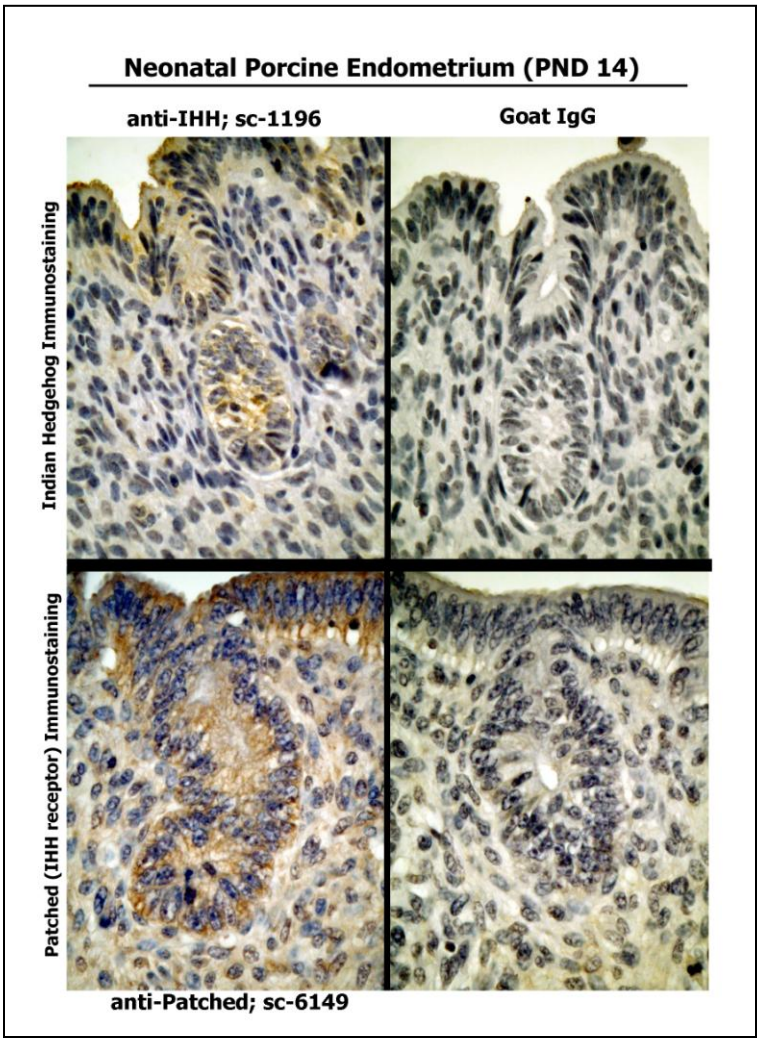
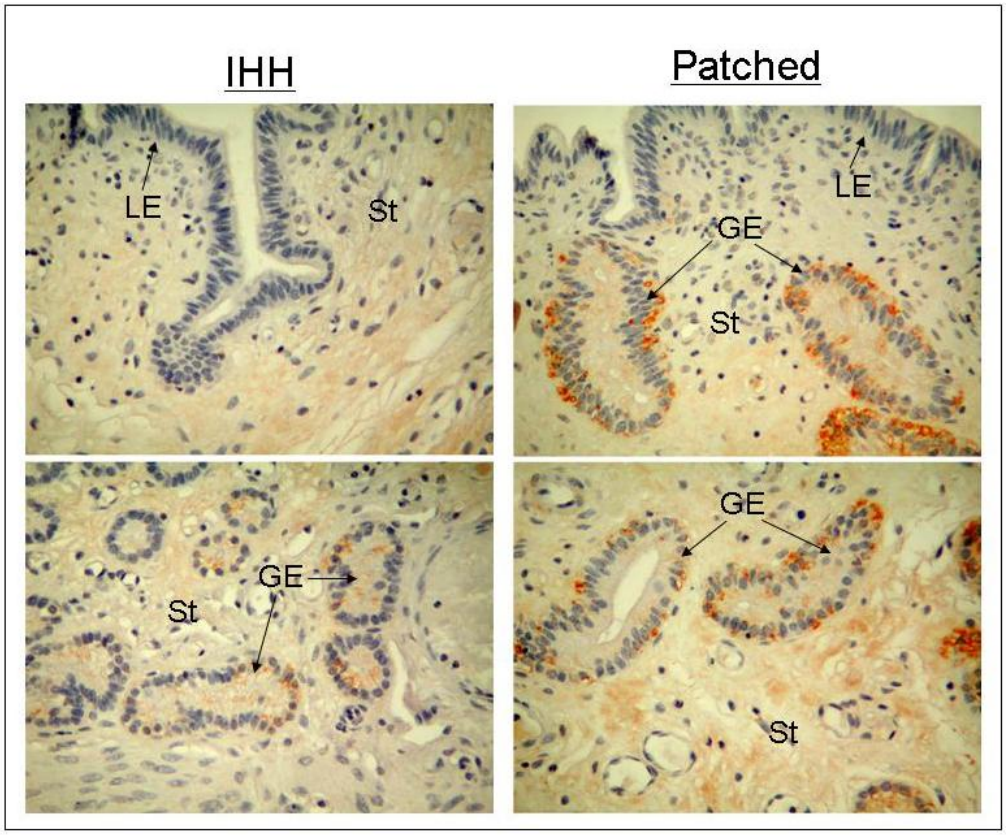

Neonatal Porcine Endometrium (PND 14)
anti-IHH; sc-1196
Goat IgG
Indian Hedgehog Immunostaining
Patched (IHH receptor) Immunostaining
anti-Patched; sc-6149

Figure 4.



CHAPTER II

Porcine Uterine Expression of Msx1 and Msx2: Effects of Relaxin, Estrogen and ICI 182,780 in the Neonatal Endometrium

Expression of *Msx*1 and *Msx*2 can affect other morphoregulatory genes, including *Hedgehog, Wnt* and *Hox* family members, required for uterine patterning and tissue programming. Factors affecting activation of the neonatal uterine estrogen receptor (ER) system directly, as with estradiol-17β (E) or the antiestrogen ICI 182,780 (ICI), or indirectly, as with peptide growth factors such as relaxin (R), can alter such expression patterns with lasting consequences for uterine form and function. Here, objectives were to determine if *Msx*1 or *Msx*2 expression occurs in porcine endometrium and if their expression patterns in neonatal endometrium are affected by E, R or ICI, given alone or in combination, during a critical period for E-sensitive uterine programming in the pig. Uteri (n = 3-5/group) were obtained from crossbred gilts on postnatal day (PND; birth = PND 0) 14 after administration of vehicle (C; DMSO: ETOH, 4:1), E (50μg/kg/day, ip), R (20μg/kg 4x/day, im) or ICI (1 mg/kg, im) given alone or 2h prior to E (IE) or R (IR) beginning on PND 12. *Msx*1 and *Msx*2 cDNAs, generated by RT-PCR from endometrial RNA obtained from cyclic adult gilts on day 12 post-estrus, were cloned, sequenced (NCBI Accession No.: pMsx1 DY453022, pMsx2 DY453023) and used to generate

[^{35}S]UTP-labeled antisense and sense cRNA probes for in situ hybridization. Antibodies for Msx1 (Covance, CA; MMS-261R) and Msx2 (Stressgen Bioreagents Corp, BC; AAM-010) were used in immunohistochemical (IHC) analyses. Tissues from all groups were processed together. *Msx*1 and *Msx*2 expression was detected in adult and neonatal uterine tissues. In situ expression of both transcripts was predominantly epithelial. For Msx1, IHC revealed staining in luminal (LE) and glandular epithelium (GE) that was pronounced relative to the stroma in all treatment groups. For Msx2, nuclear staining was seen intermittently in LE and GE for C, E, R, and IE tissues. In striking contrast, strong nuclear and cytoplasmic Msx2 staining was observed consistently in LE, GE and stroma for ICI and IR tissues. Thus, in the absence of exogenous E, ICI stimulated Msx2 expression. Data indicate that suppression of ER activity may affect critical morphoregulatory events in the neonatal uterus. (Support USDA-NRI 2003-35203-13572 and NSF EPS0447675)

Introduction

In the pig (*Sus scrofa domesticus*), the uterus is functionally and morphologically immature at birth. During the first two weeks of life, the porcine uterine wall undergoes a remodeling process where glandular epithelium (GE) that differentiates from luminal epithelium (LE) begins to proliferate and form coiled tubular glands that penetrate deep into the stroma. Gland formation is associated with the appearance of nuclear estrogen receptor α (ER) expression during the first two weeks of life in stroma cells as well as glandular epithelial cells, but ER expression is not apparent in cells of the luminal epithelium [1]. Recently, it was shown that ER protein is expressed by the neonatal

porcine uterus as early as postnatal day (PND) 2 [230]. The success of estrogen-sensitive, ERα–dependent organizational events associated with development of the uterine wall during perinatal life dictates the extent to which adult tissues are able to function optimally [231, 232].

Relaxin (RLX) is a polypeptide hormone that has uterotrophic effects in neonatal pigs [9, 230] as well as in prepubertal [233] and pregnant pigs [234, 235]. Uterotrophic effects include an increase in LE height as well as advanced endometrial growth [230, 236]. The cognate receptor for RLX, LGR7, is present in the stroma of the neonatal uterus at birth and LGR7 expression increases by PND 14 [9, 236]. Because RLX is present in porcine milk during lactation, neonatal animals are exposed to the polypeptide hormone from birth [236], but uterotrophic effects of the hormone are more pronounced when animals are exposed to RLX during the second week of life when compared with animals exposed during the first week of life [230]. The ER antagonist, ICI 182,780, inhibits the uterotrophic effects of RLX when it is administered in combination with RLX on PND 12 and PND 13 [230]. Therefore, RLX-induced uterotrophic affects maybe due, in part, to crosstalk with the ER signaling system [149, 230].

Interactions between stroma and epithelium are essential to the formation of many organs including the bladder, prostate, mammary gland, uterus and many others [10, 152, 156-158, 237]. Not only are stromal-epithelial interactions central to the success of endometrial development mediated by products of highly conserved morphoregulatory genes, but stromal-epithelial interactions also mediate the effects of estrogenic agents [10]. Morphoregulatory genes expressed in the neonatal porcine endometrium include *Wnt 7a*, *Wnt 5a*, *Wnt 4*, *Hoxa10* and *Hoxa11* [231]. Patterns of morphoregulatory gene

expression in the porcine endometrium are disrupted with transient exposure of neonates to estrogen or factors that affect the state of ER activation. Disruption of the developmental program not only alters the morphology of the neonatal uterus [13], but it also has long term consequences that include altering the endometrial proteome associated with pregnancy during the periattachment period (pregnancy day 12) and reducing conceptus survival in adult gilts [232].

Highly conserved muscle segment homoeobox-like (Msx) genes localized to areas of stromal-epithelial interactions act as transcription factors in the development of limb and tooth buds, heart, nervous system, mammary tissue, uterus and other stromal-epithelial tissues [168, 196, 200, 238]. The mouse has three muscle segment homeobox (Msx) genes (Msx1, Msx2, Msx3) that are homologous to the *Drosophila* muscle segment homeobox gene (*msh*). Stromal-epithelial interactions are essential to the development of the mouse mammary gland. During murine mammary gland development, Msx1 and Msx2 were both expressed in the glandular epithelium [198]. When mice were ovariectomized, Msx2 expression was downregulated while Msx1 expression did not change in response to the decrease in circulating estrogen [198]. It is not known how estrogen regulates Msx2 expression because there are no classical estrogen responsive elements in the Msx2 promoter region [200].

The present study was conducted to gain additional insight into mechanisms through which E and peptides with the potential to act via crosstalk with the ER system, such as RLX [149, 230], affect patterns of uterine development in the neonatal pig. Objectives of this study were to determine if (1) Msx1 and Msx2 were expressed in the neonatal porcine endometrium; (2) and if E, RLX, and ICI 182,780 (ICI), administered

alone or in combination during the second week of neonatal life changes the expression patterns of Msx1 and/or Msx2.

Materials and Methods

Animals and tissue collection

Yorkshire-Landrace gilts used in these experiments were housed at the Swine Unit of the New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick, NJ, USA and the Mississippi Agricultural and Forestry Experiment Station, Mississippi State University, Starkville, MS, USA. Gilts were randomly assigned to one of six treatment groups as follows: (1) control (C; vehicle alone; n = 7); (2) ICI 182,780 (ICI; 1 mg/kg BW, given i.p. in DMSO:ETOH, 4:1 vehicle; given as a single injection 2 h prior to start of vehicle or hormone treatment; n = 5); (3) estradiol-17β (E; 50µg/kg BW, i.p., every 24 h for 48 h; n = 5); (4) ICI/estradiol (ICI+E; n = 5); (5) porcine relaxin (RLX; 20µg/kg BW, given i.m. in PBS every 6 h for 48 h; n = 6); and (6) ICI/relaxin (ICI+RLX; n = 10). Gilts in this study were treated for two days beginning on PND 12, and uteri were obtained on PND 14. Timing, dosage and route of ICI administration were identical to those described previously [230]. Gilts were euthanized 3 h after the last injection on PND 14. Reproductive tracts were excised and trimmed free of connective tissue. Segments from the midportion of each uterine horn from each animal were fixed in 4.0% paraformaldehyde (w/v), embedded in Paraplast-Plus and sectioned at 6µm. Sections were analyzed by *in situ* hybridization for Msx1 and Msx2 as well as stained immunohistochemically for Msx1 and Msx2 analyses. All procedures involving animals were conducted in accordance with the Guide for the Care and Use of Agricultural

Animals in Agricultural Research and Teaching [239] and were approved by all Institutional Animal Care and Use Committees as appropriate.

Immunohistochemistry

Porcine uterine tissue sections were affixed to SuperFrost plus slides, deparaffinized, rehydrated, and subjected to heat-induced epitope retrieval (Zymed Laboratories, Inc., CA). Antibody to Msx1 (Covance, CA; MMS-261R) was used at 1:500 and antibody to Msx2 (Stressgen Bioreagents Corp, BC; AAM-010) was used at 5µg/mL. Sections representing all animals and categories from each study were processed together using the Vecta-Stain ABC elite kit (Vector Laboratories, CA) with color developed using DAB substrate. Irrelevant mouse IgG/IgM isotype (Zymed) was used in place of the primary antibody to serve as a negative control.

RNA Isolation

RNA was isolated from frozen endometrium of cyclic adult gilts on day 12 post-estrus using TRIzol reagent (Invitrogen) as directed by the manufacturer. RNA was evaluated spectrophotometrically and integrity of ethidium bromide-stained RNA was assessed following electrophoresis through 1.25% agarose gels [240].

Generation of cDNA Templates

Primers specific to Msx1 and Msx2 were designed using Vector NTI. Total pooled RNA (1µg) from cyclic adult gilts on day 12 post-estrus was reverse transcribed to generate pMsx1 and pMsx2 cDNA templates using the QIAGEN OneStep RT-PCR kit. The ethidium bromide stained pMsx1 amplicon (276bp) and pMsx2 amplicon (162bp) were evaluated after being electrophoresed through 2% agarose gels. pMsx1 and

pMsx2 cDNAs were submitted to NCBI and assigned the following accession numbers respectively, DY453022 and DY453023.

In situ hybridization

In situ hybridization (ISH) [76] was performed, using [^{35}S]UTP-labeled (specific activity: 1200 Ci/mmol; Amersham, Arlington Heights, IL) antisense and sense cRNA probes produced by in vitro transcription from the *KpnI* and *XhoI-linearized* pMsx1 and pMsx2 cDNA templates, with a MaxiScript kit (Ambion, Austin, TX). T7 and SP6 RNA polymerases were used to transcribe antisense and sense probes, respectively. Procedures were essentially identical to those previously described [52, 76]. Each slide included tissue sections that received either radiolabeled sense (negative control) or antisense cRNA probe. Hybridization was carried out overnight at 55°C. After posthybridization processing, slides were dipped in NTB liquid photographic emulsion (Eastman Kodak) and exposed at 4°C for 4 weeks. Slides were developed at 15°C using D19 developer (Eastman Kodak).

Microscopy

Sections were examined by bright field microscopy for analysis of the immunocytochemical tests on an Olympus BHS microscopy with a 1.30 n.a. condenser, and lenses ranging from 4X to 20X. In situ hybridization samples were examined by darkfield illumination using an Olympus oil immersion ultracondenser providing 1.2-1.4 n.a.; slides were examined with lenses ranging from 4-20X magnification for most samples; high magnification required use of an Olympus planapochromatic 100X UV objective with a built – in iris to limit the numerical aperture as needed for the darkfield condition. Images were collected with either a Dage-MTI VE1000 video camera, with

Image-Pro software (Media Cybernetics Corporation, Bethesda, MD), using digital background subtraction, or with a SIT 68 camera (also Dage-MTI, Roeske City, IN). Later microscopical analyses used the same microscopical optics coupled to a MicroPublisher 3.3 digital CCD camera (Cooled model, 3.3 megapixel, QImaging Corporation, Vancouver, BC).

QPCR

The Applied Biosystems Gene Amp 7000 Sequence Detection System and SYBR Green method were used to perform real time quantitative RT-PCR (qRT-PCR). Universal thermal cycling parameters recommended by the manufacturer were used. Primers for Msx1 were designed by Primer Express Software (Applied Biosystems), and primers for Msx2 and S15 were designed by Dr. Jacek Wower (Auburn University). Primers for Msx1 and Msx2 were synthesized by Operon Biotechnologies (Huntsville, AL). Porcine Msx1 (Accession no. DY453022) forward—5'-AGAGACTGCAGGAGGCCGA-3' and reverse—5'- TGGGTTTAGCGGCCATCTT-3' primers were used to generate a 51bp amplicon. Porcine Msx2 (Accession no. DY453023) forward—5'-GCAGAGTTCTCCAGCTCTCTGAAC-3' and reverse—5'-CCAGTTCTGCCTCCTGCAGTC-3' primers were used to generate a 100 bp amplicon. A 114 bp amplicon was generated using S15 (Accession no. NM_214334) forward—5'-GGTAGGTGTCTACAATGGCAAGG-3' and reverse—5'-GGCCGGCCATGCTTC-3'. Primer concentrations were optimized and relative efficiency of the target genes as well as S15 was evaluated by amplifying serial dilutions of the cDNA template (1, 1:2, 1:5, 1:10). Using dissociation curves, the absence of primer-dimer amplification was verified. To normalize cDNA input, porcine S15 was used as an internal control. Relative

quantitation of gene expression was determined using a comparative CT method ($\Delta\Delta C_T$) in which the sample having the minimum expression level was chosen as the reference calibrator [241]. Data are therefore presented as relative expression levels.

Statistical Analysis

Uterine Msx1 and Msx2 expression data were subjected to analyses of variance using General Linear Models procedures available with SAS [242]. Statistical models considered variation due to treatment. Treatment effects were identified by performing a set of pre-planned contrasts that included comparisons of specific groups as follows: control vs E; control vs RLX; E vs ICI; R vs ICI+R; and, E vs ICI+E. All error terms were identified based upon the expectations of the mean squares for error and data were expressed as least squares means with standard errors.

Results

Immunohistochemistry

Figure 1 and Figure 2 display photomicrographs depicting immunostaining of Msx1 and Msx2 in uterine sections from animals treated with CO, E, RLX, ICI, ICI+E and ICI+RLX. For Msx1 (Figure 1), immunostaining is more pronounced in LE and GE than in the stroma. No changes in immunostaining patterns were observed among treatment groups. In contrast, immunostaining patterns for Msx2 (Figure 2) did not remain the same. Msx2 immunostaining patterns were localized intermittently in the nucleus of LE and GE in C, E, RLX and ICI+E. Relative to the control, immunostaining was pronounced in the nucleus and cytoplasm of LE, GE and stroma in tissue sections from animals treated with ICI and ICI+RLX.

In situ hybridization

Figure 3 depicts Msx1 transcript expression in the LE and GE and faintly in the stroma of tissue sections from all treatment groups. There were no obvious changes in Msx1 expression due to treatment effects. Msx2 transcript expression in tissue sections from all treatment groups is depicted in figure 4. Similar to Msx1 expression, Msx2 signal was localized mainly to the LE and GE as well as slightly in the stroma. Relative to the control, Msx2 expression appeared to be downregulated following E treatment (Figure 4B) and ICI (Figure 4E) attenuated this effect.

QPCR

Data illustrating Msx1 and Msx2 expression in the neonatal uterus are presented in figures 5 and 6. Treatment for two days from PND 12 with C, E, RLX, ICI, ICI+E, and ICI+RLX did not alter the relative expression of Msx1 (Figure 5) or Msx2 (Figure 6) in the neonatal porcine uterus.

Discussion

Msx1 and Msx2 are homeobox containing genes important in development and function of stromal-epithelial organs such as the mammary gland and uterus [168, 198], and they have been linked to cell proliferation and differentiation in developing organs and tissues [243-246]. During the first two weeks of neonatal life, the porcine uterus undergoes rapid cell proliferation and differentiation as GE begins to differentiate from the LE, proliferate, and penetrate deep into the uterine stroma [3, 4, 13]. Organizational programming events in the uterus include gland genesis which is an estrogen-sensitive estrogen receptor α (ER) event. This organizational program can be altered by estrogen

or estrogen-like factors [13, 14, 91]. For example, administration of estrogen or relaxin on postnatal day (PND) 12 and 13 increased uterine gland proliferation in the neonatal porcine uterus [91]. In the porcine uterus, transient exposure to estrogen or estrogen-like factors also alters morphoregulatory gene expression [3, 4, 11, 247].

Developmental programming of the uterus involves correct temporal and spatial expression of morphoregulatory genes including *Wnt4, Wnt 5a, Wnt 7a, Hoxa10* and *Hoxa11*. In the uterus, a critical Wnt/Hox expression axis is evident to maintain an organizationally correct and functional uterus. These genes have been implicated in cell differentiation and tissue cell boundaries in the female reproductive tract. Using gene knock-out technology in mice, it was determined that in adult mice *Wnt7a* expression is required to support and maintain *Hoxa10* and *Hoxa11* expression [176]. The uterus of the *Wnt7a* mutant no longer expresses *Hoxa10* or *Hoxa11*and undergoes posteriorization frequently observed in *Hoxa* mutants [176]. In the absence of Hoxa10 and Hoxa11, stromal expression of Wnt4 and Wnt5a is lost in the murine uterus [176, 216]. Wnt5a mutants lack defined cervical and vaginal structures and therefore works with Wnt7a to support proper anteroposterior development of the uterus. Similar results were observed in mice that had been exposed to diethylstilbestrol (DES), a synthetic estrogen. In the porcine uterus, Wnt and Hox gene expression was reduced in the neonatal porcine uterus after transient exposure to estrogen during the first two weeks of life.

Highly conserved Msx genes also play a role in maintaining the Wnt/Hox expression axis in the uterus [219]. Msx2 appears to regulate the expression of Wnt5a and Wnt7a in the neonatal murine uterus [219]. Normally, Wnt7a is expressed in the LE, but in the absence of Msx2, Wnt7a expression is significantly expressed in the LE.

Exposure to DES repressed Wnt7a expression in both the uterus of the control mice as well as in the uterus of the Msx2 mutant mice. In contrast, Wnt5a is normally expressed in the stromal compartment of the Msx2 null murine uterus, but after DES exposure, Wnt5a expression was shifted to the LE of the uterus of the Msx2 null mice at a greater extent than that of the control mice [219]. DES exposure also downregulates the LE expression of Msx2 in the neonatal murine uterus [219]. Wnt5a also plays a role in Msx1 expression [168]. During embryonic development, Wnt5a and Msx1 are colocalized in the limb, brachial arches and genital eminence of the mouse [135, 168]. Similar colocalization expression was observed in the adult murine uterus. Using tissue recombinant studies, it was determined that stromal expression of Wnt5a is necessary to support LE expression of Msx1 [168]. During pregnancy, both Wnt5a and Msx1 expression significantly decreases in the murine uterus at the time of implantation [168]. Taken together, it is apparent that Msx1 and Msx2 are integral components of the murine uterine Wnt-Hox expression axis. Because there is evidence of Wnt and Hox gene expression in the developing neonatal porcine uterus, it was hypothesized that not only does the porcine uterus express Msx1 and Msx2, but that administration of estrogen or relaxin could alter their expression patterns.

In this study, expression of Msx1 and Msx2 mRNA was observed predominantly in the GE and LE of the developing porcine uterus similar to Msx1 expression observed in the adult murine uterus [168] and Msx2 in the neonatal murine uterus [219]. Compared with the control, treatment with E, RLX, ICI, ICI+E and ICI+RLX on PND 12 and 13 did not have an effect on Msx1 expression in the porcine uterus as evidenced by *in situ* and relative QPCR. Localization of Msx1 protein mirrors that of the uterine

mRNA expression. For Msx2, treatment with E downregulated the epithelial expression similar to that seen in the neonatal mouse exposed to DES [219]. Downregulation of Msx2 expression was attenuated by ICI as evidenced by in situ, but not by relative QPCR. Lack of treatment effects for Msx2 expression was not expected. To analyze relative Msx2 expression, RNA extracted from whole uterine tissue was used. Expression of Msx2 in the uterine stroma and myometrium may have overshadowed the treatment effects observed in the epithelium by *in situ* in the QPCR reaction. Immunohistochemical analysis also revealed treatment effects in the protein localization. Msx2 immunostaining patterns were localized intermittently in the nucleus of LE and GE in C, E, RLX and ICI+E. Treatment with ICI in the absence of estrogen (RLX and ICI+RLX), significantly increased the immunostaining intensity for Msx2 in the nucleus and cytoplasm of LE, GE and stroma. It is unknown how estrogen regulates Msx2 expression because the promoter of the Msx2 gene lacks an estrogen response element [200]. It is possible that expression of Msx2 may involve non-classical ER signaling. It has been determined that ICI can alter the transcription
of estrogen-responsive genes via activation of Sp1 promoter elements [248, 249], and the Msx2 gene contains an Sp-1 promoter [250].

Developmental programming of the uterus involves correct temporal and spatial expression of morphoregulatory genes including *Wnt4*, *Wnt 5a*, *Wnt 7a*, *Hoxa10* and *Hoxa11*. Data from this study indicates that Msx1 and Msx2 may also play a role in development of the neonatal porcine endometrium as a constituent of the Wnt/Hox expression axis. With the use of laser microdissection technology, uterine epithelium can

be analyzed separately from the stroma to determine treatment effects on Msx1 and Msx2 expression in the porcine uterus.

Figure 1: Msx1 Immunohistochemistry 20x, 50 microns

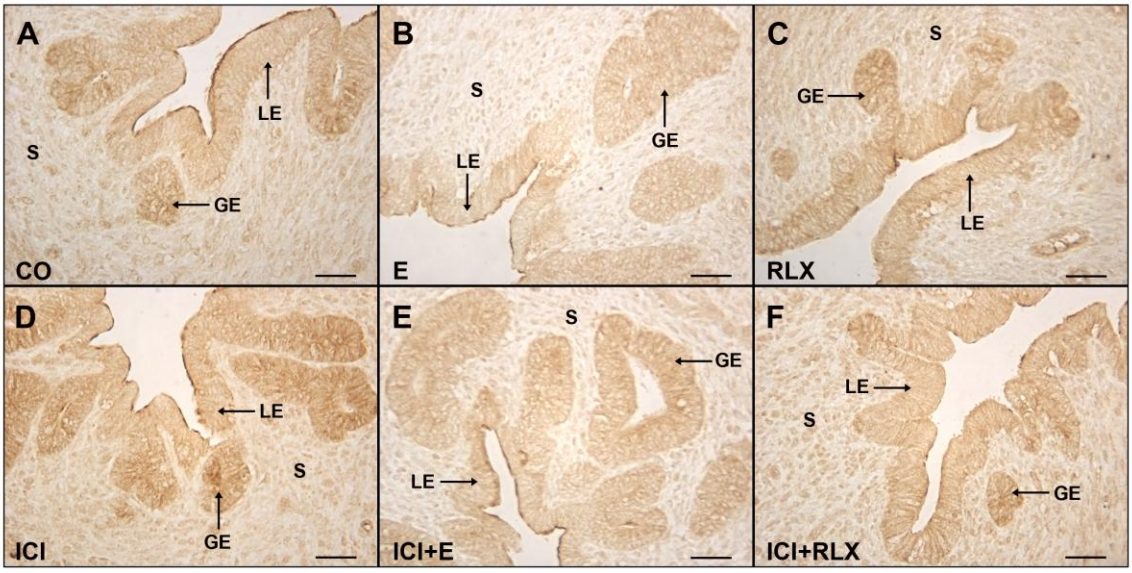


Figure 2: Msx2 Immunohistochemistry 20x, 50 microns

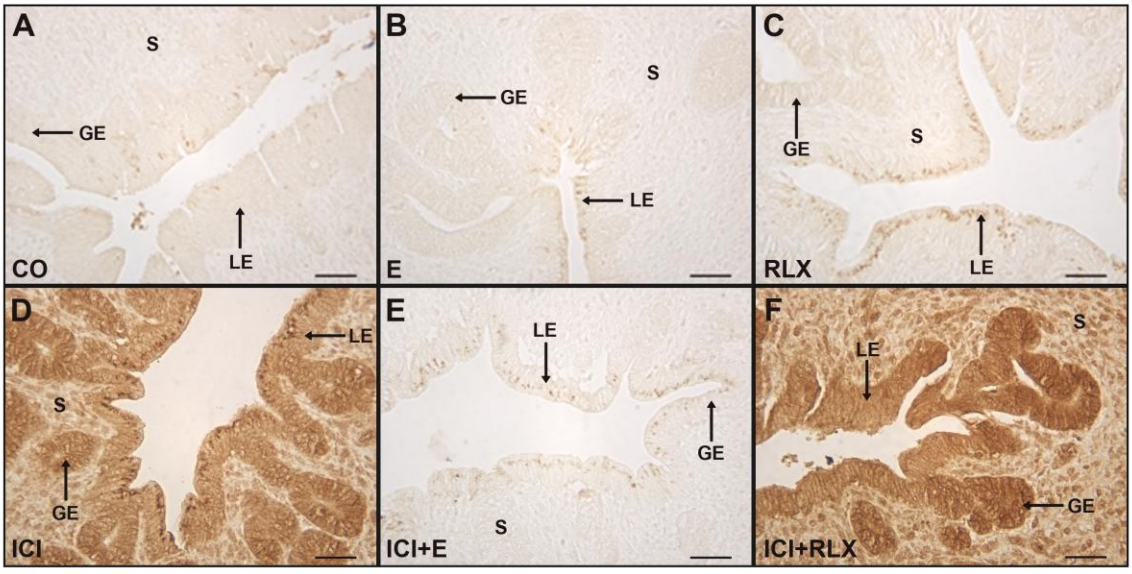


Figure 3: Msx1 ISH 10x, 50 microns

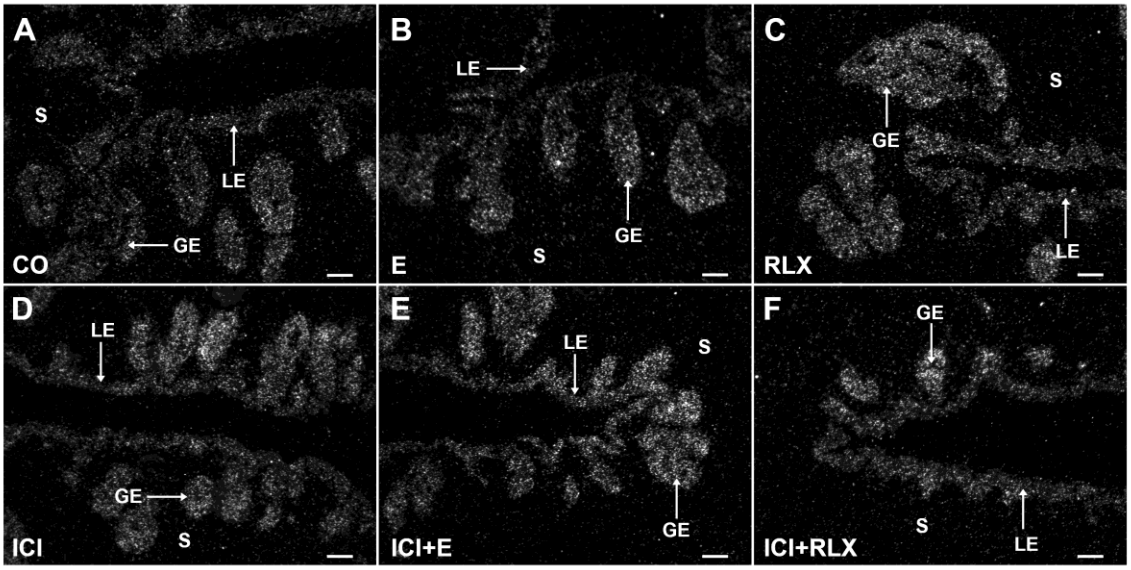


Figure 4: Msx2 ISH 10x, 50 microns

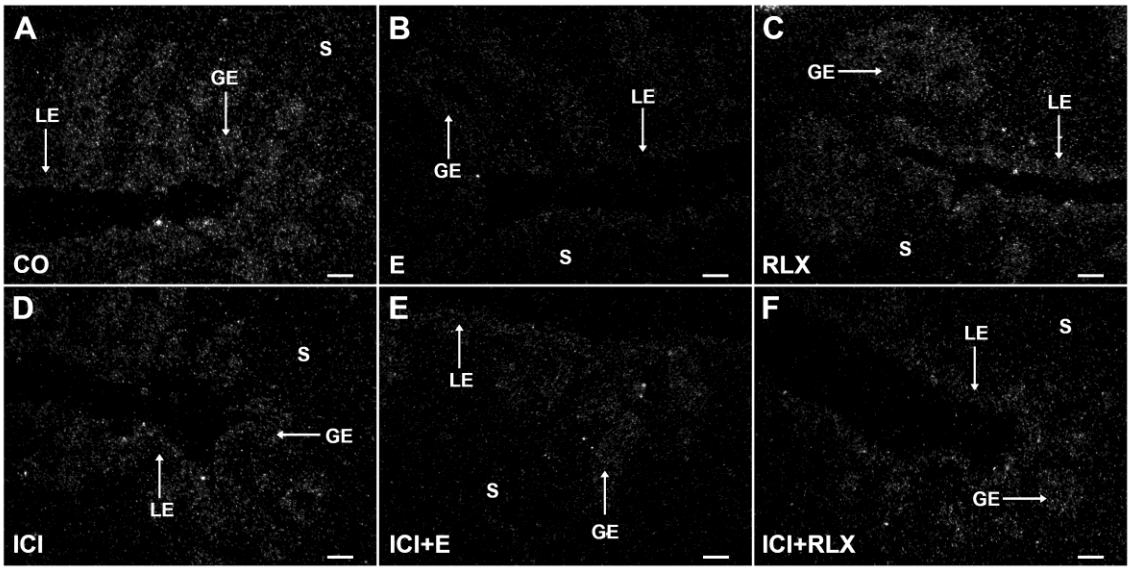


Figure 5. Msx1 Transcript Expression

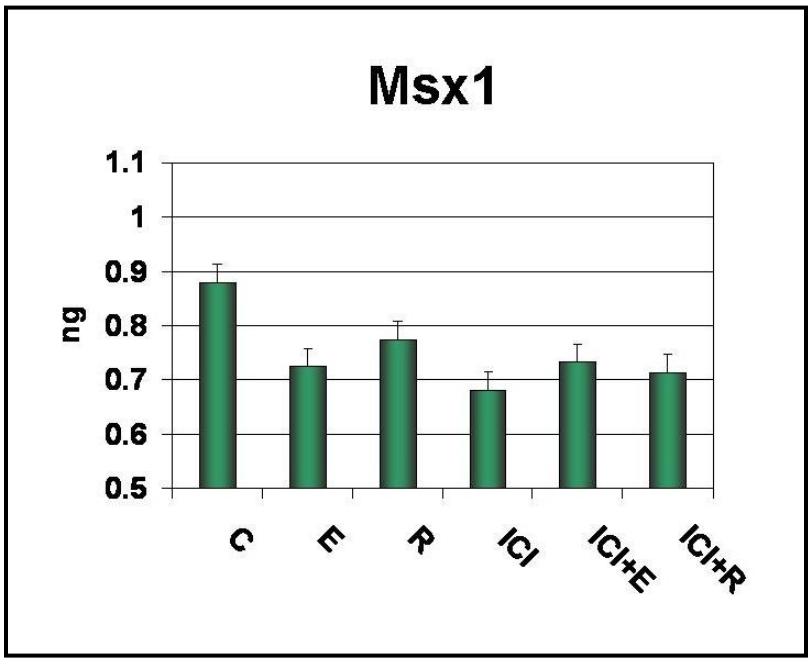
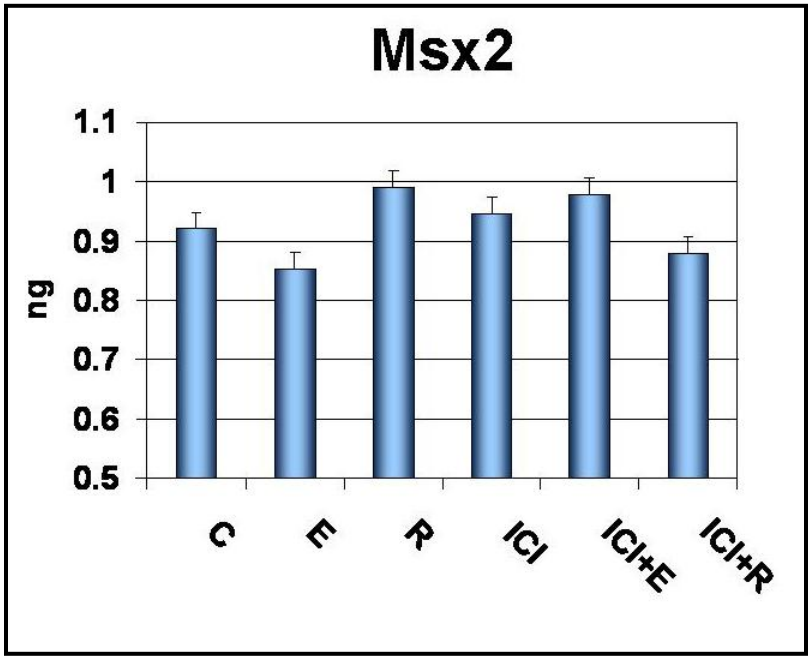


Figure 6. Msx2 Transcript Expression



CHAPTER III

Proteomic Analyses of Porcine Endometrial Proteins Governing Embryo Survival

In mammals, the uterus provides an embryotrophic environment for conceptus (embryo) development and integrates maternal and conceptus signals needed for maintenance of pregnancy. Uterine development begins prenatally, but is completed postnatally. In the pig (*Sus scrofa*), the first two weeks of postnatal life are an important period for developmental programming of the endometrium (uterine mucosa). Disruption of this program by exposure of neonates to estradiol valerate (EV; 50µg/kg BW/day) for two weeks from birth affects adult uterine morphology, endometrial function and, ultimately, decreases embryo survival. These developmentally induced effects on uterine phenotype are pronounced on day 12 post-mating (GD 12). Molecular comparisons of normal (control or CO) and neonatally EV exposed adult uterine tissues from GD 12 should reveal factors necessary for uterine function and embryo survival. The endometrial proteome is the complete set of proteins expressed by this tissue under defined physiological conditions. Objectives were to determine effects of neonatal EV exposure for two weeks from birth on the adult endometrial proteome on GD 12. Effects of treatment (EV vs CO; N=4 gilts/group) on elements of the endometrial proteome were evaluated by two-dimensional electrophoresis (2DE). Proteins extracted from endometrial tissues were separated first by isoelectricfocusing on immobilized pH

gradients (IPG, pH 4-7), followed by electrophoresis in 10% polyacrylamide gels with sodium dodecylsulfate. Gels representing all animals from both treatments were processed together in a Criterion DodecaCell (Bio-Rad, CA). Gels were stained with SYPRO Ruby (Bio-Rad), images generated using a Typhoon 9410 (Amersham, NJ), and 2DE patterns were evaluated using PDQuestsoftware (Bio-Rad). The adult porcine endometrial tissue proteome of GD 12 was estimated to contain over 2000 unique elements. Systematic image analyses will permit assessment of potential differences between EV and CO 2DE fingerprints. Procedures represent foundational elements of a novel strategy for identification of uterine proteins required for the success of pregnancy. Supported by USDA-NRI 98-3203-6198, 2003-3520313572; AU-CMB USRS funds.

Introduction

In mammals, the uterus provides an embryotrophic environment for conceptus (embryo) development and integrates conceptus and maternal signals necessary for embryo survival and fetal growth [5]. These important functions are born by the uterine mucosa or endometrium. The capacity of adult endometrial tissues to support these processes is dictated, in part, by developmental events that program endometrial functionality [210].

Uterine development begins prenatally and is completed postnatally [4]. In the pig (*Sus scrofa*), postnatal development of the uterine wall is significant. Between birth (postnatal day = PND 0) and PND 14, nascent endometrial glands differentiate from luminal epithelium to form coiled, branched structures that penetrate endometrial stroma as uterine tissues continue to differentiate biochemically [1, 4, 13]. Genesis of normal

adult uterine histoarchitecture and function depends upon the success of estrogen sensitive, estrogen receptor-α (ER)–dependent morphogenetic and cytodifferentiative events associated with endometrial development between birth and PND 14 [1, 13].

Disruption of the normal course of uterine development by exposure of neonatal pigs to estradiol valerate (EV) during the first two weeks of postnatal life alters adult uterine morphology and compromises uterine functionality [4, 13, 14, 224, 251]. When compared to vehicle-treated controls, effects of transient neonatal estrogen exposure (PND 0-13) were most pronounced in pregnant adult, neonatally EV-exposed gilts on day 12 postmating [14]. Neonatal EV treatment did not affect the ability of adult gilts to display estrous cycles of normal duration, ovulation rates or conception rates. However, neonatal EV inhibited uterine growth response to local conceptus signals, reduced uterine luminal protein content and endometrial protein biosynthetic activity, and altered endometrial mRNA expression patterns for several primary endometrial proteins including keratinocyte growth factor, retinol binding protein and uteroferrin [14]. In addition, EV administered from PND 0-13 increased embryo mortality by 22% in adult, neonatally EV-exposed gilts on gestational day 45 [4]. Data indicate that the neonatally EV-exposed adult endometrium is functionally lesioned. Therefore, comparisons of normal adult endometrium to neonatally EV-exposed endometrium may provide important insights into aspects of endometrial function that are essential for embryo survival. Early embryonic mortality is one of the principle causes of reproductive inefficiency [252].

Objectives for this study were to determine effects of neonatal EV exposure from birth through postnatal day 13 on the adult endometrial proteome in tissues obtained from pregnant gilts on day 12 post-mating using 2DE and image analysis.

Materials and Methods

Animals and Tissue Collection

Procedures for animal care, administration of treatments and tissue collection were approved by the Auburn University institutional animal care and use committee. Details are described elsewhere [8].

Briefly, 36 white crossbred gilts were assigned randomly at birth (PND 0) to one of two treatment groups (N=18/group). Half of the gilts were treated with estradiol-17β valerate (EV; 50ug/kg/day; im) beginning on PND 0 through PND 13, while the other half were given corn oil (CO) vehicle as the control group. All gilts were allowed to cycle at least twice before tissue collection. Ultimately, 9 EV and 9 CO gilts were bred and determined to be pregnant based upon the presence of embryos in uterine flushings. Gilts were hysterectomized on day 12 postmating. Endometrial proteins from a subset of 4 CO and 4 EV gilts, chosen at random, were evaluated in this study.

Extraction of Endometrial Proteins

Adult endometrial tissues stored at -80º C were subjected to sonication in reagent 3 of the Bio-Rad (Hercules, CA) ReadyPrep Sequential Extraction Kit. This reagent was used to help solubilize and stabilize extracted endometrial proteins.

Protein Quantification

The Bio-Rad RC-DC Protein Assay was employed to quantify proteins extracted from endometrial tissues. Use of this assay was necessary to accommodate the presence of reducing agents in the extraction buffer. Standard curves were prepared using bovine serum albumin (BSA).

Two-Dimensional Electrophoresis (2DE)

Endometrial proteins were separated by two-dimensional electrophoresis (2DE). Equal amounts of total protein (70μg/gel) from each sample were subjected to isoelectric focusing (IEF) in the first dimension using immobilized pH gradient strips (ReadyStrip IPG strips, 11cm, pH4-7; Bio-Rad) and a Bio-Rad Protean IEF Cell. Second dimension separations were performed in 10% (w/v) polyacrylamide gels with sodium dodecyl sulfate (SDS-PAGE; Criterion Pre-cast 11cm gels; Bio-Rad). Duplicate 2-DE gels were run for each animal. A 'master' reference sample was prepared by combining proteins from all gilts (CO+EV) and subjecting this protein mixture to 2DE with each set of eight individual samples. Individual samples representing the master mix and proteins from the eight individual animals [4 CO (A-D) and 4 EV (E-H)] were electrophoresed together using the Bio-Rad Criterion Dodeca Cell. Gels were stained with SYPRO® Ruby (Bio-Rad) and images were produced using a Typhoon 9410 (Amersham, NJ). Images were analyzed using PDQuest software (Bio-Rad).

Results

2DE Analysis of proteins extracted from endometrium obtained on gestational day 12 in CO and EV gilts

Figure 1 depicts representative 2DE gel images depicting the array of proteins present in: (A) the 'master' mix of all endometrial protein extracts used in the study; and (B) gels representing an individual CO and EV-treated gilt. Proteins were separated by isoelectric point in the 1st dimension (pH 4-7) and by molecular weight in the 2nd dimension using SDS-PAGE. A liberal PDQuest analysis of the master image indicated that the GD12 porcine endometrial proteome could contain 2000 elements as detected using Sypro® Ruby staining and the Typhoon 9410 imager system.

Quantitative analysis of 2DE images generated from CO and EV gilts using PDQuest

Quantitative analysis of 2DE image sets using PDQuest permits evaluation of fold changes in spot intensity. Three types of analyses were performed to determine if : **(1)** EV spot intensity is at least 2X that of the corresponding CO spot (upper limit analysis); **(2)** spot intensity in EV < 0.5x quantity in CO (below lower limit analysis); and **(3)** spot intensity in EV was not different from CO (between limits analysis). Results of these analyses for a subset of 717 spots determined to be consistent elements of the GD12 endometrial proteome are summarized in Table 1 and illustrated in Figure 2. Image reports illustrate distribution of spots (black) in each category on 2DE master gel images by pI (top; left to right) and molecular weight (left; Kd $x10^{-3}$).

Discussion

In this study, two-dimensional electrophoresis was used to evaluate endometrial proteins extracted from uterine tissues obtained on gestational day (GD) 12, a critical time period for maternal recognition of pregnancy, after exposure to EV for two weeks from birth. During the first two weeks of neonatal life, the porcine uterus undergoes an estrogen-sensitive, estrogen receptor (ER)-dependent organizational process where ER-positive endometrial glands differentiate, proliferate, coil and extend through the stroma towards the myometrium. In previous studies, the neonatal organizational program was disrupted by exposure of gilts to EV for two weeks from birth with lasting effects on endometrial function in adult gilts.

In previous studies, administration of EV for 7 days prior to hysterectomy on PND 7, 14, or 49 was uterotrophic and increased uterine weight and advanced gland genesis [4, 253]. In contrast to these results for the neonatal uterus, administration of EV from birth through PND 14, decreased uterine weight, uterine horn length, uterine luminal fluid protein content by PND 100 [224] and caused a 22% decrease in embryo survival on GD 45 [4]. Administration of EV for two weeks from birth also altered endometrial steroid responses in prepubertal gilts [224].

One of the main functions of the uterus is to recognize conceptus signals to establish pregnancy and provide an environment for conceptuses to grow and develop. In the pig, conceptuses secrete estrogen as the signal for maternal recognition of pregnancy at GD 12. Once pregnancy has been established, the uterus begins to secrete "histotroph" which contains peptides, proteins, enzymes, protease inhibitors, growth factors and cytokines that are necessary for conceptus growth, development, and attachment. It is

possible that the endometrial proteome on GD 12 is altered by transient neonatal exposure to EV from two weeks from birth.

The main objective of this study was to determine if there were any changes in the endometrial proteome on GD 12 after neonatal exposure to EV for two weeks from birth. Although 2000 spots were detected in the adult endometrial proteome, a subset of 717 polypeptides were determined to be consistent elements of the GD12 endometrial proteome. Image analysis revealed that although a majority of the conserved subset of protein spots were present in approximately equal abundance, 99 protein spots were present in relatively lower abundance and 201 protein spots were present in relatively higher abundance in neonatally estrogen exposed tissues. Results indicate that disruption of the neonatal uterine organizational program by exposure of gilts to EV for two weeks from birth has lasting effects on endometrial function in adult gilts that are reflected by dramatic changes in the endometrial proteome on GD12. These changes are likely to affect conceptus-maternal interactions necessary for establishment and maintenance of pregnancy and embryo survival.

Figure 1A. Representative 2DE gel image depicting the 'master' mix of all endometrial protein extracts used in the study.

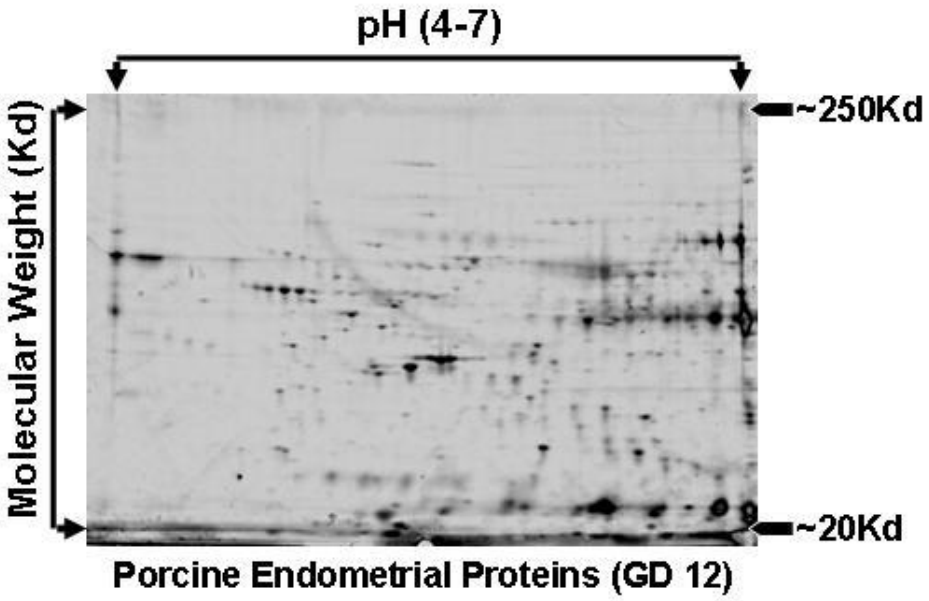


Figure 1B. Representative 2DE gel image depicting gels representing an individual CO and EV-treated gilt.

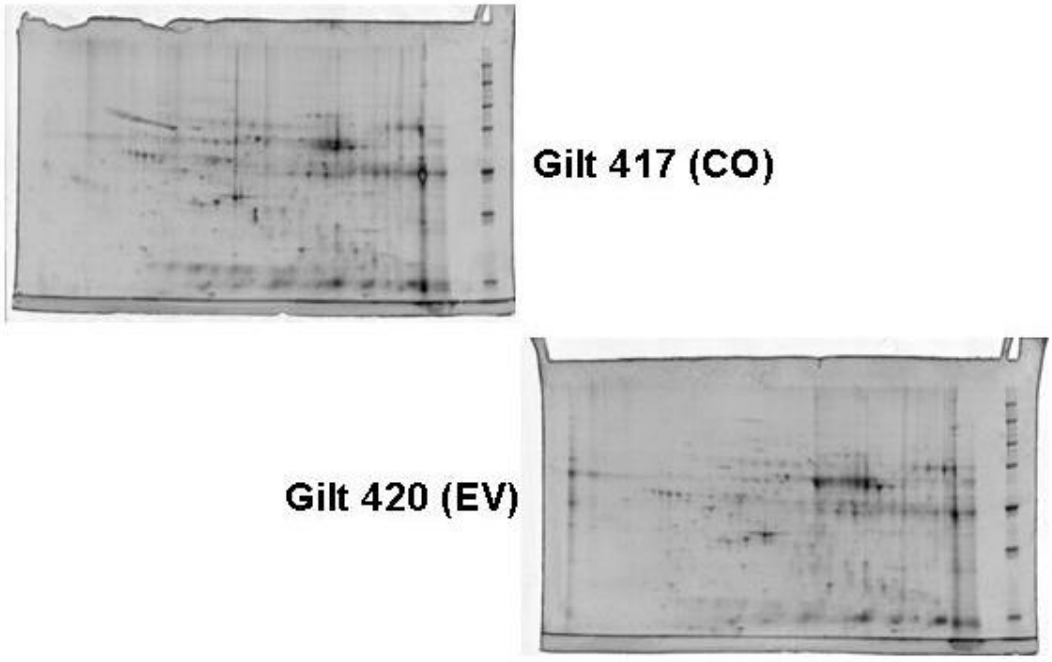


Figure 2.

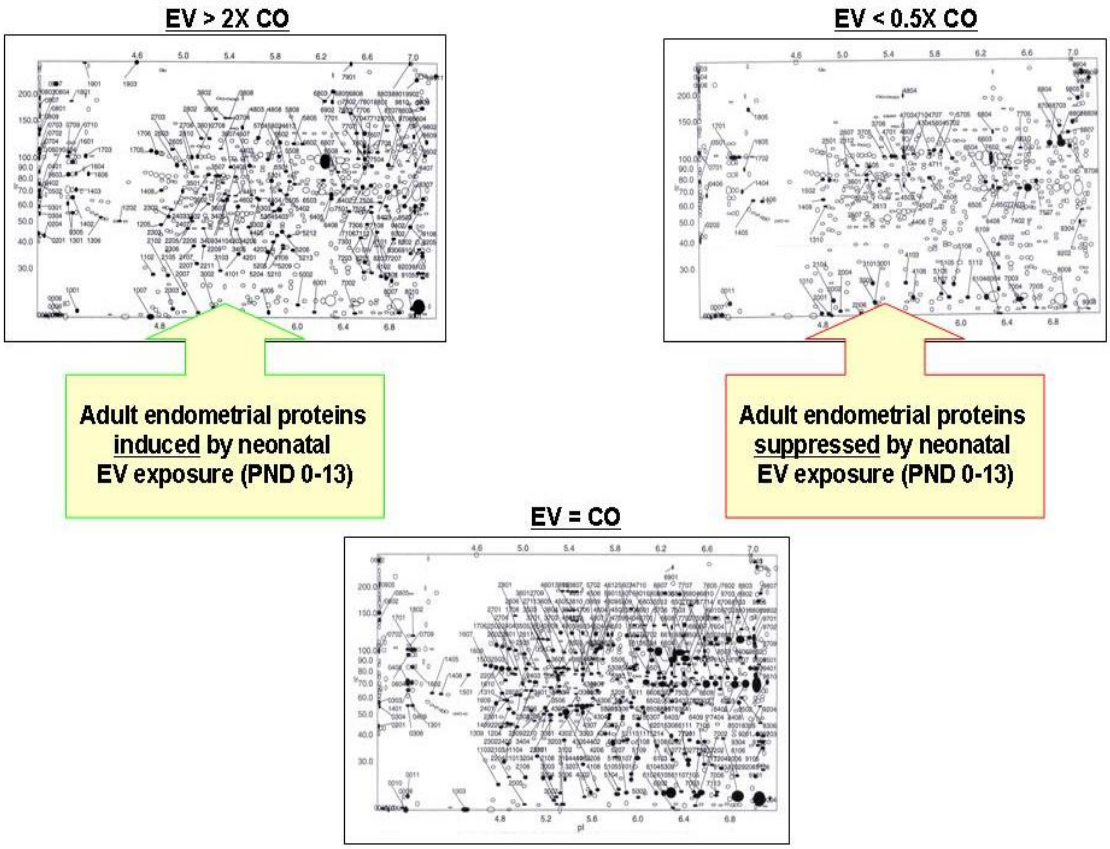


Table 1. 2DE Image Analysis

Spot Intensity Relationship	Number of Spots Identified	Image Analysis
EV ≥ 2X CO	201	Upper Limit
EV < 0.5X CO	99	Lower Limit
EV = CO	326	Between Limits

SUMMARY AND CONCLUSIONS

Development of the porcine uterus is a complex process that when altered can compromise adult form and function [4]. At birth, the uterus consists of myometrium, stroma and luminal epithelium (LE), but during the first two weeks of neonatal life, the uterine wall undergoes rapid developmental changes. Glandular epithelium proliferates and differentiates from LE to form coiled, tubular glands that penetrate deep into the stroma [3, 4, 13]. Formation of endometrial glands is associated with the appearance of nuclear estrogen receptor α (ER) expression in stromal cells as well as in GE, but is not generally apparent in LE [1]. Data for the pig indicate that the success of estrogen-sensitive, ER–dependent organizational events associated with development of the uterine wall during the early neonatal period dictate the extent to which adult tissues are able to function optimally in support of reproduction [3, 4].

Organization of the neonatal porcine uterus is dependent on interactions between the stroma and the epithelium. These interactions play a pivotal role in mediating steroid and peptide horomone effects [10] as well as actions of morphoregulatory gene products. Previous studies have indicated that morphoregulatory genes play a role in patterning and development of the neonatal porcine uterus. These genes include members of the Wnt [3, 11] gene family as well as the abdominal-B Hoxa [3, 12] gene family. Morphoregulatory gene expression patterns can be disrupted by transient exposure of neonates to estrogen or factors that affect the state of ER activation.

Members of the Wnt gene family identified in the developing porcine uterus include Wnt4, Wnt5a and Wnt7a. Vertebrate Wnt genes are related to *wingless* (*wg*), a segment polarity gene first discovered in *Drosophila*. In the developing porcine endometrium, Wnt7a is expressed in the LE and Wnt4 and Wnt5a are expressed in the stroma beginning at PND 7 and increasing by PND 14 [3]. Previous studies have indicated that *Hedgehog* (*hh*), a gene discovered in *Drosophila* that encodes secreted glycoproteins, regulates *wg* expression. For example, expression of *wg* in the *Drosophila* embryo was induced in cells adjacent to Hh-expressing cells [228, 229]. Therefore, it is possible that Indian hedgehog (Ihh), a vertebrate homologue of *hh*, regulates Wnt gene expression in the neonatal porcine endometrium.

Consequently, the first study, presented in chapter 1, investigated whether Ihh and its receptor, Patched-1 (Ptc), were present in the neonatal and adult porcine endometrium. Also, the effects on endometrial Ihh and Ptc expression after estrogen was administered for two weeks from birth were observed. At birth, Ihh mRNA expression was observed in the LE, stroma and myometrium of the neonatal porcine uterus. By PND 7 and 14, Ihh mRNA was expressed in GE as well as in the LE, stroma and myometrium. Pronounced in the LE, expression of Ihh mRNA in the neonatal uterus on PND 14 was significantly decreased after treatment with EV for two weeks from birth. Like Ihh expression, after transient exposure to EV for two weeks from birth, Wnt7a expression is significantly decreased in the LE by PND 14 [3]. This leads to the possibility that Ihh regulates Wnt7a expression in the neonatal porcine endometrium. Immunohistochemical localization of Ihh mirrored the expression pattern observed with *in situ hybridization*. Protein

localization for Ptc was also observed in the LE, GE and stroma of PND 14 uterine tissue. Unlike the neonate, Ihh mRNA was evident in the stroma, pronounced in the GE, and was minimal or absent in LE in the adult uterus. Localization of Ihh and Ptc protein in the adult uterus is weak in the stroma, minimal or absent in LE and pronounced in GE. Data from study one indicated that Ihh could play a role in neonatal porcine uterine development because Ihh and Ptc are expressed during a critical time period for development.

Members of the abdominal-B Hoxa gene family observed in the neonatal porcine uterus include Hoxa10 and Hoxa11. Studies involving mice and gene-knockout technology discussed in chapter 2 indicated a role for a Wnt/Hox axis to maintain a functionally correct uterine organizational program. In the mouse, highly conserved homeobox genes, Msx1 and Msx2, have been implicated in maintaining the uterine Wnt/Hox axis.

Therefore, the objectives of study two (chapter 2) were to determine if the porcine neonatal endometrium expressed Msx1 and Msx2 mRNA/protein and if treatment with estrogen and estrogen agonists and antagonists on PND 12 and 13 would affect these expression patterns on PND 14. Expression of Msx1 and Msx2 mRNA was observed predominantly in the GE and LE of the porcine uterus on PND 14. Treatment with E, RLX, ICI, ICI+E and ICI+RLX on PND 12 and 13 did not have an effect on Msx1 expression in the porcine uterus as evidenced by *in situ* and relative QPCR when compared with the control. Localization of Msx1 protein mirrors that of the uterine mRNA expression. For Msx2, treatment with E downregulated the epithelial expression

similar to that seen in the neonatal mouse exposed to DES [219]. Downregulation of Msx2 expression was attenuated by ICI as evidenced by *in situ*, but not by relative QPCR. Lack of treatment effects for Msx2 expression was not expected. To analyze relative Msx2 expression, RNA extracted from whole uterine tissue was used. Expression of Msx2 in the uterine stroma and myometrium may have overshadowed the treatment effects observed in the epithelium by *in situ* in the QPCR reaction.

Immunohistochemical analysis also revealed treatment effects in the protein localization. Msx2 immunostaining patterns were localized intermittently in the nucleus of LE and GE in C, E, RLX and ICI+E. Treatment with ICI in the absence of estrogen (RLX and ICI+RLX), significantly increased the immunostaining intensity for Msx2 in the nucleus and cytoplasm of LE, GE and stroma. It is unknown how estrogen regulates Msx2 expression because the promoter of the Msx2 gene lacks an estrogen response element [200]. It is possible that expression of Msx2 may involve non-classical ER signaling. It has been determined that ICI can alter the transcription of estrogen-responsive genes via activation of Sp1 promoter elements [248, 249], and the Msx2 gene contains an Sp-1 promoter [250].

In previous studies, administration of EV for 7 days prior to hysterectomy on PND 7, 14, or 49 was uterotrophic and increased uterine weight and advanced gland genesis [4, 253]. In contrast to these results for the neonatal uterus, administration of EV from birth through PND 14, decreased uterine weight, uterine horn length, uterine luminal fluid protein content by PND 100 [224] and caused a 22% decrease in embryo

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APPENDICES

PRIMER DESIGN

Materials:

- VectorNTI Software (Invitrogen)
- Template sequence

Procedure:

1. Locate template sequence in NCBI database (http://www.ncbi.nlm.nih.gov/) and note Accession number. Also, determine coding sequence (CDS) from NCBI.

2. Open sequence in VectorNTI:
    a. Tools→Open→Retrieve DNA-RNA by GenBank NID.
    b. Insert Accession number for NID and click OK.

3. Select several hundred base pairs within the coding sequence by highlighting and then copy the selected bases by going to:
    a. Edit→Copy Sequence

4. Paste sequence into http://seq.yeastgenome.org/cgi-bin/web-primer and click submit.

5. Click submit again to accept the default parameters.

6. A list of valid primers will be generated. If a list of primers was not available, try choosing a different set of bases within the coding sequence of the template.

7. Click on "This is the BEST pair of primers"

8. Once primers have been generated, make sure they align with the template sequence.

9. Go back to VectorNTI and create a new sequence:
    a. File→Create new sequence→Using sequence editor (DNA/RNA)
    b. Under the General Tab, name the sequence
    c. Click on the DNA/RNA molecule tab and select DNA and linear
    d. Click on the Sequence and Maps tab, then Edit sequence.
    e. Go back to website with best primers and highlight and copy the one of the primers.
    f. Click paste—the primer that you have copied should show up in the white box.
    g. Click OK and the molecule will me saved. To open this molecule go to:
        i. File→Open→Choose Database DNA/RNAs tab→Select molecule and click OK
10. With template sequence still open, go to:
    a. Align→AlignX – Align selected molecules
    b. A new window will open. In the new window go to:
        i. Project→Add files→Select primer
        ii. Highlight both file names. Go to:
            1. Align→Align selected sequences

11. Review alignment and make sure primer sequence matches the template sequence.

12. Repeat this process with the reverse primer.

RNA ISOLATION

Materials:

- TRIzol Reagent (Invitrogen, Cat. no. 10296-010)
- Tissue Homogenizer (PowerGen 125, Fisher Scientific)
- 15mL polypropylene tubes
- 15mL Corex tubes (Fisher Scientific, Cat. no. 05-505-05)
- Adapter rubber for Corex tubes (Fisher, Scientific, Cat. no. 75-002-971)
- Chloroform
- Isopropanol
- 75% ethanol
- Nuclease-free H_2O

Use baked glassware, wear gloves, and make sure all equipment is RNase-free

Procedure:

1. Clean tissue homogenizer with 1mL bleach + 4mL distilled H_2O by turning on homogenizer while immersed in solution.

2. Rinse tissue homogenizer with DEPC-treated H_2O by turning on homogenizer while immersed in DEPC-treated H_2O.

3. Wipe off the tissue homogenizer and turn it on a couple of times to blow the H_2O out, and then wrap a Kimwipe around the homogenizer until you are ready to use it.

4. Add 5mL of TRIzol to 15mL polypropylene tubes. Use one tube per animal/tissue that RNA will be extracted from. Label, weigh and record weights of tubes.

5. Keep TRIzol filled tubes on ice.

6. Collect ~190mg of frozen endometrial tissue (~a scoop and a half of frozen powdered endometrial tissue) and add it to the tubes with TRIzol. Keep the spatula in the freezer between samples.

7. Immerse homogenizer in TRIzol + tissue and run until tissue has been completely homogenized and no pieces of tissue are seen.

8. Incubate at room temperature for 5 minutes.

9. Add 1mL of chloroform to each tube and vortex for 15 seconds.

10. Transfer mixture to corex tubes and add rubber adapters for centrifuging.

11. Before putting tubes into the centrifuge, add chloroform (small amount at a time) to each tube until they are balanced.

12. Make sure the TRIzol and chloroform have not separated into distinct layers. If there are two layers, vortex again for 15 seconds before putting into centrifuge.

13. Centrifuge at 12,000 x g for 15 minutes at 4ºC.

14. Remove most of the clear layer with a plastic pipette and place in a fresh corex tube.

15. Add 2.5mL isopropanol and vortex briefly.

16. Incubate at room temperature for 10 minutes.

17. Before centrifuging, balance tubes with isopropanol.

18. Centrifuge at 12,000 x g for 10 minutes at 4ºC.

19. Pour off supernatant and add 5mL of 75% ethanol.

20. Before centrifuging, balance tubes with 75% ethanol.

21. Centrifuge at 7,500 x g for 5 minutes at 4ºC.

22. Pour off supernatant, quick-spin and pipette off last drop, and let the resulting pellet dry for 5-10 minutes.

23. Add 100µl of nuclease-free H_2O and swirl to make sure everything is removed from the sides of the tube.

24. Centrifuge for 2-3 minutes at >400 x g at 4ºC.

25. Determine volume and transfer to a microfuge tube.

26. Determine concentration by spectrophotometer.
    40 x A260 x dilution factor = X/1000 → Xµg/µL

27. Determine quality of RNA by running it on a Formaldehyde gel (Protocol in next section).

RNA GEL ELECTROPHORESIS

Materials:

- 1.1 M Formaldehyde (~pH4, 12.32 M)
- 10x MOPS Stock (500mL)
    - MOPS (200mM) 20.93g
    - NaAc (50mM) 2.05g
    - EDTA (50mM) 10mL
- DEPC-treated water
- 1.0-1.5 g agarose (1 – 1.5% total; DNase and RNase free)
- Gel Running Buffer = 1x MOPS

Procedure:

1. Make 100mL Formaldehyde gel.
    a. Add 10mL of 10x MOPS to 81mL DEPC-treated H2O.
    b. Add agarose and melt with stirring.
    c. When flask is cool enough to handle, add 8.93mL formaldehyde (37%, 12.32 M) under a hood.
    d. Stir well without introducing bubbles and pour gel into gel holder.
    e. Allow to polymerize for at least an 1 hour.

2. Make sample buffer (per sample).
    a. 10x MOPS 2µL
    b. 50% Formamide 10µL
    c. 2.2M Formaldehyde 3.5µL
    d. 1mg/mL Ethidium bromide 1µL

3. Make a 10x gel loading dye stock for RNA.
    a. 25% glycerol
    b. 0.025% Bromophenol blue

4. Add RNA sample to 16.5µL Sample Buffer.

5. Heat RNA samples in buffer at 65º C for 5 minutes.

6. Chill on ice for 2 minutes.

7. Add 2μL of 10x RNA loading dye.

8. Load gel and run at 150V until dye front reaches bottom or sufficient separation occurs.

MOLECULAR CLONING

RT-PCR

Materials:

- One-Step RT-PCR kit (Qiagen, Cat. no. 210210)
- Forward and Reverse primers for target gene
- Extracted RNA
- Agarose
- 1x TBE
- 1x TE
- Ethidium bromide
- 100 bp DNA ladder (Amresco, Cat. no. K180-250UL)
- 2x Loading dye (Ambion)
- QIAquick PCR Purification kit (Qiagen, Cat. no. 28104)

Procedure:

1. For the forward primer, determine the number of A&T and G&C. Multiply the number of A&T by 2 and the number of G&C by 4. This will give you the melting temperature of the forward primer. Repeat the same process for the reverse primer.

2. Determine the average temperature of the two primers.

3. Follow the instructions for the One-Step RT-PCR kit to create a master mix. In the master mix, use 0.6μM of the forward primer and 0.6 μM of the reverse primer.

4. For the reaction, use 1μg of RNA in 5μl of H_2O and make sure to include a No Template control (H_2O only).

5. Create a PCR program. If there is a 2º difference between the forward and reverse melting temperatures, a touch down program is normally used. If the melting temperatures are the same, then only one temperature is used. For example:

--One Temperature--

1. 50º C 30 minutes Reverse transcription
2. 95º C 15 minutes Activates HotStarTaq DNA polymerase
3. 94º C 30 seconds Denaturation
4. XXº C 30 seconds 5º C below the melting temp of the primers Annealing
5. 72º C 30 seconds Extension
6. Go to:

   Step 3 36 more times
7. 72º C 10 minutes Final extension
8. 4º C 000 Hold step
9. End

--Touchdown--

1. 50º C 30 minutes Reverse transcription
2. 95º C 15 minutes Activates HotStarTaq DNA polymerase
3. 94º C 30 seconds Denaturation
4. Option:

   Increment step

   XXº C 30 seconds 5º C above the average melting temp of the primers

   -1º C/cycle
5. 72º C 30 seconds Extension
6. Go to Step 3 10 more times
7. 94º C 30 seconds Denaturation
8. 55º C 30 seconds Annealing

9. 72º C 30 seconds Extension

10. Go to:

    Step 7 25 more times

11. 72º C 10 minutes Final extension

12. 4º C 000 Hold step

13. End

6. Electrophorese PCR product on an agarose gel to determine if a product was generated and if it is the correct size. *Use a 2% gel for small PCR products and a 1% gel for large products.*
   - **2% Gel:**
     - 0.8g agarose
     - 40mL 1x TBE
     - Heat mixture together until agarose has melted, and let cool slightly
     - Add 1.5µL ethidium bromide and swirl to mix
     - Pour mixture into gel cast, add well combs and let polymerize (~15-30 minutes in fridge)

7. Once the gel has polymerized, load samples and DNA ladder combined with loading dye into wells. Load 5µL DNA ladder + 2µL 2x loading dye into first well. Load 12µL PCR product + 4µL 2x loading dye into wells starting with the 2nd well.

8. Run gel at 125V for ~20 minutes. (Run to the red)

9. If PCR product was generated and is the predicted size, purify remaining product that was not run on a gel using the QIAquick PCR Purification Kit and follow manufacturers instructions.

10. Run purified product on gel (Step 6-8).

11. Sequence remaining purified product. (Use AU Sequencing Lab).

LIGATION AND TRANSFORMATION OF COMPETENT *E. coli* CELLS

Materials:

- QIAGEN PCR Cloning^{plus} Kit (QIAGEN, Cat. no. 231222)
    - DH5α cells (Invitrogen, Cat. no. 18258-012) can be used in place of the EZ Competent cells that come with the QIAGEN kit
- Purified PCR product
- 100 mM IPTG
- XGal (20mg/mL)
- LB Agar plates
- Ampicillin [50mg/mL], (sterilize with syringe tip filter)

Procedure:

1. Ligate purified PCR product into pDrive vector using the PCR cloning kit per manufacturer's instructions. Can ligate longer than 30 minutes (sometimes ligated overnight). Ligate at 14ºC in thermalcycler.

2. Run ligation reaction on 1% agarose gel.

3. Add 40µL XGal and 50µL of 100mM IPTG to prepared LB agar plates, spread with a flamed glass rod and let it soak in.

4. Transform EZ Competent cells per manufacturer's instructions.

5. Plate 100µL transformed EZ Competent cells on the LB agar plates. Spread around with flamed glass rod and let soak into plate.

6. Incubate the plate upside down overnight in incubator at 37º C.

7. To store plates, remove from incubator, seal edges with parafilm and refrigerate upside down.

500 mL LB Agar Plates:

- Bacto-tryptone 5g
- Yeast extract 2.5g
- NaCl 5g

Adjust pH to 7.0 and bring volume up to 500mL with distilled H_2O.

- Agarose 7.5g

1. Add agarose to large flask and then pour in the liquid.
2. Cover loosely and autoclave.
3. Let cool to about 55ºC in water bath.
4. Add 2mL Ampicillin to mixture.
5. Pour liquid into petri dishes, put lids on part way and let solidify.
6. Once solidified, close lids, place back into plastic sleeve and store upside down at 4ºC until ready to use.

PCR SCREENING

Materials:

- HotStarTaq Master Mix Kit (QIAGEN, Cat. no. 203443)
- LB Agar plate
- Sterile wooden sticks

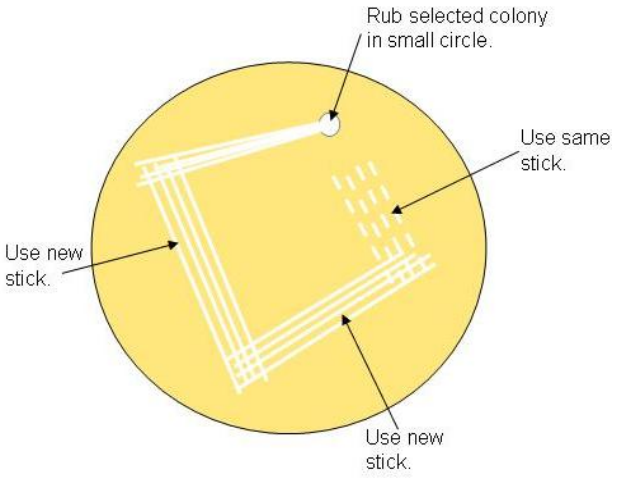
Procedure:

1. Add 40µL XGal and 50µL of 100mM IPTG to prepared LB agar plate and let it soak in.

2. Prepare PCR reaction:
   <u>One Tube (1.1)</u>

   - HotStarTaq 4.8µL
   - PrimerF 0.24µL
   - PrimerR 0.24µL
   - H_2O 4.3µL

3. Pick one white colony from plate grown overnight with wooden stick and touch bottom of PCR tube, streak prepared plate and incubate at 37ºC upside down overnight.



4. Run PCR Screen program:

    1. 95º C 15 minutes Activates HotStarTaq DNA polymerase
    2. 94º C 30 seconds Denaturation
    3. 55º C 30 seconds Annealing
    4. 72º C 30 seconds Extension
    5. Repeat steps 2-4 for 34 more times
    6. 72º C 10 minutes Final extension
    7. 4º C 000 Hold step
    8. End

5. Run PCR screen on 2% gel to determine if product has been incorporated into cells.

SMALL-SCALE PREPARATIONS OF PLASMID DNA (MINI-PREP)

Materials:

- QIAprep Spin Miniprep Kit (QIAGEN, Cat. no. 27104)
- LB Broth
- EcoR1 (New England BioLabs, Cat. no.R0101L)
- NEBuffer EcoR1 (New England BioLabs, Cat. no. B0101S)

Procedure:

1. Remove plate streaked during PCR screening from incubator, seal edges with parafilm and refrigerate upside down until ready to grow miniprep.

2. Pick several white colonies and inoculate test tubes filled with 5mL of 37ºC LB Broth (1 colony/culture tube). After inoculating culture tube, streak another plate prepared with IPTG and X-gal and incubate upside down overnight at 37º C.

3. Let grow 12-16 hours at 37º C in rotating incubator.

4. Place culture tubes on ice.

5. Swirl to resuspend cells and pour into a 1.5mL microfuge tube.

6. Centrifuge microfuge tube for 4 minutes at 10,000 x g.

7. Discard supernatant.

8. Refill same tube with more broth.

9. Repeat steps 5-7 until all broth has been centrifuged.

10. After incubating for 12-16 hours, follow manufacturer's instructions in QIAprep Spin Miniprep Kit to purify the plasmid DNA.

11. Determine resulting DNA concentration by spectrophotometer.

12. Cut ~1µg resulting DNA with EcoR1:

DNA volume	H_2O	10x Buffer	Enzyme	Total Volume
2µL	15µL	2µL	1µL	20µL

13. Incubate at 37º C for 1 hour.

14. Run both cut and uncut product on 1% agarose gel.

15. Sequence EcoR1 cut DNA. (Use AU Sequencing Lab)

1L LB Broth:

- Bacto-tryptone 10g
- Yeast extract 5g
- NaCl 10g

1. Adjust to pH 7.0 and bring volume up to lL with distilled H_2O.
2. Pour into 1L bottle and make sure lid is loose before autoclaving.
3. Let cool to about 55ºC.
4. Add 4mL Ampicillin to broth.
5. Store at 4ºC.

LARGE-SCALE PREPARATIONS OF PLASMID DNA (MAXI-PREP)

Materials:

- QIAGEN Plasmid Maxi Kit (Qiagen, Cat. no. 12162)
- LB Broth
- Sterile Glycerol
- Large plastic bottles
- EcoR1 (New England BioLabs, Cat. no.R0101L)
- NEBuffer EcoR1 (New England BioLabs, Cat. no. B0101S)

Procedure:

1. From mini-prep streak plate, choose colony and inoculate 5mL of 37º C LB Broth.

2. Incubate at 37º C in rotating incubator for several hours. Grow to late log phase (OD_{600} of ~0.6).

3. Pour mini-prep into 100mL 37º C LB Broth. (Use 500mL erlenmyer flask. Bottle needs to be at least 5 volumes larger than the size of the culture).

4. Incubate at 37º C on rotary shaker (300 cycles/minute for 12-16 hours).

5. Make sure cells are in suspension and take 850µL of broth and add it to 150µL of sterile glycerol. Vortex mixture to evenly disperse glycerol. Store at -80º C.

6. Pour broth into large plastic bottles and centrifuge at 6,000 x g for 15 minutes.

7. Follow manufacturer's instructions for Maxiprep Kit.

8. Determine DNA concentration with a spectrophotometer.
    a. A_{260} x 50 x dilution factor = x/1000 → xµg/µL

9. Cut ~ 1µg DNA with EcoR1.

DNA volume	H_2O	10x Buffer	Enzyme	Total Volume
2µL	15µL	2µL	1µL	20µL

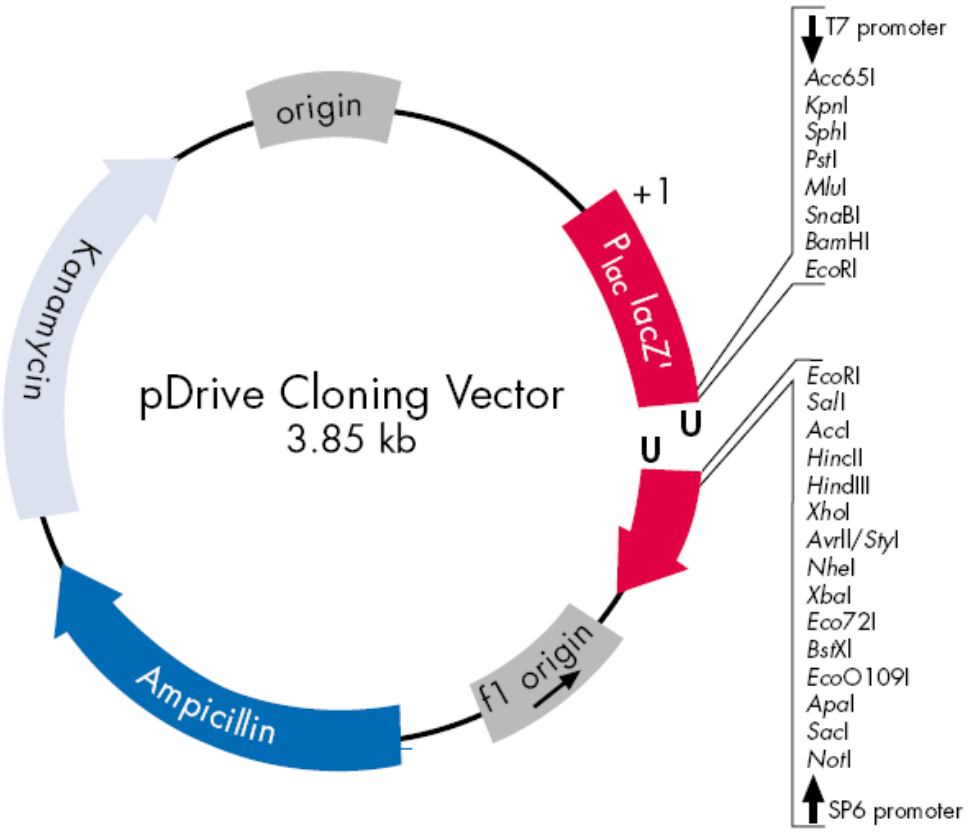
10. Run cut and uncut DNA on 1% agarose gel.

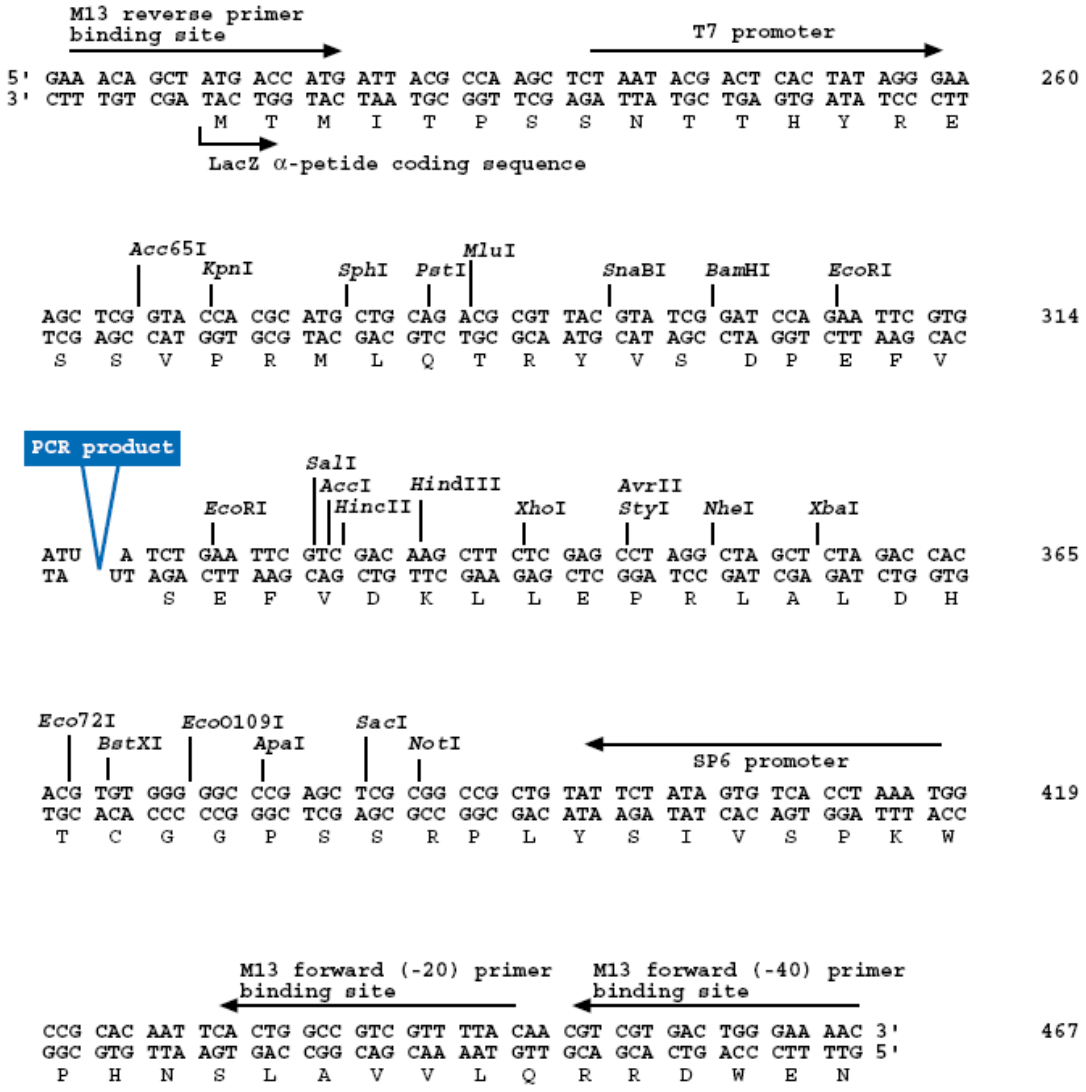
11. Sequence EcoR1 cut DNA. (Use AU Sequencing Lab)

DNA DIGESTION WITH RESTRICTION ENZYMES

Materials:

- Selected Restriction Enzyme and corresponding buffer (New England BioLabs)
- Chloroform:Isoamyl Alcohol 24:1 (Amresco, Cat. no. X205)
- Phenol, Buffer Saturated pH 6.6/7.9 (Amresco, Cat. no. 0945)
- 3M NaOAC
- 1x TE
- 100% EtOH
- 70% EtOH





Procedure:

1. Determine the appropriate restriction enzymes needed to linearize the DNA without cutting the PCR product. Use http://tools.neb.com/NEBcutter2/index.php to find appropriate enzyme. Paste PCR product sequence into white box, choose linear and NEB enzymes, then click submit. In the results, select two enzymes from the noncutters list that are found on either side of the PCR product.

2. Cut 30µg of DNA in both directions. For example, cut 30µg of DNA with Kpn1 and another 30µg of DNA with Xho1. Follow the manufacturer's instructions for the Restriction Enzyme and coordinating buffer.

3. Once DNA has been linearized, extract it with phenol:chloroform.
   Chloroform:Phenol Extraction

   1. Bring volume of linear reaction up to 200µL with 1x TE.
   2. Add 200µL chloroform:phenol (bottom layer of bottle).
   3. Vortex for 1 minute.
   4. Centrifuge at 10,000 x g for 10 minutes at room temperature.
   5. Transfer the aqueous phase to a new tube leaving about 10% behind.
   6. Add 200µL 1x TE to beginning tube.
   7. Vortex for 1 minute.
   8. Centrifuge at 10,000 x g for 10 minutes at room temperature.
   9. Remove aqueous layer and combine with the 1st aqueous layer.
   10. Record volume of combined aqueous layers.
   11. Add 0.1 volume of 3M NaOAC and 2 volume of 100% EtOH (precipitate DNA) and incubate at -20º C for 30 minutes.
   12. Centrifuge at 14,000 x g for 10 minutes at 4º C.
   13. Remove supernatant.

14. Wash pellet with 500µL of 70% EtOH.

15. Vortex and centrifuge at 14,000 x g for 5 minutes at 4º C.

16. Remove liquid and quick spin to remove last of liquid.

17. Leave tube open and let EtOH evaporate, but don't let the pellet dry out.

18. Dissolve pellet in 30µL of nuclease-free H_2O.

19. Determine DNA concentration with spectrophotometer.
    a. A_{260} x 50 x dilution factor = X/1000 → Xµg/µL

20. Run phenol:chloroform extracted DNA on 1% gel.

21. Sequence linearized and unlinearized DNA. (Use AU Sequencing Lab).
    a. Sequence both, so you know which is sense and anti-sense

4. Once sequence has been returned, use BLAST to determine which linearized DNA is sense and which one is anti-sense. http://blast.ncbi.nlm.nih.gov/Blast.cgi
   a. Choose nucleotide blast
   b. Copy one linearized sequence and paste it into the Query Sequence box
   c. Click BLAST
   d. When results are displayed, scroll to where the query sequence is paired with another (subject) sequence.
   e. If the subject is aligned with the query in a descending order, that piece of linearized DNA is anti-sense. For example: Query sequence = 3-62 and Subject sequence = 501-442. If the opposite is observed, then the linearized DNA is sense.

IN VITRO TRANSCRIPTION OF cRNA

Materials:

- Maxiscript Sp6/T7 Kit (Ambion, Cat. no. AM1320)
- Linearized template cDNA for target gene
- Radiolabeled nucleotide
  [α-^{32}P]UTP, at ~800 Ci/mmol and 10mCi/mL

- DEPC-treated H_20
- Acrylamide, 40% total monomer, 19:1 Acrylamide/Bis
- Urea (crystalline)
- 5x TBE
- 10% Ammonium Persulfate (APS) – Make fresh every time!
- TEMED

Procedure:

1. Follow manufacturer's instructions for Maxiscript kit to create radiolabeled cRNA probe. This probe can now be used for *in situ* hybridization.

2. Run probe on Mini 0.75mm 4% Polyacrylamide gel to make sure radiolabeled probe is the right size.

3. Add components to flask with small stir bar.
   a. Acrylamide 1.7mL
   b. Urea 8g
   c. 5x TBE 3.33mL
   d. DEPC-H_2O 4.6mL

   Stir to solubilize components—then add APS and TEMED before pouring

   e. 10% APS 167µL
   f. TEMED 16.7µL

4. Pour gel between plates until reaches top edge of lower glass plate.

5. Insert comb.
6. Allow gel to polymerize for ~1 hour.

7. Use 10-20mL syringe to blow any dense urea solution out of wells before loading denatured IVT reaction mixture.
    a. Set Dri-block for 95ºC.
    b. Add 9µL gel loading buffer (95% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue, 18mM EDTA, 0.025% SDS) to a microfuge tube.
    c. Add 1µL IVT reaction to buffer.
    d. Incubate at 95º C for 5 minutes.

8. Run gel at 200-300 V until tracking dye reaches within 1cm of bottom. Tank buffer is 1x TBE.

In Situ Hybridization **(ISH)**

Tissue preparation--paraplast embedded tissue:

1. Fix tissue in 4% PAF--overnight--small pieces may take less time.
2. Dehydrate tissues in ethanol (70-100%) --tissue can be stored indefinitely in 70% ethanol.
3. Clear tissues in xylene or xylene substitute, Hemo-De, 2-3 changes.
4. Infiltrate tissues with melted paraplast, 2 changes.**
5. Embed tissues fresh melted paraplast, tissues can be stored at room temperature indefinitely.

**Our lab uses an autotechnicon to dehydrate, clear and infiltrate tissue with paraplast using a routine schedule.

Slide preparation:

1. Remove slides from new package and clean in 1% HCl in 70% ethanol, dry. Use baked glassware, slide carriers and wear gloves.
2. Dip clean, dry slides in freshly prepared 2% solution of 3-amino-propyltriethoxysilane in acetone for 5 secs.
3. Rinse briefly, twice in acetone, and once in depc-treated H2O.
4. Dry slides overnight at 42 C, and store at room temperature.

Tissue sections:

1. Place tissue blocks in refrigerator to cool, this makes sectioning easier.
2. Use a new disposable blade or clean blade with RNase away.
3. Wearing gloves section tissue at 5-6um.
4. Float sections in water bath filled with depc-treated water and place on prepared slides.
5. Dry sections completely at 42-45 C, overnight.
6. Place dry slides in new slide box , include some dry-rite in a kimwipe, and place in plastic bag in -20 freezer. Alternatively slides may be kept at room temperature.

Tissue processing:

1. Hemo-De 2x 3-5 min
2. 100% EtOH 2x 2 min (discard)
3. Rehydrate through :

   100% 2 min

   100% 2 min

   95% 2 min

   85% 2 min

   70% 5 min

   --Make dilutions of EtOH in DEPC treated H2O.
4. 0.5X SSC- 5.0 min
5. Post-fix in freshly prepared 4% PAF 10 min.

6. Wash in0.5X SSC 2 x 5 min each.
7. Incubate sections in Proteinase-K 20 ug/ml in0.5M NaCl and 0.1M Tris(pH 7.5) for 7.5 min @ RT. (Stock P-ase-K=10mg/ml--dilute 1:500).This step can vary depending on tissue(ie time and concentration).
8. Rinse sections in 0.5X SSC, 5 min.
9. Post-fix in 4% PAF for 5 min (reuse solution from step 5).
10. Wash sections in DEPC H2O 1 min.
11. Wash in PBS--5.0 min.
12. Dehydrate in graded EtOH --same as in step 3(70%-100%).
13. Air dry and use slides on the same day for hybridization or store acetylated slides at -80 C in a tightly sealed clean slide box. Warm slide box before opening.

Hybridization

1. Determine probe concentration and dilute both to the same concentration. For small sections 30-50 ul/section should be sufficient.
2. Heat probe in hybridization mixture to 80 C for 5 min.
3. Place hyb. Mix on sections which have been previously encircled with PAP pen. Do not coverslip.
4. Place slides in a humid chamber lined with paper soaked in 5X SSC and 50% formamide.
5. Incubate in a 55 C oven overnight--16 hrs.

Washing--Day 2

Wash one rack (25 slides) in 250-500 ml.

1. Wash in 5X SSC , 10 mM BME (beta mercaptoethanol) for 30 min at RT.

2. Wash in 5X SSC, 10mM BME for 30 min at 55 C, prewarmed.
3. Wash in 2X SSC, 50% formamide, 100mM BME for 30 min at 65 C, prewarmed.
4. Wash in prewarmed .5M NaCl, 10 mM Tris pH 8.0, 5 mM EDTA(TEN) once for 10 min at RT.
5. Wash 3 more times in TEN, 10 min each at 37 C.
6. Treat with RNase 20 ug/ml in TEN for 30 min at 37 C.
7. Rinse in TEN for 15 min at 37 C.
8. Wash in 2X SSC, 50% formamide, 50mM BME for 30 min at 65 C.
9. Wash in 2X SSC for 15 min at 65 C.
10. Wash in .1X SSC for 15 min at 37 C.
11. Dehydrate by quickly processing through 70%, 80%, 95% ethanol made with 0.3 M ammonium acetate, then through 100% ethanol twice RT.
12. Air dry and expose to X-ray film overnight at RT.

Autoradiography:

1. The following steps must be carried out in a darkroom. Melt the NTB-2 emulsion in original bottle at 42 C for approximately 20 mins. Pour slowly into a large flask containing an equal amount of water (118ml). Swirl gently to mix, avoid causing bubbles, aliquot in conical tubes to contain ~ 10-15ml. Wrap tightly with aluminum foil and store away from all sources of radiation.
2. Melt an aliquot of emulsion at 42 C for 20 min and pour into a dipping chamber-a slide mailer will work- keep emulsion dipping chamber in water bath set to 42 C.
3. Dip each experimental slide, withdrawing evenly and quickly. Drain the bottom edge against the top of the dipping chamber and wipe off the back of each slide.

4. Allow each slide to dry vertically for 5-10 min against a test tube rack. Transfer to open slide boxes in a light-tight box containing desiccant and leave to dry at room temperature for 2 hours.

5. Close slide boxes, wrap tightly with aluminum foil and store at 4C for the desired exposure time. Include a few slides in a separate box to be developed before the others to determine how long an exposure is necessary.

6. Develop slides as follows:

    1. In the dark, remove slides from box and place into slide carriers.
    2. Place slides into Kodak D-19 (diluted 1:1 with water, 15 C)--3 min***
    3. Wash in water 30 secs--15 C
    4. Fix in Kodak Rapid Fixer (with hardener) 5 min 15 C

    The following steps can be done in the light:

    5. Wash in water 5 min twice
    6. Stain with filtered hematoxylin --30 secs or less
    7. Wash with water
    8. Dehydrate in 70, 80, 95, 100% ethanol
    9. Clear in hemo-de and coverslip with permount.

***Keep developer, wash water and fixer at 15 C. This will result in finer grain size.

Preparation of Hybridization Buffer :

(50% Formamide, 10% Dextran Sulfate, 1X Denhardt's, 4X SSC, 500 ug/ml Heparin Sodium Salt, 0.5 mg/ml Yeast tRNA, 0.4 mg/ml Salmon Sperm DNA)

Mix 2 g dextran sulfate with 4.78 ml water in a 50 ml sterile conical tube. Vortex and let stand at room temperature for 10 min. Add 10 ml formamide, vortex, and heat this mixture in a shaker bath at 37 C until dextran sulfate is in solution. Add 4 ml 20X SSC, 400 ul 50X Denhardt's solution, 400 ul tRNA* (25mg/ml), 10 mg (1760 USP units*) heparin sodium salt and 800 ul sheared single-stranded salmon sperm DNA* (10 mg/ml). Bring volume to 20 ml with DEPC-treated water, vortex and store at -20 C. Just before applying buffer containing probe to tissue, add 1:100 (v/v) 5 M DTT in 0.01 M sodium acetate. (Note: DTT is needed for radioactive probes only).

50% Formamide decreases the melting temperature of hybrids-- each 1 % increase in formamide concentration reduces the melting temperature by 0.35 C for RNA:RNA hybrids.

Dextran sulfate (or other polymers) accelerates hybridization rates by volume exclusion, which increases the effective probe concentration.

Increased ionic strength (sodium concentration) of the hyb. Buffer stabilizes the hybrids. By varying the salt concentration it is possible to alter the stringency of the buffer.

Heparin is especially effective in reducing the background in detection of nonradiolabeled probes. It is also an inhibitor of RNases.

Salmon sperm DNA may not change the signal:background ratio, (it is added to occupy the majority of the non-specific binding sites) but it will increase the viscosity of the buffer which will facilitate the application and removal of coverslips.

*Concentrations of these stocks may vary--check label and adjust accordingly.

The following solutions can be made up in untreated bottles. Treat with 0.1% DEPC overnight at 37 C and then autoclave:

EDTA, NaCl, MgCl2, NaOAC

The following solutions cannot be autoclaved. Make these up in DEPC-treated water in glass bottles that have been baked at 170 C for 8 hours, and whose caps have been soaked in water containing 0.1% DEPC overnight at 37 C and then autoclaved:

Tris, SDS, Denhardt's any buffers with Tween, or Triton, Hybridization buffer

Proteinase K: Stock solution-- 10mg/ml in 0.1M Tris-HCl, 50mM EDTA, pH 8.0. Aliquot in smallest volume store at -20 C, use once and discard. Previously (TAMU) we used 20 ug/ml, 7.5 min. at 37 C. Another recommendation is 1 ug/ml for 30 min at 37 C. -- and yet another 3.5 ug/ml, 15 min at 37 C.

Buffer: .5M NaCl, .01M Tris,pH 7.5

For 20 ug/ml in 200 ml use 400 ul of stock

Note: proteinase K improves penetration of probe in formaldehyde fixed tissues also it does not have to be pre-digested as does pronase.

RNase A Buffer: **0.5 M NaCl**

10 mM Tris-HCl

1 mM EDTA pH 8.0

Stock solution-- 10 mg/ml

Working solution-- 15ug/ml (TAMU)

20X SSC: **Sodium chloride, sodium citrate**

NaCl--175.3g

Sodium citrate-- 88.2g

Dissolve in 800 ml milli-Q H2O adjust pH to 7.0 bring to 1 L, add 1.0 ml DEPC, let stand overnight and autoclave next day. Dilute as needed with DEPC H2O.

0.5 M EDTA **Stock solution**

(pH 8.0) Add 186.1 g of disodium ethylenediaminetetra acetate 2 H2O to 800 ml of milli-Q H2O. Stir vigorously on a magnetic stirrer. Adjust pH to 8.0 with NaOH (~20 g of NaOH pellets) Add 0.1% DEPC stir overnight and autoclave. The disodium salt of EDTA will not go into solution until the pH is adjusted to ~ 8.0 by the addition of NaOH.

Reference: Millen KJ and Hui C. In: (Krieg PA., Ed.). *A Laboratory Guide to RNA - Isolation, Analysis, and Synthesis.* 1996. Wiley-Liss, Inc. Chapter. 18. Pp. 339-355.

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IHH AND PTC IMMUNOSTAINING

Materials:

- IHH: Santa Cruz Goat Polyclonal (Santa Cruz Biotechnology, c-terminus, SC-1196; store at 2-8ºC)
- Ptc: Santa Cruz Goat Polyclonal (Santa Cruz Biotechnology, n-terminus, SC-6149; store at 2-8ºC)
- Goat IgG
- VECTASTAIN ABC Elite Kit (Goat) (Vector Labs)
- 1x PBS
- Hemo-De
- EtOH
- 3% Hydrogen peroxide
- Citric Acid-mono hydrate
- Sodium Citrate dihydrate
- DAB
- BSA

Procedures:

1. Deparaffinize and rehydrate. (Using Hemo-De, 100%, 100%, 95%, 80%, 70% EtOH, water; 3 minutes in each and shake well in the Hemo-De).

2. Boil in 10mM (600ml) Citrate pH 6.0 20 minutes.
    a. Citrate Buffer (pH6.0): Stock solutions (store at 4º C)
        i. 1M Citric Acid-mono hydrate--Solution A
            1. 105.07 g in 500ml

        ii. 0.1M Sodium citrate-dihydrate --Solution B
            1. 14.7 g in 500ml

    b. Working solution—10mM Citrate buffer, pH 6.0
        i. 10.8 ml Solution A
        ii. 49.2 ml Solution B
        iii. Bring to 600mL (this is the volume necessary to keep slides submerged while boiling in a 1L beaker) and pH to 6.0.

3. Cool in citrate 20 minutes.

4. Block tissue sections with normal serum 20 minutes at room temperature (Use ABC Elite Kit and directions).

5. Apply primary IHH antibody, diluted 1µg/mL in PBS with 1% BSA or apply primary Ptc antibody, diluted to 1µg/mL in PBS with 1% BSA. Use Goat IgG as negative control for polyclonal IHH or Ptc antibody diluted1µg/mL in PBS with 1% BSA. Incubate overnight in humid box at 4ºC.

6. Rinse slides in PBS 5 minutes.

7. Apply biotinylated secondary antibody (ABC Elite Kit, follow directions). Incubate 40 minutes at room temperature. Prepare ABC reagent at least 30 minutes prior to using (see ABC Elite Kit directions).

8. Rinse in PBS 5 minutes.

9. Block endogenous peroxidase activity by placing slides in 3% hydrogen peroxide, 5 minutes.

10. Rinse in H2O and place in PBS for 5 minutes.

11. Apply ABC reagent and incubate slides for 40 minutes at room temperature.

12. Rinse slides in PBS for 5minutes.

13. Develop slides in 0.1% DAB in 0.1M Tris pH 7.5 and 0.2%hydrogen peroxide(dilute hydrogen peroxide from 30% stock) in H2O. (Use 100ml of the DAB solution and 100ml of the hydrogen peroxide solution; make these solutions just before use).

MSX IMMUNOSTAINING

Materials:

- Msx1: Covance Mouse Monoclonal Clone 4F11 (Covance, CA; MMS-261R; store at 2-8ºC)
- Msx2: Stressgen Mouse Monoclonal (Stressgen Bioreagents Corp, BC; AAM-010; store at 2-8ºC)
- Mouse Primary Antibody Isotype Control (Zymed Lab. Cat. no. 08-65990)
- VECTASTAIN ABC Elite Kit (Mouse) (Vector Labs)
- 1x PBS
- Hemo-De
- EtOH
- 3% Hydrogen peroxide
- Citric Acid-mono hydrate
- Sodium Citrate dihydrate
- DAB
- BSA

Procedures:

1. Deparaffinize and rehydrate. (Using Hemo-De, 100%, 100%, 95%, 80%, 70% EtOH, water; 3 minutes in each and shake well in the Hemo-De).

2. Boil in 10mM (600ml) Citrate pH 6.0 15 -20 minutes.
    a. Citrate Buffer (pH6.0): Stock solutions (store at 4º C)
        i. 1M Citric Acid-mono hydrate--Solution A
            1. 105.07 g in 500ml

        ii. 0.1M Sodium citrate-dihydrate --Solution B
            1. 14.7 g in 500ml

    b. Working solution—10mM Citrate buffer, pH 6.0
        i. 10.8 ml Solution A
        ii. 49.2 ml Solution B
        iii. Bring to 600mL (this is the volume necessary to keep slides submerged while boiling in a 1L beaker) and pH to 6.0.

3. Cool in citrate 15 minutes.

4. Block tissue sections with normal serum 20 minutes at room temperature (Use ABC Elite Kit and directions).

5. Apply primary Msx1 antibody, diluted 1:500 in PBS with 1% BSA or apply primary Msx2 antibody, diluted to 5µg/mL in PBS with 1% BSA. Use Mouse Primary Antibody Isotype Control (Zymed Lab. Cat# 08-65990) as negative control for monoclonal Msx1 or Msx2 antibody (do not dilute, use as is). Incubate overnight in humid box at 4ºC.

6. Rinse slides in PBS 5 minutes.

7. Apply biotinylated secondary antibody (ABC Elite Kit, follow directions). Incubate 40 minutes at room temperature. Prepare ABC reagent at least 30 minutes prior to using (see ABC Elite Kit directions).

8. Rinse in PBS 5 minutes.

9. Block endogenous peroxidase activity by placing slides in 3% hydrogen peroxide, 5 minutes.

10. Rinse in H2O and place in PBS for 5 minutes.

11. Apply ABC reagent and incubate slides for 40 minutes at room temperature.

12. Rinse slides in PBS for 5minutes.

13. Develop slides in 0.1% DAB in 0.1M Tris pH 7.5 and 0.2%hydrogen peroxide(dilute hydrogen peroxide from 30% stock) in H2O. (Use 100ml of the DAB solution and 100ml of the hydrogen peroxide solution; make these solutions just before use).

REAL TIME PCR (qRT-PCR)

Materials:

- High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Cat. no. 4368814)

- Extracted RNA
- Forward and Reverse Primers for Target Gene
- cDNA at 1ng/µl
- MicroAmp Optical tube and cap strips, 8 tubes or caps/strip (Applied Biosystems, Cat. no. N8010933 (tubes) and 4323032 (caps))
- Power SYBR Green PCR Master Mix, 5mL (Applied Biosystems, Cat. no. 4368577)

All qRT-PCR reactions are performed in triplicate and prepared on ice

Procedure:

1. Dilute RNA to 100ng/µL.

2. Convert 500ng of RNA to cDNA. Follow manufacturer's instructions for the High Capacity cDNA Reverse Transcription kit. Use a 20µL reaction volume → 10µL master mix + 5µL RNA + 5µL H_20.

3. Once cDNA is generated, dilute it to 1ng/µL. This may not be ideal for some samples.

4. Reconstitute lypholized primers to 1mM with 1x TE. Dilute reconstituted primers to 500µl of 2µm.

   Example: 1mM → 0.002mM = 500x dilution

   $\frac{500\mu l}{} = 1\mu l$ of 1mM Primers + 499µl of 1x TE

5. Refer to ABI User Bulletin #2 for Primer Validation and SYBR Green PCR Master Mix protocol for primer optimization.

6. Create a standard curve, using cDNA from the same tissue type that is being assayed. We typically use 10x, 5x, 2.5x, 1.25x and a no template control (NTC). 10x = 10ng

7. For each 25µl reaction, there is 10µl of sample (usually 10ng) and 15µl of master mix. Create master mix for all samples + standard curve + NTC. Remember that everything is done in triplicate, so multiply number of samples, standard curve, and NTC by 3 when calculating master mix. Also, add 3-4 extra reactions of master mix to account for pipetting errors.

Master Mix (1 reaction)

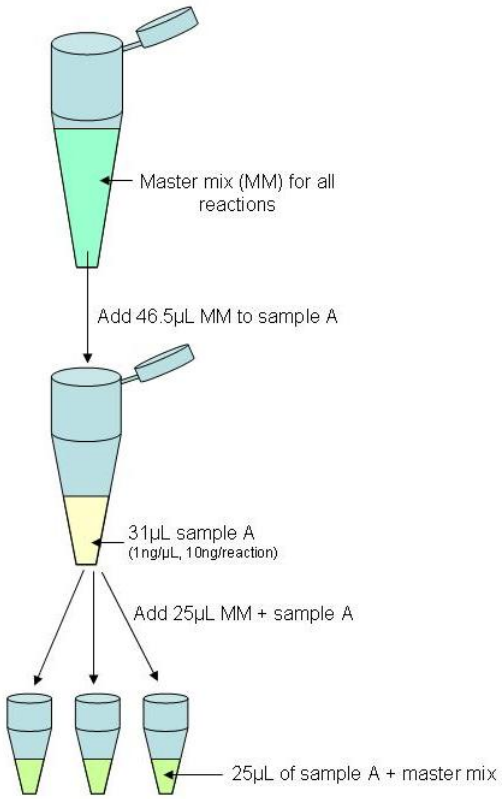
SYBR Green	12.5µl	
Primer F	1.25µl	Final concentration is 100nM
Primer R	1.25µl	Final concentration is 100nM
Total	15µl	

8. Mix master mix by pipetting up and down or tip the tube back and forth. Do not vortex to prevent bubbles. Quick-spin to get rid of any bubbles.

9. Reactions are prepared in one tube and then aliquot into 25µl reaction volumes. For a triplicate reaction, 31µl (10µl x 3.1, the 0.1 adds a little extra to account for pipetting errors) is in one tube. The master mix (46.5µl) is then added to tube with sample (15µl master mix x 3.1).

10. Mix reaction by pipetting up and down. Quick-spin to get rid of any bubbles.

11. Aliquot 25µl of reaction into separate optical tubes.



12. After all samples have been aliquoted, apply optical caps. Make sure that the cap strips do not touch other tubes except for the tube that they are to be placed on.

13. Quick-spin to get rid of any bubbles.

14. Use Applied Biosystems 7500 Real Time PCR system to run assay. Refer to instruction manual to set up PCR program.

PORCINE ENDOMETRIAL PROTEIN EXTRACTION

Materials:

- ReadyPrep Reagent 3 from Sequential Extraction Kit (Bio-Rad; Cat no. 163-2104)

- Reducing Agent TBP, 200 mM, 0.6 mL (Bio-Rad; Cat. no. 163-2101)
- Phenylmethanesulfonyl fluoride (PMSF), 4mM
- Serine and Cysteine protease inhibitor
- DNase 1, 12.5 units/mL
- RNase A, 15µg/mL

Procedure:

1. Prepare ReadyPrep Reagent 3 by adding TBP at 1:100 to ReadyPrep Reagent 3.

2. Before collecting frozen endometrial tissue, label, weigh, and record weight of 1.5mL microfuge tubes for each animal.

3. Collect frozen endometrial tissue. Only want ~25 mg/500µl of buffer.

4. Weigh and record weight for tube plus tissue. If tissue weighs less than 20mg, add a little more tissue.

5. Determine volume of ReadyPrep Reagent 3 needed for each tissue. For example, with 30 mg of tissue:

6. $\frac{25\ mg}{500\mu l} = \frac{30mg}{X}$ $\quad X = \frac{30 \times 500}{25}$, so X = 600µl of ReadyPrep Reagent 3

7. Add PMSF to each tube. If using a 100mM stock, 24µl of 100mM PMSF should be added to the tube with 600µl ReadyPrep Reagent 3 and 30mg of tissue for a final concentration of 4mM of PMSF.

8. Sonicate tissues on ice in short bursts to avoid foaming. Sonicate until solution is clear and tissue is dissolved.

9. Add DNase1 and RNaseA.

10. Incubate at room temperature for 15 minutes.

11. Add PMSF again as in #5 (24µl of PMSF for 600µl of ReadyPrep Reagent 3 and 30mg tissue).

12. Vortex for 5 minutes.

13. Centrifuge at 15,000 rpm for 10 minutes.

14. Collect supernatant which contains extracted endometrial protein.