Uterine development and endometrial programming

F.F. Bartol[1], A.A. Wiley[1] and C.A. Bagnell[2]

[1]Department of Animal Sciences, Cellular & Molecular Biosciences Program, Auburn University, Auburn, Alabama 36849; [2]Department of Animal Sciences, Rutgers University, New Brunswick, New Jersey 08901, USA

Structural patterning and functional programming of uterine tissues are mechanistically coupled. These processes ensure anteroposterior differentiation of uterine tissues from adjacent segments of the developing female reproductive tract (FRT) and radial patterning that establishes uterine-specific histoarchitecture and functionality. Uterine organogenesis begins prenatally and is completed postnatally. Genes required for FRT development include *Pax2*, *Lim1* and *Emx2*, genes in the abdominal-B *Hoxa* cluster, and members of both *Wnt* and *Hedgehog (Hh)* gene families. Disruption of morphoregulatory gene expression patterns can prevent FRT development entirely or compromise uterine organogenesis specifically. Oestrogen receptor-α (ER) -dependent events associated with development of the neonatal porcine uterus can be altered by administration of oestrogen (E) or relaxin (RLX). Expression of the RLX receptor is detectable in porcine endometrium at birth, before onset of ER expression and uterine gland genesis. Uterotrophic effects of both E and RLX can be inhibited with the ER antagonist ICI 182,780, indicating that RLX may act via crosstalk with the ER system in neonatal tissues. Exposure of neonatal gilts to E alters temporospatial patterns of *Hh*, *Wnt* and *Hoxa* expression in the uterine wall. Oestrogen given for two weeks from birth produced hypoplastic adult porcine uteri that were less responsive to periattachment conceptus signals as reflected by reduced growth response and luminal fluid protein accumulation, altered endometrial gene expression, and reduced capacity for conceptus support. Data reinforce the concept that factors affecting signalling events in uterine tissues that produce changes in morphoregulatory gene expression patterns during critical organisational periods can alter the developmental trajectory of the uterus with lasting consequences. Thus, uterine tissues can be programmed epigenetically for success or failure during perinatal life.

Introduction

The uterus is a mesodermally derived specialisation of the Müllerian ducts. In mammals, including the pig (*Sus scrofa*), these paired tubular structures arise from invaginations of coelomic epithelium on the lateral aspects of the urogenital ridges, grow caudally and begin to fuse

E-mail: bartoff@auburn.edu

(Bartol, 1999). The degree of fusion can be complete, partial or incomplete and defines gross morphological characteristics (simplex, bicornuate or duplex phenotypes) of adult uteri. Anteroposterior patterning of the Müllerian ducts results in their segmentation into structurally and functionally unique parts of the female reproductive tract (FRT) including the oviducts, uterus, cervix and anterior vagina. Radial patterning begins prenatally, is completed postnatally and establishes the principal histological elements of the uterine wall including the endometrium, myometrium, and perimetrium (Bartol *et al.*, 1993; Bartol 1999; Gray *et al.*, 2001a). Uterine functions in mammals include: (1) transport, storage and maturation of spermatozoa; (2) embryo spacing, recognition, reception and support; and (3) foetal and placental expulsion during parturition. Additionally, endometrial prostaglandin production is required for luteolysis in many animals, including the pig (Bartol, 1999).

Uterine abnormalities and dysfunction can compromise fertility, increase embryonic mortality, contribute to intrauterine growth retardation and associated complications and potentiate dysplasia and disease (Bartol *et al.*, 1999; Gray *et al.*, 2000b; Gray *et al.*, 2001b; Kitajewski and Sassoon, 2000; Ashworth *et al.*, 2001; Greenwood and Bell, 2003; Hales and Ozanne, 2003; Kobayashi and Behringer, 2003; Tarleton *et al.*, 2003b). The aetiologies of such problems are complex. However, it is clear that the integrity, stability and functionality of adult uterine tissues are defined, to a significant extent, by the course of events associated with development of uterine tissues during perinatal life (Bartol *et al.*, 1999; Kobayashi and Behringer, 2003).

Morphogenesis (structural patterning) and cytodifferentiation (functional programming) are coupled processes. For epithelial-mesenchymal organs including the uterus, these processes involve the progressive generation of increasingly complex and specific cellular relationships and interactions (Bartol *et al.*, 1993; Bartol *et al.*, 1999; Kitajewski and Sassoon, 2000; Gray *et al.*, 2001b; Kobayashi and Behringer, 2003). These interactions are accompanied by and drive the evolution of organisationally critical, temporally and spatially unique morphoregulatory gene expression domains that direct and specify cell fate, define patterns of development, and determine cell and tissue identity. Genes most centrally involved in tissue patterning and cell fate specification include those that encode transcription factors and signalling ligands, their receptors and downstream elements of signalling pathways (Davidson, 2001; Hu *et al.*, 2004). Here, elements of the primary organisational palette of factors governing uterine organisation will be described and the consequences of disruption of critical organisational mechanisms governing uterine development and endometrial programming will be discussed, with emphasis given to recent observations involving the pig.

Genesis of the female reproductive tract

Efforts to identify genes and gene networks required for development of the mammalian FRT have been aided by advances in molecular profiling techniques, the power of murine genetics and inferences drawn from murine phenotypes produced through targeted mutagenesis. From such studies it is clear that expression of genes encoding the transcription factors *Pax2*, *Lim1* and *Emx2*, as well as *Wnt4*, a secreted morphoregulatory glycoprotein, is required for FRT formation (Kobayashi and Behringer, 2003). The fact that mice with compound mutations for retinoic acid receptor (RAR) genes can lack either all or caudal portions of the FRT (Mendelsohn *et al.*, 1994; Kastner *et al.*, 1997) indicates that complex RAR signalling is also required for the formation of these tissues.

Genes governing FRT development

Pax2-null murine females lack kidneys and reproductive tracts, owing to Müllerian duct degeneration during embryogenesis. Normally, *Lim1* is expressed in Müllerian epithelium, the meso-

nephros, metanephros and foetal gonads (Kobayashi *et al.*, 2004). Female *Lim1*-null mutants have normal ovaries, but lack Müllerian derivatives. Additionally, Lim-1-null Müllerian epithelial cells do not contribute to uterine epithelium, but can contribute to uterine stroma, indicating a requirement for epithelial Lim-1 expression in Müllerian epithelium as a prerequisite to successful uterine development (Kobayashi *et al.*, 2004). *Emx2*-null mutants lack kidneys, gonads and reproductive tracts (Kobayashi and Behringer, 2003).

Wnt4 is one of several *Wnt* genes implicated in patterning and function of the FRT (Miller *et al.*, 1998; Sassoon, 1999; Vainio *et al.*, 1999; Carta and Sassoon 2004; Mericskay *et al.*, 2004). Müllerian ducts do not form in male or female *Wnt4*-null mutants (Vainio *et al.*, 1999). Female *Wnt4*-null mice lack female, but contain male reproductive tract tissues (Vainio *et al.*, 1999) due to the presence of androgen-active ovarian cells that support mesonephric duct development (Kobayashi and Behringer, 2003). Consistently, a woman with symptoms of Mayer-Rokitansky-Küster-Hauser syndrome, who lacked a uterus and displayed clinical signs of androgen excess, was found to have a loss-of-function mutation in *Wnt4* (Biason-Lauber *et al.*, 2004; Hughes, 2004). Thus, *Wnt4* is required for Müllerian duct development and ovarian programming.

Morphoregulatory genes and uterine patterning

Patterning events required for differentiation of the uterine segment of the FRT occur in both anteroposterior and radial axes (Fig. 1). Anteroposterior patterning establishes histologically distinct boundaries between the oviducts, uterus and cervix. Radial patterning establishes uterine histoarchitecture. Temporospatial coordination of these processes is governed locally by a group of highly conserved morphoregulatory genes, including members of both *Hox* and *Wnt* gene families, expression of which may be affected by up-stream regulatory factors that could include Hedgehog gene products.

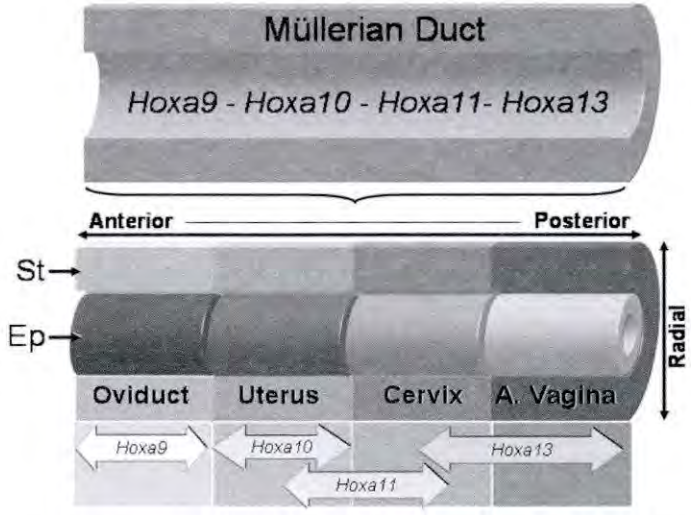

Fig. 1 Patterning of the female reproductive tract (FRT) and uterine differentiation. Prior to differentiation of FRT tissues, genes in the abdominal-B *Hoxa* cluster, including *Hoxa*9, *Hoxa*10, *Hoxa*11 and *Hoxa*13 are expressed uniformly along the anteroposterior axis of the developing Müllerian duct (top). Segmentation of the Müllerian duct, leading to differentiation of stromal (St) and epithelial (Ep) tissues and histoarchitectural relationships unique to the oviduct, uterus, cervix and anterior vagina, requires that *Hoxa* expression domains develop along this axis, establishing a '*Hox* code' specific to each tissue segment (bottom). Radial patterning and programming of FRT tissues, including the uterus, requires stromal-epithelial interactions supported by the local actions of both *Hoxa* and *Wnt* gene products (not shown).

Homeobox genes

Homeobox or *Hox* genes encode nuclear proteins that act as transcription factors (Mark *et al.*, 1997; Cillo *et al.*, 2001). *Hox* genes share a common sequence, the homeobox, which codes for the DNA-binding homeodomain. *Hox* gene products affect gene expression events required to establish positional identity of cells along anteroposterior axes in developing tissues (Cillo *et al.*, 2001). Human and murine Class I *Hox* genes are organised into four genomic clusters on different chromosomes (Mark *et al.*, 1997). Displaying colinear expression, the 3′-5′ chromosomal arrangement of these genes defines the relative timing and order in which they are expressed along the anteroposterior body axis. Thus, 5′ vertebrate *Hox* genes control development of the lumbo-sacral region, including the genitalia (Taylor *et al.*, 1997; Cillo *et al.*, 2001). Products of the reproductive homeobox (R*Hox*) gene cluster have yet to be associated with uterine organisational mechanisms (Maclean *et al.*, 2005).

Prior to differentiation, *Hox* genes in the abdominal-B *Hoxa* cluster, including *Hoxa*9, -10, -11 and -13, are expressed uniformly along the anteroposterior axis of the Müllerian ducts (Taylor *et al.*, 1997; Kitajewski and Sassoon, 2000). Segmentation of the Müllerian ducts along this axis requires restricted, overlapping expression of these genes (Fig. 1) such that *Hoxa*9 is expressed in oviduct, *Hoxa*10 and -11 are expressed in the uterine segment, and *Hoxa*11 and -13 are expressed in the cervix and anterior vagina (Taylor *et al.*, 1997; Kitajewski and Sassoon, 2000). Spatially restricted expression of these genes is required to define and stabilise tissue boundaries along the anteroposterior axis of the FRT. Thus, a segment-specific *Hox* code (Krumlauf, 1994) must be established and maintained to insure FRT patterning success.

Uterine segmentation requires stable expression of *Hoxa*10 and -11, which define the uterine *Hox* code (Mark *et al.*, 1997). Both functional redundancies and interactions were described for these two uterine *Hox* genes (Branford *et al.*, 2000). However, disruption of the uterine *Hox* code can produce homeotic transformations in which boundaries between the uterus and adjoining tissue segments are poorly defined. For example, targeted disruption of *Hoxa*10 expression produced a murine FRT phenotype characterised by dissolution of histological and functional boundaries between the oviducts and uterus (Benson *et al.*, 1996). In contrast, disruption of *Hoxa*11 expression affects radial patterning in the uterine segment due to dysregulation of an organisationally critical *Hox*/*Wnt* expression axis described below (Gendron *et al.*, 1997; Mericskay *et al.*, 2004).

Functional redundancies and overlapping expression domains documented for *Hox* genes governing FRT patterning may explain how mutations in genes not strictly associated with the uterine *Hox* code can affect uterine phenotype. Uterine anomalies in women with the hand-foot-genital syndrome, in which a nonsense mutation of *Hoxa*13 truncates the homeodomain and inhibits DNA binding, involve defects in Müllerian duct fusion such that the normal simplex human uterus is absent, and partially (bicornuate) or completely (didelphic) divided uterine morphologies are seen (Mortlock and Innis, 1997). Observations reinforce the importance of *Hox* genes in uterine patterning and indicate a role for *Hoxa*13 in orchestration of Müllerian duct fusion, a process central to the genesis of diversity in uterine forms found in nature.

Wnt genes

Vertebrate *Wnt* genes encode secreted glycoproteins related to wingless, the Drosophila segment polarity gene (DasGupta *et al.*, 2005). Mammalian *Wnt* gene products regulate patterning events associated with establishment of cell boundaries, mediate cell-cell interactions that determine cell fate, and contribute to adult tissue homeostasis (Carta and Sassoon, 2004; Logan and Nusse, 2004; Wang and Wynshaw-Boris, 2004). Three *Wnt* genes; *Wnt4*, *Wnt*5a and *Wnt7*a are coordinately expressed during FRT development and continue to be expressed in

adult uterine tissues where they may affect cyclical endometrial patterning (Vainio *et al.*, 1999; Tulac *et al.*, 2003; Carta and Sassoon, 2004; Mericskay *et al.*, 2004). Classically, *Wnt* proteins act via a canonical signalling pathway involving frizzled receptors (Wodarz and Nusse, 1998; Nusse, 2003; Logan and Nusse, 2004). Non-canonical *Wnt* signalling systems are also recognised (Veeman *et al.*, 2003) and may be more important in normal *Wnt*-dependent uterine development (Miller *et al.*, 1999; Topol *et al.*, 2003; Mericskay *et al.*, 2004). However, canonical *Wnt* signalling was implicated in oestrogen-induced uterine growth (Hou *et al.*, 2004) and may facilitate disruption of oestrogen-sensitive uterine organisational events.

Coordinated expression of *Wnt*5a and *Wnt7*a is also essential for FRT development (Carta and Sassoon, 2004; Mericskay *et al.*, 2004). Murine *Wnt*5a mutants have short uterine horns of normal diameter, but lack defined cervical and vaginal structures (Mericskay *et al.*, 2004). Similarities between this phenotype and the FRT phenotype noted for *Hoxa*13/d13 mutants (Warot *et al.*, 1997) indicated that these gene products act through a common pathway to support posteriorisation of the FRT (Mericskay *et al.*, 2004). In contrast, the FRT in *Wnt7*a mutants displays normal posterior development, but atrophic uterine horns (Miller and Sassoon, 1998). Epithelial *Wnt7*a expression is required to maintain, but not to induce uterine stromal expression of *Hoxa*10 and -11 (Miller and Sassoon, 1998). Loss of *Wnt7*a expression results in loss of uterine *Hoxa*10 and *Hoxa*11 expression in adult mice, and produces a homeotic transformation characterised by posteriorisation of uterine tissues (Kitajewski and Sassoon, 2000). Thus, *Wnt*5a and *Wnt7*a act to support complete anteroposterior development of the FRT, and *Wnt7*a influences organisationally critical cellular interactions in the uterine wall by enforcing positional signals dictated by *Hoxa*10 and *Hoxa*11 (Miller and Sassoon, 1998).

While basic histological elements of the uterine wall are present at birth (postnatal day = PND 0), substantial radial patterning occurs after birth in most mammals, including the pig (Bartol *et al.*, 1993; Gray *et al.*, 2001a). Postnatal endometrial development is marked by differentiation of glandular epithelium (GE) from luminal epithelium (LE) and rapid proliferation of nascent GE (Gray *et al.*, 2001a). In pigs and other mammals, initiation of uterine gland genesis is ovary-independent and occurs in the absence of substantial levels of circulating steroid hormones (Bartol *et al.*, 1993; Bartol *et al.*, 1999). Consistently, murine endometrial gland genesis was retarded when aglandular neonatal uterine tissue grafts were placed into intact adult female hosts, but proceeded when hosts were ovariectomised two weeks prior to grafting (Mericskay *et al.*, 2004). These observations reinforce the importance of paracrine mechanisms supporting the evolution of organisationally critical microenvironmental conditions in developing epithelial-mesenchymal organs such as the uterus.

Tissue recombination experiments showed that epithelial expression of *Wnt7*a and stromal expression of *Wnt*5a are required for uterine gland genesis (Mericskay *et al.*, 2004). Persistent stromal expression of *Wnt*5a is required for GE differentiation, which requires down regulation of *Wnt7*a expression in invaginating LE from which nascent GE arises. Additionally, down regulation of *Wnt7*a in LE requires stromal expression of *Wnt*5a (Mericskay *et al.*, 2004). Thus, *Wnt*-mediated interactions determine the fate of luminal epithelial cells, allowing them to invaginate, differentiate and form glands.

Uteri of neonatal *Wnt7*a mutants appear normal and effects of the xenoestrogen diethylstilbestrol (DES) on uterine cell proliferation were similar in wild-type and *Wnt7*a mutants, indicating that *Wnt7*a does not mediate the proliferative response to DES (Carta and Sassoon, 2004). However, DES-exposed uteri in *Wnt7*a-null mice displayed high levels of apoptosis not observed in wild-type animals. Thus, *Wnt7*a may function as a negative regulator of apoptosis and contribute to stability of LE (Carta and Sassoon, 2004). Loss of uterine *Hoxa*10 and *Hoxa*11 expression after ablation or oestrogen-induced suppression of *Wnt7*a expression, precedes loss of *Wnt4* and *Wnt*5a expression in murine uterine stroma (Miller and Sassoon, 1998; Kitajewski

and Sassoon, 2000). Oestrogen-induced suppression of *Wnt7a* expression is both ERα- and *Wnt5a*-dependent (Couse *et al.*, 2001; Mericskay *et al.*, 2004). Loss of perinatal *Wnt7a* expression sufficient to alter the uterine *Hox/Wnt* expression axis produced adult murine uteri that were hypoplastic and displayed disorganised, glandless histoarchitecture (Miller and Sassoon 1998; Kitajewski and Sassoon, 2000). *Wnt5a* mutants maintain columnar uterine epithelium but fail to form endometrial glands (Mericskay *et al.*, 2004), indicating that *Wnt7a* is necessary to maintain normal uterine epithelial phenotype.

Recently, Msx2, an epithelial morphoregulatory factor, was implicated as an upstream regulator of *Wnt* gene expression in the neonatal uterus (Huang *et al.*, 2005). In Msx2-null mutants patterns of *Wnt5a* expression changed from stromal to epithelial, a condition also induced by DES exposure in normal mice. Additionally, uterine epithelial *Wnt7a* expression was elevated in the Msx2-null uterus (Huang *et al.*, 2005). Thus, epithelial Msx2 expression appears to stabilise spatial patterns of *Wnt5a* expression and to control levels of epithelial *Wnt7a* expression necessary to insure a normal developmental trajectory for the endometrium.

Collectively, current data suggest a mechanism whereby epithelial *Wnt7a* acts to stabilise stromal expression of *Hoxa10* and *Hoxa11* which, in turn, insures stromal expression of *Wnt4* and *Wnt5a* in the developing uterine wall. These factors cooperate as primary elements of a temporally and spatially dynamic morphoregulatory programme governing uterine developmental events that determine the functional potential of uterine tissues. Evidence that a *Hoxa/Wnt* expression axis develops in neonatal porcine uterine tissues supports this concept (Figs. 2 and 3).

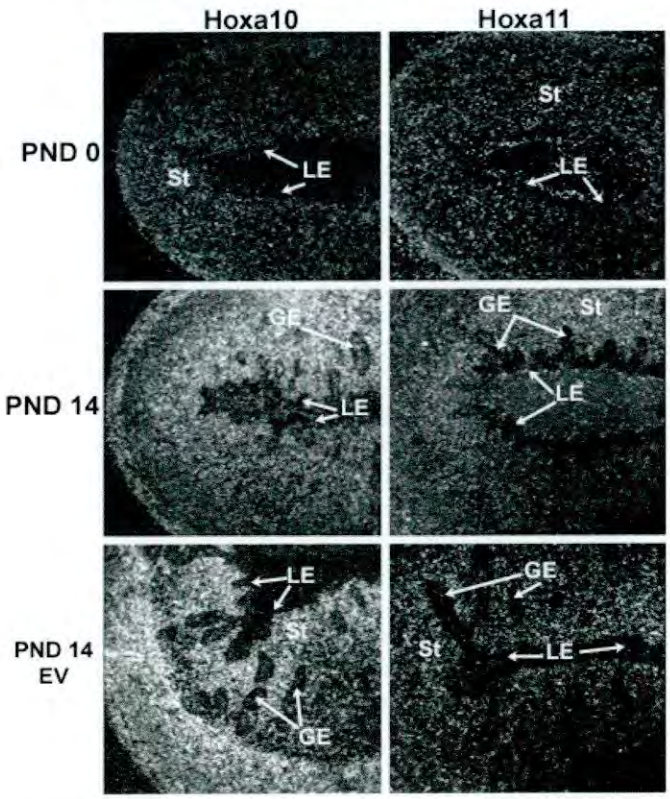

Fig. 2 Effects of age and administration of oestradiol valerate (EV; 50μg/kg bw/d) for 14 days from birth (postnatal day = PND 0) on patterns of *Hoxa10* (left) and *Hoxa11* (right) expression in neonatal porcine uterine tissues. Uteri obtained from 4-6 gilts on each day and

Fig. 2 Contd. after treatment with EV on PND 14, were fixed in 4% (w/v) paraformaldehyde, embedded in paraplast-plus, sectioned (5 μm), and processed together under identical conditions for hybridisation of cRNA probes. Antisense (α-^{35}S)UTP-labelled cRNA probes, generated by *in vitro* transcription from linearised porcine cDNA templates for *Hoxa*10 (NCBI accession number: AF281156) and *Hoxa*11 (AF453292), were used to localise targeted transcripts *in situ*. Darkfield photomicrographs show signal (white grains) above background, determined by subtraction of negative control images generated using corresponding (α-^{35}S)UTP-labelled sense cRNA probes (not shown). Signal indicative of expression of both genes was detected in tissues from PND 0 (top), increased to PND 14 (middle) and was predominantly stromal. Effects of EV administered from birth were most pronounced for *Hoxa*10 (PND 14 vs PND 14EV). [Original magnification = 10X; GE = glandular epithelium, LE = luminal epithelium, St = stroma]

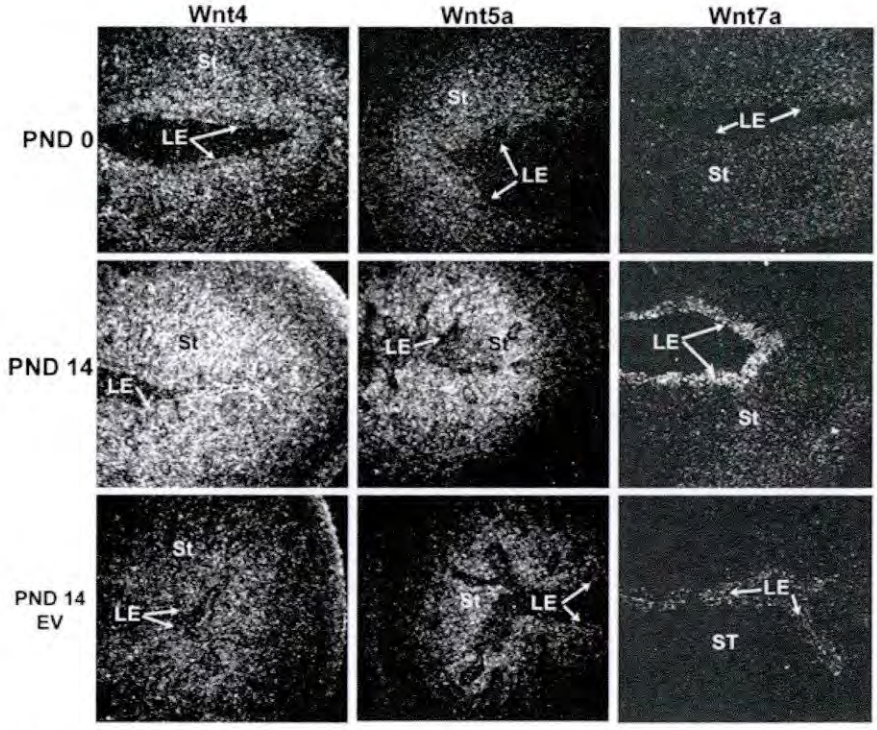

Fig. 3 Effects of age and administration of oestradiol valerate (EV; 50μg/kg bw/d) for 14 days from birth (postnatal day = PND 0) on patterns of *Wnt4* (left), *Wnt*5a (middle) and *Wnt*7a expression in neonatal porcine uterine tissues. Uteri obtained from 4-6 gilts on each day and after treatment with EV on PND 14, were fixed in 4% (w/v) paraformaldehyde, embedded in paraplast-plus, sectioned (5 μm), and processed together under identical conditions for hybridisation of cRNA probes. Antisense (α-^{35}S)UTP-labelled cRNA probes, generated by *in vitro* transcription from linearised porcine cDNA templates for *Wnt4* (NCBI accession number: CA997682), *Wnt*5a (CA997683), and *Wnt*7a (CA997684) were used to localise targeted transcripts *in situ*. Darkfield photomicrographs show signal (white grains) above background, determined by subtraction of negative control images generated using corresponding (α-^{35}S)UTP-labelled sense cRNA probes (not shown). Signal indicative of expression of all *Wnt* genes was detected in uterine stroma on PND 0 (top). Stromal signals were most pronounced for *Wnt4* and *Wnt*5a and both increased by PND 14 (middle). Signal indicative of *Wnt*7a expression (right) was absent in luminal epithelium (LE) at birth, but pronounced in LE by PND 14. Administration of EV from birth reduced signal for all genes (PND 14 vs PND 14EV). [Original magnification = 10X; LE = luminal epithelium, St = stroma]

Hedgehog genes

Genes encoding the vertebrate hedgehog (Hh) proteins, including Sonic (Shh), Desert (Dhh) and Indian hedgehog (Ihh), are homologues of the Hh gene discovered in Drosophila (Cohen, 2003). Secreted vertebrate Hh proteins display inductive properties at epithelial-mesenchymal boundaries (Walterhouse *et al.*, 2003) and affect cellular differentiation, proliferation and survival via a complex signalling network initiated by direct interaction with the Patched (Ptch1) receptor (Cohen, 2003). Downstream targets of Hh signalling include *Wnt* genes.

A progesterone-inducible gene, Ihh mRNA and protein, as well as Ptch1 were localised in murine endometrial LE and GE (Takamoto *et al.*, 2002), suggesting potential for autocrine signalling. Additionally, epithelial Ihh was proposed to act as a paracrine mediator of stromal proliferation in the endometrium, where it may also induce expression of Ptch1 (Goodrich 1997; Matsumoto *et al.*, 2002). Established roles for Hh proteins in mediation of organisationally critical epithelial-mesenchymal interactions (Chuang *et al.*, 2003; McMahon *et al.*, 2003) and documentation of Ihh and Ptch1 expression in the neonatal porcine endometrium (Fig. 4) suggest a potential role for Ihh in perinatal uterine development.

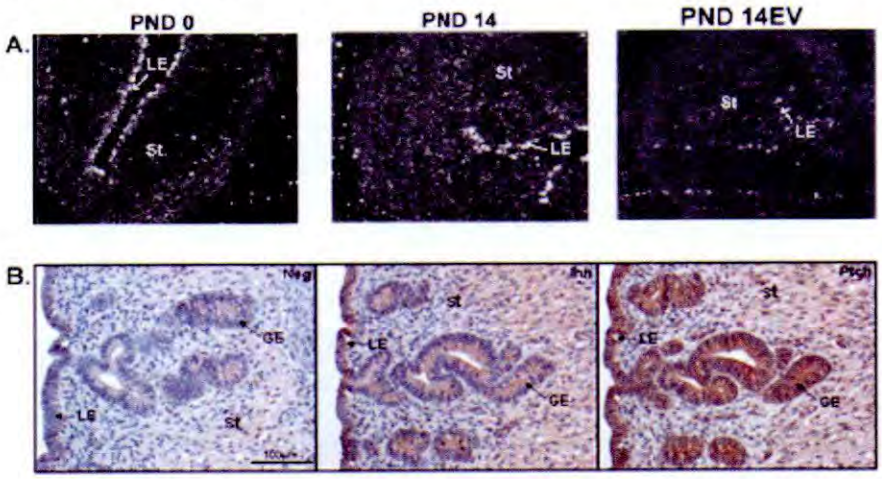

Fig. 4 Effects of age and administration of oestradiol valerate (EV; 50μg/kg bw/d) for 14 days from birth (postnatal day = PND 0) on patterns of Indian Hedgehog (Ihh) expression (A), and immunlocalisation of Ihh and the Ihh receptor Patched (Ptch) in PND 14 endometrium (B). In A - Uteri obtained from 4-6 gilts on each day and after treatment with EV on PND 14, were fixed in 4% (w/v) paraformaldehyde, embedded in paraplast-plus, sectioned (5 μm), and processed together under identical conditions for hybridisation of cRNA probes. An antisense (α-^{35}S)UTP-labelled cRNA probe, generated by *in vitro* transcription from a linearised porcine cDNA template for Ihh (NCBI accession number: CK172438), was used to localise the targeted transcript *in situ*. Darkfield photomicrographs show signal (white grains) above background, determined by subtraction of negative control images generated using corresponding (α-^{35}S)UTP-labelled sense cRNA probes (not shown). Signal indicative of Ihh expression was detected in both stroma and developing epithelium on PND 0 (left) and PND 14 (middle). Administration of EV from birth reduced the Ihh signal (PND 14 vs PND 14EV). In B – Immunolocalisation of Ihh protein and Ptch1 in neonatal porcine uterine tissues (PND 14 shown). Tissues were subjected to heat-induced epitope retrieval in boiling citrate buffer (10mM, pH 6.0 for 20 min). Antibodies directed against the c-terminus of Ihh (SC-1196) and the n-terminus of Ptch1 (SC-6149) were obtained from Santa Cruz Biotechnolgoy (CA). Negative control sections (Neg, left) received irrelevant IgG for the primary antibody. Both Ihh (middle) and Ptch1 (right) proteins were detected in stromal and epithelial compartments. Signal for both was most pronounced for epithelium. [Original magnification = 20X; GE = glandular epithelium, LE = luminal epithelium, St = stroma hematoxylin counterstain]

Tissue programming and developmental disruption

It is well known that aberrant stimuli encountered during critical periods of development can affect organisational programmes, alter normal developmental trajectories and induce permanent changes in the structure and function of tissues and organs. Recently, the term programming has been adopted to describe long-term effects of exposure to environmental, nutritional or xenobiotic factors that affect the course of development with lasting consequences (Godfrey and Barker, 2001; Rhind *et al.*, 2001). Tissues of the FRT are potential targets for such factors.

Prenatal exposure of human foetuses to DES alters the organisational programme of FRT tissues and sets the stage for cervicovaginal cancer and other complications (Herbst *et al.*, 1979; Cunha *et al.*, 1999; Iguchi and Sato, 2000). These observations provided important insights into the roles of the steroid hormone superfamily of receptors and related ligands in normal and aberrant FRT programming. Loss-of-function studies showed that oestrogen receptor-α (ER) expression is required for normal uterine growth, while both the progesterone receptor (PR) and ER are required for normal uterine function (Conneely *et al.*, 2001; Emmen and Korach, 2003). Neither ER nor PR expression is necessary to support primary uterine patterning events in pre- and/or perinatal life. However, aberrant activation of these and related receptor systems during critical organisational periods can affect the developmental programme with significant consequences for uterine function (Bartol *et al.*, 1999; Cunha *et al.*, 1999; Gray *et al.*, 2000a; Iguchi and Sato, 2000; Taylor *et al.*, 2001; Hendry *et al.*, 2002; Huang *et al.*, 2005).

Risks of exposure to compounds with the potential to disrupt steroid-sensitive uterine programming events are real. Categories of developmentally disruptive environmental chemicals likely to be encountered by animals include: (1) pharmaceuticals designed for therapeutic purposes, such as growth promotants or agents used to control timing of ovulation; (2) bioactive dietary factors and endocrine modulating chemicals found in feedstuffs; and (3) industrial xenochemicals that act as hormonal mimics or selective steroid receptor modulators (SSRMs).

In laboratory animals, perinatal oestrogen exposure produced lesions in adult uteri that included altered steroid receptor concentration and responsiveness; changes in oestrogen metabolism and protein synthesis; persistent induction or de-regulation of gene expression; de-regulation of protooncogene expression affecting uterine epithelial cell proliferation and apoptosis; and structural lesions including cystic endometrial hyperplasia, squamous metaplasia, adenomyosis, myometrial hypoplasia and general uterine hypoplasia (Bartol *et al.*, 1999; Newbold, 1999; Iguchi and Sato, 2000; Huang *et al.*, 2005). Complementary data involving ungulate models clearly indicate that adult uterine phenotype can be programmed by targeted disruption of hormone-sensitive postnatal organisational events (Bartol *et al.*, 1999; Gray *et al.*, 2000b; Gray *et al.*, 2000a; Gray *et al.*, 2001b; Carpenter *et al.*, 2003; Tarleton *et al.*, 2003a).

Porcine uterine development and endometrial programming

In the pig, as in other mammals, radial patterning of the uterine wall is incomplete at birth (Bartol *et al.*, 1993). Uterine morphogenetic events characteristic of the first 60 days of postnatal life in the pig, including appearance and proliferation of uterine glands (Fig. 5), development of endometrial folds, and differentiation and growth of myometrial smooth muscle layers, occur normally following bilateral ovariectomy at birth, whereas ovaries are required for normal uterine growth past PND 60 (Bartol *et al.*, 1993). Thus, as reported for other species (Gray *et al.*, 2001a), early postnatal events associated with radial patterning of the porcine uterine wall are ovary- and, most likely, steroid hormone-independent. Consistently, oestrogen and progestin sensitivities develop postnatally in these tissues (Vallet *et al.*, 1995; Groothuis *et al.*, 1997; Tarleton *et al.*, 1998).

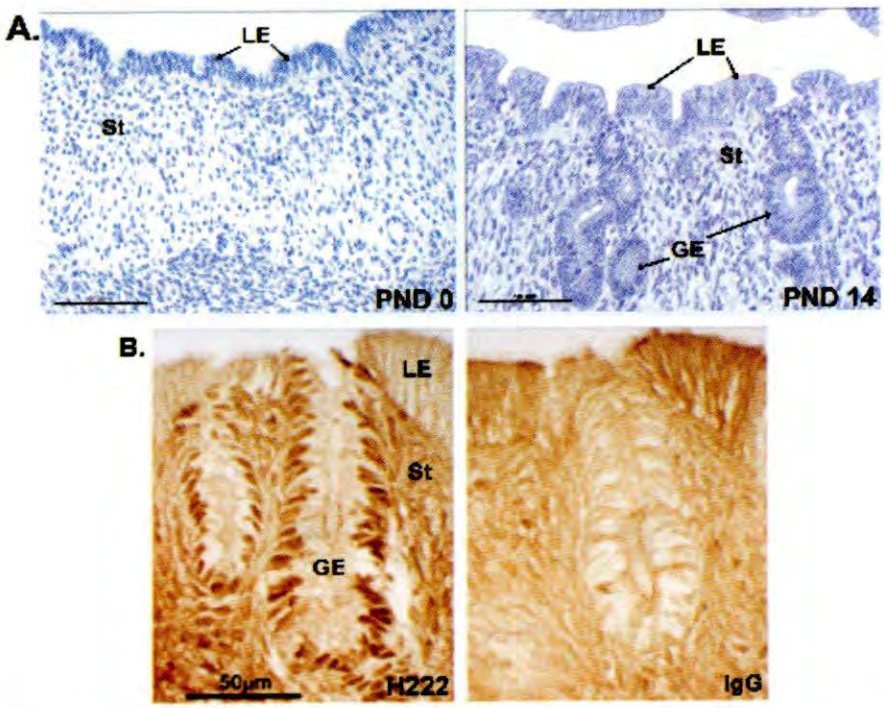

Fig. 5 Endometrial histogenesis (A) and oestrogen receptor-α (ER) expression (B) in the neonatal porcine uterus. Brightfield photomicrographs depicting normal endometrial histology for uterine tissues obtained from gilts at birth (postnatal day = PND 0) and on PND 14 (A, hematoxylin). During this period luminal epithelium (LE) ultimately gives rise to glandular epithelium (GE). Differentiation of GE from LE is associated with onset of ER expression in nascent GE (B). Immunohistochemical localisation of ER protein in porcine endometrium from PND 14, as described elsewhere (Tarleton *et al.*, 1998), using the H222 monoclonal antibody (left) reveals clear nuclear staining in GE and stroma (St), but not in LE. Irrelevant IgG was used in place of H222 in negative control sections.

The porcine uterus is ER-negative at birth (Tarleton *et al.*, 1998). Onset of ER expression between PND 0 and 15 in uterine stroma and glandular epithelium (GE), but not in luminal epithelium (LE; Fig. 5), is associated with appearance and proliferation of endometrial glands (Tarleton *et al.*, 1998). The ER antagonist ICI 182,780 retards endometrial development and inhibits gland genesis during this period when administered from birth (Tarleton *et al.*, 1999). Thus, the ER is both a marker and mediator of GE differentiation and radial patterning of the neonatal porcine uterine wall.

Temporally aberrant activation of the ER system by administration of oestradiol valerate (EV) to gilts from PND 0-13, while acutely uterotrophic (Spencer *et al.*, 1993), is ultimately both anti-uterotrophic and anti-embryotrophic (Bartol *et al.*, 1993; Tarleton *et al.*, 2001; Tarleton *et al.*, 2003b). The hypoplastic, neonatally EV-exposed adult porcine uterus does not respond normally to signals associated with the periattachment stage of early pregnancy. Numbers of corpora lutea and uterine luminal fluid (ULF) oestrogen content were similar in control and neonatally EV-exposed pregnant adult gilts on day 12 post-mating (gestational day = GD 12), suggesting similar levels of maternal and conceptus signalling in both groups. However, ULF protein content was reduced, uterine growth responses to early pregnancy were inhibited, and endometrial gene expression patterns were altered in neonatally EV-exposed gilts on GD 12 (Tarleton *et al.*, 2003b). Treatment

effects on the endometrial proteome for GD 12 (Crean *et al.*, 2004) were also pronounced (Fig. 6), and embryo survival in neonatally EV-exposed gilts was reduced by 22% when assessed on GD 45 (Bartol *et al.*, 1993). Thus, transient, oestrogen-induced disruption of the neonatal uterine organisational programme has marked and lasting effects on uterine form and function in the pig.

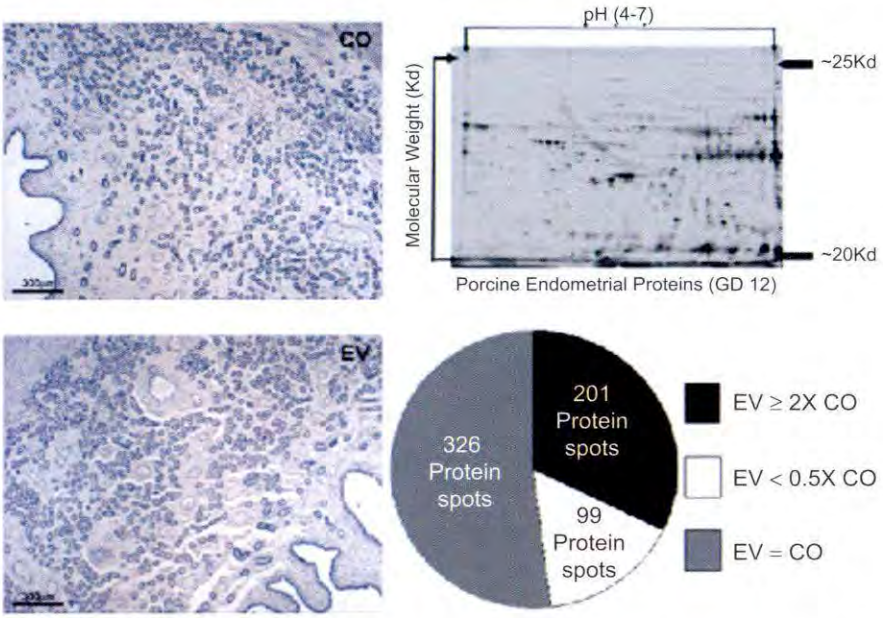

Fig. 6 Effects of administration of oestradiol valerate (EV; 50μg/kg bw/d, i.m.) for 14 days from birth (postnatal day = PND 0) on adult endometrial histology (left) and relative quantitative changes in a subset of conserved peptides representative of the endometrial tissue proteome on day 12 postmating in pregnant adult gilts (gestational day = GD 12). Pregnant gilts (n = 4/group), treated with either EV or corn oil vehicle alone (CO) for 14 days from birth, were hysterectomised on GD 12. Tissue from individual uteri were fixed in 4% (w/v) paraformaldehyde, embedded in paraplast, sectioned (5μm), stained (hematoxylin) and processed for histology (left) and histomorphometric analyses. Endometrium from each gilt was sonicated in reagent 3 of the Bio-Rad (Hercules, CA) ReadyPrep Sequential Extraction kit. Equal amounts (70 μg) of total endometrial tissue protein from each sample were applied to ReadyStrip immobilised pH gradient strips (11cm, pH 4-7; Bio-Rad) for separation in the first dimension, followed by separation in the second dimension by SDS-PAGE using Criterion Pre-cast, 11 cm gels (10% total monomer; Bio-Rad). Duplicate two-dimensional (2DE) gels were run for each animal. A master reference gel (image top right) was prepared by combining proteins from all gilts (CO and EV). Individual samples representing the master mix and proteins from the eight gilts were electrophoresed on separate gels run simultaneously using a Criterion Dodeca cell (Bio-Rad). Gels were stained with SYPRO® Ruby (Bio-Rad), images were generated using a Typhoon 9410 (Amersham, NJ), and image analyses were performed using PDQuest software (Bio-Rad). A subset of 717 spots determined to be consistent elements of the GD 12 endometrial tissue proteome was identified and PDQuest analyses were performed to determine if spot intensity for EV tissues was: (1) at least twice (2X; upper limit analysis); (2) less than half (0.5X; lower limit analysis); or (3) approximately equal to (between limits analysis) that observed for CO tissues. Endometrial histoarchitecture and glandularity were similar for CO (top left) and EV (bottom left) tissues. Image analyses (bottom right) revealed that, while the majority of proteins in the subset of conserved peptide spots were present in approximately equal abundance, 201 peptide spots were present in greater abundance and 99 were present in relatively lower abundance in adult, neonatally oestrogen-exposed tissues.

Given that the first two weeks of neonatal life represent a critical period for ER-dependent, oestrogen-sensitive programming of porcine uterine tissues, it follows that factors affecting ER activation during this period can alter critical uterine programming events. Recent data indicate that long-term effects of neonatal oestrogen exposure on uterine phenotype reflect dysregulation of primary morphoregulatory gene expression events in developing porcine endometrium similar to that observed in murine systems described above.

In situ hybridisation (ISH) studies indicate that a *Hoxa/Wnt* expression axis develops in the porcine uterine wall between birth and PND 14 (Figs. 2 and 3). Normally, stromal expression of *Hoxa*10 and *Hoxa*11 (Fig. 2), as well as *Wnt4*, *Wnt*5a and *Wnt7*a (Fig. 3), is detectable at birth and increases by PND 14. Epithelial expression of *Wnt7*a, undetectable at birth, is clearly evident by PND 14 in LE, but not in GE (Fig. 3). Up-regulation of *Wnt7*a expression in LE coincides with differentiation of *Wnt7*a-negative GE. Thus, in the pig, *Wnt7*a expression marks LE differentiation just as ER expression marks GE differentiation. Observations are consistent with those reviewed above for the mouse and support the idea that down-regulation of *Wnt7*a is necessary for differentiation of GE (Mericskay *et al.*, 2004). Treatment of gilts with EV from birth reduced the signal for *Wnt7*a in LE and *Wnt4* in stroma, altered stromal *Wnt*5a expression patterns, and increased stromal *Hoxa*10 signal by PND 14 (Figs. 2 and 3). Collectively, data reinforce the idea that dysregulation of the organisationally critical neonatal uterine *Hoxa/Wnt* expression axis can alter the developmental trajectory of these tissues with negative reproductive consequences.

Expression of Ihh in porcine uterine tissues at birth that persisted on PND 14 was recently documented by ISH (Fig. 4). Expression of Ihh was pronounced in LE on PND 0 (Fig. 4), in advance of detectable *Wnt7*a expression (Fig. 3). Immunohistochemical localisation of Ihh and Ptch1 (Fig. 4) revealed the ligand and its primary receptor to be associated with endometrial epithelium and stroma. Thus, essential elements of the Hh signalling system are in place at or shortly after birth. In gilts exposed to EV for 14 days from birth, Ihh expression was reduced on PND 14 (Fig. 4), following a pattern similar to that observed for epithelial *Wnt7*a (Fig. 3). Thus, Ihh may be another up-stream regulator of epithelial *Wnt7*a expression, as well as an element of the primary organisational palette of factors governing development and stabilisation of the uterine *Hoxa/Wnt* axis.

Postnatal uterine patterning and peptide signalling

The uterine gland knock-out (UGKO) phenotype (Bartol *et al.*, 1999) provides definitive evidence of the consequences of SSRM-induced disruption of the neonatal uterine developmental programme in ungulates. In sheep, induction of the UGKO phenotype is associated with loss of uterine epithelial ER expression and changes in expression patterns for paracrine-acting growth factors and/or their receptors, including uterine hepatocyte growth factor and fibroblast growth factor receptor 2IIIb, now implicated in postnatal uterine patterning events (Gray *et al.*, 2000a; Taylor *et al.*, 2001). In addition, ovine uterine gland genesis was inhibited by neonatal administration of bromocryptine and stimulated by administration of prolactin (Carpenter *et al.*, 2003). Data provide evidence that peptide growth factor signalling can affect ungulate uterine patterning and tissue programming.

Relaxin and neonatal uterine development

Peptide hormone signalling can affect uterine development directly and indirectly, via crosstalk with steroid hormone signalling systems (Smith 1998). Supportive evidence in the pig comes from studies of relaxin (RLX), a member of the insulin-like growth factor family. Like oestrogen, uterotrophic effects of RLX in the neonatal pig are age-specific (Spencer *et al.*, 1993; Bagnell *et al.*, 2005). Administered for two days from birth, prior to onset of endometrial ER expression, RLX

increased uterine LE height, but not uterine weight on PND 2 (Bagnell *et al.*, 2005; Yan *et al.*, 2005a). However, when administered for two days from PND 12, after onset of endometrial ER expression, RLX increased both uterine LE height and uterine weight on PND 14, effects that were inhibited with ICI 182,780 (Bagnell *et al.*, 2005). Thus, effects of RLX in the neonatal pig are determined, in part, by the relative presence of uterine ER and may involve crosstalk with the ER signalling system (Pillai *et al.*, 1999; Bagnell *et al.*, 2005).

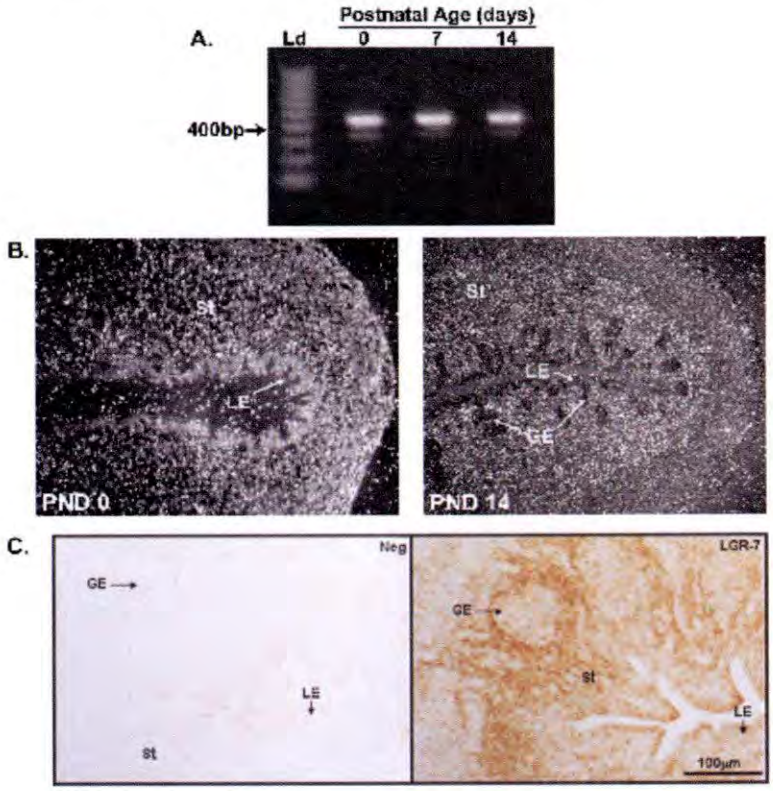

Fig. 7 Relaxin receptor (LGR7) expression in the neonatal porcine uterus detected by RT-PCR (A), *in situ* hybridisation (B) and immunohistochemistry (C). Uteri (n = 4-6/day) were obtained from neonatal gilts at birth (postnatal day = PND 0) and on PND 7 and 14. Primers designed based on human sequence data for LGR7 were used to generate a 431 bp porcine cDNA amplicon by RT-PCR, identified in tissues from all days (A). The amplicon from PND 14 was cloned, sequenced (NCBI accession number: CA994862) and determined to have 88% similarity with the human sequence for LGR7. An antisense (α-^{35}S)UTP-labelled cRNA probe, generated by *in vitro* transcription from the linearised porcine cDNA template for LGR7, was used to localise the targeted transcript *in situ* (B). Darkfield photomicrographs show signal (white grains) above background, determined by subtraction of negative control images generated using corresponding (α-^{35}S)UTP-labelled sense cRNA probes (not shown). Signal indicative of LGR7 expression was detected primarily in the endometrial stroma and increased from PND 0 to PND 14. Immunohistochemical localisation of LGR7 in uterine tissues (C; PND 14 shown), using the L7 antibody provided as a gift by Dr. Richard Ivell (University of Adelaide, Adelaide, Australia), revealed some signal above background (left; IgG substituted for L7) in epithelium, but corroborated the stromal expression signal observed by *in situ* hybridisation.

The RLX receptor, LGR7, and a related RLX-sensitive receptor, LGR8 (Hsu *et al.*, 2002), are expressed by porcine uterine tissues from birth (Bagnell *et al.*, 2005). Stromal LGR7 expression increases from PND 0 to PND 14 (Fig. 7). Immunolocalisation studies (Fig. 7) corroborated ISH results, revealing LGR7 staining similar to that reported for adult primate and human endometrium (Ivell *et al.*, 2003). Because endometrial LGR7/8 expression precedes ER expression in the neonatal pig, subtle uterotrophic effects observed for RLX administered from birth could be LGR7/8-specific, while pronounced effects seen in week two could reflect amplification of the RLX signal through crosstalk with ER. Given that RLX can augment oestrogen-stimulated uterine *Hoxa*10 expression (Gui *et al.*, 1999), RLX signalling could affect ER-dependent events governing morphoregulatory gene expression in the neonatal uterus.

Given evidence of a functional RLX signalling system in porcine uterine tissues from birth, a source of RLX in the neonatal pig was sought. Not withstanding the potential for an endogenous source, milk is a recognised source of peptide growth factors in young animals (Donovan *et al.*, 1994). Because the immature GI tract is permeable to macromolecules for 24-36 h prior to gut closure, bioactive milk-borne agents may enter the neonatal circulation to influence growth and development of peripheral organs (Burrin *et al.*, 1997). Lactational exposure is also a well recognised route for delivery of xenobiotic compounds with the potential to affect development of reproductive tissues (Hughes *et al.*, 2004).

Both human (Eddie *et al.*, 1989) and canine (Goldsmith *et al.*, 1994) colostrum and milk contain RLX, which can be absorbed into the neonatal circulation through the gut (Goldsmith *et al.*, 1994). In sows, RLX is present at highest levels in colostrum within 48 h of parturition, before gut closure, and is transmitted into the circulation of nursing pigs (Yan *et al.*, 2005b). Thus, milk-borne RLX has the potential to play a role in programming of uterine tissues during the early neonatal period.

Conclusions

The uterus, an epithelial-mesenchymal organ, develops through processes involving increasingly complex and specific cellular interactions that are accompanied by and support the evolution of organisationally critical, temporally and spatially unique patterns of primary and secondary morphoregulatory gene expression domains. Structural patterning and functional programming of uterine tissues are coupled processes. Elements of the primary organisational palette of factors required for, and mechanisms governing the success of, these processes have only just begun to be defined precisely.

Temporospatial expression patterns for factors implicated in porcine endometrial development between birth and PND 14 are summarised in Fig. 8. Evidence of the evolution of a *Hoxa/Wnt* axis in the neonatal porcine endometrium shortly after birth, including stromal *Wnt4* and *Wnt*5a expression preceding up-regulation of *Wnt7*a in LE and the associated up-regulation of ER expression in nascent *Wnt7*a-negative GE, supports murine data and reinforces the idea that these events are fundamental to uterine developmental success. The fact that expression patterns for uterine *Hoxa*, *Wnt* and Hh genes can be altered by factors that affect ER activation during a critical period for porcine uterine development emphasises the importance of these genes, as well as their yet-to-be-defined up-stream regulators and down-stream targets, in both normal and aberrant organisational processes. Evidence that RLX can not only affect uterine development in the neonatal pig directly, through LGR7/8, and indirectly via crosstalk with the ER, but can be presented to this system normally via colostrum, indicates that maternal factors governing FRT development extend into the postnatal period and should not be ignored. Clearly, factors affecting signalling events in uterine tissues which produce changes in morphoregulatory gene expression patterns during critical organisational periods can alter the developmental trajectory of the uterus with lasting consequences. Thus, uterine tissues can be programmed epigenetically for success or failure during perinatal life.

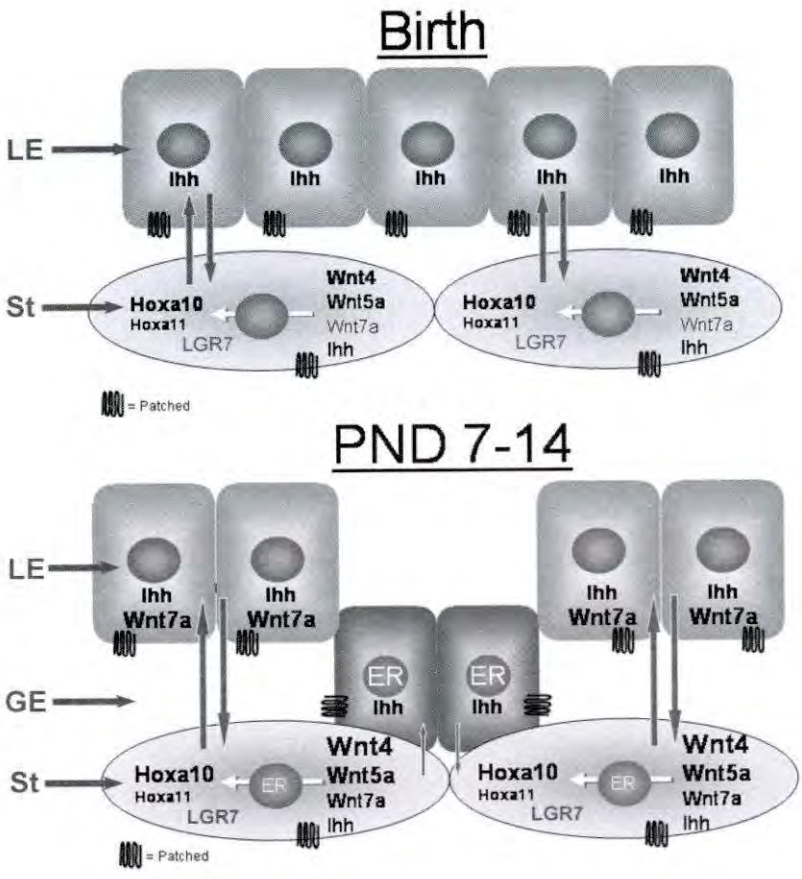

Fig. 8 Temporospatial patterns of morphoregulatory gene expression in the porcine endometrium from birth to week two of postnatal life associated with onset of uterine gland genesis. At birth (top; postnatal day = PND 0) the endometrium consists principally of a simple luminal epithelium (LE) supported by uterine stroma (St). Indian hedgehog (Ihh) expression is detectable in LE and, to a lesser extent, in St on PND 0. Similarly, expression of *Hoxa*10 and *Hoxa*11, *Wnt4*, *Wnt*5a and *Wnt*7a, as well as LGR7, the relaxin receptor, is detectable. Differentiation of nascent glandular epithelium (GE) from LE is marked and possibly mediated by the onset and increased expression of *Wnt*7a in LE and oestrogen receptor-α (ER) in GE, in which *Wnt*7a expression is not detected.

Acknowledgements

This work was supported by USDA-NRICGP grants 98-3203-6198 (FFB), 99-35203-7812 (CAB) and 2003-35203-13572 (FFB/CAB), the Alabama Agricultural Experiment Station and the New Jersey Agricultural Experiment Station. The authors acknowledge Ms. Bethany D. Crean, Ms. Regina A. Masters and Mr. Wenbo Yan for their important contributions to work reviewed here, and thank Dr. Anthony G. Moss for his assistance with imaging procedures.

References

Ashworth CJ, Finch AM, Page KR, Nwagwu MO and McArdle HJ (2001) Causes and consequences of foetal growth retardation in pigs *Reproduction* Supplement **58** 233-246.

Bagnell CA, Yan W, Wiley AA and Bartol FF (2005) Effects of relaxin on neonatal porcine uterine growth and development *Annals of the New York Academy of Sciences* **1041** 248-255.

Bartol FF (1999) Uterus, Nonhuman. In *Encyclopedia of Reproduction* pp 950-960, Eds. E Knobil & JD Neill. Academic Press, San Diego, CA.

Bartol FF, Wiley AA, Spencer TE, Vallet JL and Christenson RK (1993) Early Uterine Development in Pigs *Journal of Reproduction and Fertility* **48** 99-116.

Bartol FF, Wiley AA, Floyd JG, Ott TL, Bazer FW, Gray CA and Spencer TE (1999) Uterine differentiation as a foundation for subsequent fertility *Journal of Reproduction and Fertility* Supplement 54 287-302.

Benson GV, Lim H, Paria BC, Satokata I, Dey SK and Maas RL (1996) Mechanisms of reduced fertility in *Hoxa*-10 mutant mice: uterine homeosis and loss of maternal *Hoxa*-10 expression *Development* Supplement. **122** 2687-2696.

Biason-Lauber A, Konrad D, Navratil F and Schoenle EJ (2004) A *WNT4* mutation associated with Mullerian-duct regression and virilization in a 46,XX woman *New England Journal of Medicine* **351** 792-798.

Branford WW, Benson GV, Ma L, Maas RL and Potter SS (2000) Characterization of *Hoxa*-10/*Hoxa*-11 transheterozygotes reveals functional redundancy and regulatory interactions *Developmental Biology* **224** 373-387.

Burrin DG, Davis TA, Ebner S, Schoknecht PA, Fiorotto ML and Reeds PJ (1997) Colostrum enhances the nutritional stimulation of vital organ protein synthesis in neonatal pigs *Journal of Nutrition* **127** 1284-1289.

Carpenter KD, Gray CA, Noel S, Gertler A, Bazer FW and Spencer TE (2003) Prolactin regulation of neonatal ovine uterine gland morphogenesis *Endocrinology* **144** 110-120.

Carta L and Sassoon D (2004) *Wnt7a* is a suppressor of cell death in the female reproductive tract and is required for postnatal and estrogen-mediated growth *Biology of Reproduction* **71** 444-454.

Chuang PT, Kawcak T and McMahon AP (2003) Feedback control of mammalian Hedgehog signaling by the Hedgehog-binding protein, Hip1, modulates Fgf signaling during branching morphogenesis of the lung *Genes and Development* **17** 342-347.

Cillo C, Cantile M, Faiella A and Boncinelli E (2001) Homeobox genes in normal and malignant cells *Journal of Cellular Physiology* **188** 161-169.

Cohen MM, Jr. (2003) The hedgehog signaling network *American Journal of Medical Genetics*. Part A **123** 5-28.

Conneely OM, Mulac-Jericevic B, Lydon JP and De Mayo FJ (2001) Reproductive functions of the progesterone receptor isoforms: lessons from knock-out mice *Molecular and Cellular Endocrinology* **179** 97-103.

Couse JF, Dixon D, Yates M, Moore AB, Ma L, Maas R and Korach KS (2001) Estrogen receptor-alpha knockout mice exhibit resistance to the developmental effects of neonatal diethylstilbestrol exposure on the female reproductive tract *Developmental Biology* **238** 224-238.

Crean BD, Wiley AA and Bartol FF (2004) Transient neonatal estrogen exposure from birth affects the adult porcine endometrial proteome on day 12 of pregnancy *Biology of Reproduction* **Special Issue** 159.

Cunha GR, Forsberg JG, Golden R, Haney A, Iguchi T, Newbold R, Swan S and Welshons W (1999) New approaches for estimating risk from exposure to diethylstilbestrol *Environmental Health Perspectives* **107** 625-630.

DasGupta R, Kaykas A, Moon RT and Perrimon N (2005) Functional genomic analysis of the *Wnt*-Wingless signaling pathway *Science* **308** 826-833.

Davidson EH (2001) *Genomic Regulatory Systems*, Academic Press, San Diego.

Donovan SM, McNeil LK, Jimenez-Flores R and Odle J (1994) Insulin-like growth factors and insulin-like growth factor binding proteins in porcine serum and milk throughout lactation *Pediatric Research* **36** 159-168.

Eddie LW, Sutton B, Fitzgerald S, Bell RJ, Johnston PD and Tregear GW (1989) Relaxin in paired samples of serum and milk from women after term and preterm delivery *American Journal of Obstetrics and Gynecology* **161** 970-973.

Emmen JM and Korach KS (2003) Estrogen receptor knockout mice: phenotypes in the female reproductive tract *Gynecological Endocrinology* **17** 169-176.

Gendron RL, Paradis H, Hsieh-Li HM, Lee DW, Potter SS and Markoff E (1997) Abnormal uterine stromal and glandular function associated with maternal reproductive defects in *Hoxa*-11 null mice *Biology of Reproduction* **56** 1097-1105.

Godfrey KM and Barker DJ (2001) Foetal programming and adult health *Public Health Nutrition* **4** 611-624.

Goldsmith LT, Lust G and Steinetz BG (1994) Transmission of relaxin from lactating bitches to their offspring during suckling *Biology of Reproduction* **50** 258-265.

Goodrich LV ML, Higgins KM and Scott MP (1997) Altered neural cell fates and medulloblastoma in mouse patched mutants *Science* **277** 1109-1113.

Gray CA, Taylor KM, Bazer FW and Spencer TE (2000) Mechanisms regulating norgestomet inhibition of endometrial gland morphogenesis in the neonatal ovine uterus. Molecular Reproduction and Development **57** 67-78.

Gray CA, Taylor KM, Ramsey WS, Hill JR, Bazer FW, Bartol FF and Spencer TE (2001b) Endometrial glands are required for preimplantation conceptus elongation and survival *Biology of Reproduction* **64** 1608-1613

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

Gray CA, Bartol FF, Taylor KM, Wiley AA, Ramsey WS, Ott TL, Bazer FW and Spencer TE (2000b) The ovine uterine gland knock-out model: Effects of gland ablation on the estrous cycle *Biology of Reproduction* **62** 448-456.

Greenwood PL and Bell AW (2003) Consequences of intra-uterine growth retardation for postnatal growth, metabolism and pathophysiology *Reproduction* Supplement. **61** 195-206.

Groothuis PG, Blair RM, Simmen RCM, Vallet JL, Grieger DM and Davis DL (1997) Uterine response to progesterone in prepubertal gilts *Journal of Reproduction and Fertility* **110** 237-243.

Gui Y, Zhang J, Yuan L and Lessey BA (1999) Regulation of *HOXA*-10 and its expression in normal and abnormal endometrium *Molecular Human Reproduction* **5** 866-873.

Hales CN and Ozanne SE (2003) The dangerous road of catch-up growth *Journal of Physiology* **547** 5-10.

Hendry WJ, 3rd, Sheehan DM, Khan SA and May JV (2002) Developing a laboratory animal model for perinatal endocrine disruption: the hamster chronicles *Experimental Biology and Medicine* **227** 709-723.

Herbst AL, Scully RE and Robboy SJ (1979) Prenatal diethylstilbestrol exposure and human genital tract abnormalities *National Cancer Institute Monographs* 25-35.

Hou X, Tan Y, Li M, Dey SK and Das SK (2004) Canonical *Wnt* signaling is critical to estrogen-mediated uterine growth *Molecular Endocrinology* **18** 3035-3049.

Hsu SY, Nakabayashi K, Nishi S, Kumagai J, Kudo M, Sherwood OD and Hsueh AJ (2002) Activation of orphan receptors by the hormone relaxin *Science* **295** 671-674.

Hu J, Gray CA and Spencer TE (2004) Gene expression profiling of neonatal mouse uterine development *Biology of Reproduction* **70** 1870-1876.

Huang W-W, Yin Y, Bi Q, Chiang T-C, Garner N, Vuoristo J, McLachlan JA and Ma L (2005) Developmental diethylstilbestrol exposure alters genetic pathways of uterine cytodifferentiation *Molecular Endocrinology* **19** 669-682.

Hughes CL, Liu G, Beall S, Foster WG and Davis V (2004) Effects of genistein or soy milk during late gestation and lactation on adult uterine organization in the rat *Experimental Biology and Medicine* **229** 108-117.

Hughes IA (2004) Female development - all by default? *New England Journal of Medicine* **351** 748-750.

Iguchi T and Sato T (2000) Endocrine disruption and developmental abnormalities of female reproduction *American Zoologist* **40** 402-411.

Ivell R, Balvers M, Pohnke Y, Telgmann R, Bartsch O, Milde-Langosch K, Bamberger AM and Einspanier A (2003) Immunoexpression of the relaxin receptor LGR7 in breast and uterine tissues of humans and primates *Reproductive Biology and Endocrinology* **1** 24.

Kastner P, Mark M, Ghyselinck N, Krezel W, Dupe V, Grondona JM and Chambon P (1997) Genetic evidence that the retinoid signal is transduced by heterodimeric RXR/RAR functional units during mouse development *Development* **124** 313-326.

Kitajewski J and Sassoon D (2000) The emergence of molecular gynecology: homeobox and *Wnt* genes in the female reproductive tract *Bioessays* **22** 902-910.

Kobayashi A and Behringer RR (2003) Developmental genetics of the female reproductive tract in mammals *Nature Reviews Genetics* **4** 969-980.

Kobayashi A, Shawlot W, Kania A and Behringer RR (2004) Requirement of *Lim1* for female reproductive tract development *Development* **131** 539-549.

Krumlauf R (1994) *Hox* Genes in Vertebrate Development *Cell* **78** 191-201.

Logan CY and Nusse R (2004) The *Wnt* signaling pathway in development and disease *Annual Review of Cell and Developmental Biology* **20** 781-810.

Maclean JA, 2nd, Chen MA, Wayne CM, Bruce SR, Rao M, Meistrich ML, Macleod C and Wilkinson MF (2005) R*Hox*: a new homeobox gene cluster *Cell* **120** 369-382.

Mark M, Rijli FM and Chambon P (1997) Homeobox genes in embryogenesis and pathogenesis *Pediatric Research* **42** 421-429.

Matsumoto H, Zhao XM, Das SK, Hogan BLM and Dey SK (2002) Indian hedgehog as a progesterone-responsive factor mediating epithelial-mesenchymal interactions in the mouse uterus *Developmental Biology* **245** 280-290.

McMahon AP, Ingham PW and Tabin CJ (2003) Developmental roles and clinical significance of hedgehog signaling *Current Topics in Developmental Biology* **53** 1-114.

Mendelsohn C, Lohnes D, Decimo D, Lufkin T, LeMeur M, Chambon P and Mark M (1994) Function of the retinoic acid receptors (RARs) during development (II). Multiple abnormalities at various stages of organogenesis in RAR double mutants *Development* **120** 2749-2771.

Mericskay M, Kitajewski J and Sassoon D (2004) *Wnt5a* is required for proper epithelial-mesenchymal interactions in the uterus *Development* **131** 2061-2072.

Miller C and Sassoon DA (1998) *Wnt-7a* maintains appropriate uterine patterning during the development of the mouse female reproductive tract *Development* **125** 3201-3211.

Miller C, Pavlova A and Sassoon DA (1998) Differential expression patterns of *Wnt* genes in the murine female reproductive tract during development and the estrous cycle *Mechanisms of Development* **76** 91-99.

Miller JR, Hocking AM, Brown JD and Moon RT (1999) Mechanism and function of signal transduction by the *Wnt*/beta-catenin and *Wnt*/Ca2+ pathways *Oncogene* **18** 7860-7872.

Mortlock DP and Innis JW (1997) Mutation of *HOXA13* in hand-foot-genital syndrome *Nature Genetics* **15** 179-180.

Newbold RR (1999) Diethylstilbestrol (DES) and environmental estrogens influence the developing female reproductive system In *Endocrine Disruptors*, pp 39-56 Eds RK Naz. CRC Press, Boca Raton, FL.

Nusse R (2003) *Wnts* and Hedgehogs: lipid-modified proteins and similarities in signaling mechanisms at the cell surface *Development* **130** 5297-5305.

Pillai SB, Rockwell LC, Sherwood OD and Koos RD (1999) Relaxin stimulates uterine edema via activation of estrogen receptors: Blockade of its effects using ICI 182,780, a specific estrogen receptor antagonist *Endocrinology* **140** 2426-2429.

Rhind SM, Rae MT and Brooks AN (2001) Effects of nutrition and environmental factors on the foetal programming of the reproductive axis *Reproduction* **122** 205-214.

Sassoon D (1999) *Wnt* genes and endocrine disruption of the female reproductive tract: a genetic approach *Molecular and Cellular Endocrinology* **158** 1-5.

Smith CL (1998) Cross-talk between peptide growth factor and estrogen receptor signaling pathways *Biology of Reproduction* **58** 627-632.

Spencer TE, Wiley AA and Bartol FF (1993) Neonatal Age and Period of Estrogen Exposure Affect Porcine Uterine Growth, Morphogenesis, and Protein-Synthesis *Biology of Reproduction* **48** 741-751.

Takamoto N, Zhao B, Tsai SY and DeMayo FJ (2002) Identification of Indian hedgehog as a progesterone-responsive gene in the murine uterus *Molecular Endocrinology* **16** 2338-2348.

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

Tarleton BJ, Wiley AA and Bartol FF (2001) Neonatal estradiol exposure alters uterine morphology and endometrial transcriptional activity in prepubertal gilts *Domestic Animal Endocrinology* **21** 111-125.

Tarleton BJ, Braden TD, Wiley AA and Bartol FF (2003a) Estrogen-induced disruption of neonatal porcine uterine development alters adult uterine function *Biology of Reproduction* **68** 1387-1393.

Tarleton BJ, Braden TD, Wiley AA and Bartol FF (2003b) Estrogen-induced disruption of neonatal porcine uterine development alters adult uterine function *Biology of Reproduction* **68** 1387-1393.

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

Taylor HS, VandenHeuvel GB and Igarashi P (1997) A conserved *Hox* axis in the mouse and human female reproductive system: Late establishment and persistent adult expression of the *Hoxa* cluster genes *Biology of Reproduction* **57** 1338-1345.

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

Topol L, Jiang X, Choi H, Garrett-Beal L, Carolan PJ and Yang Y (2003) *Wnt*-5a inhibits the canonical *Wnt* pathway by promoting GSK-3-independent beta-catenin degradation *Journal of Cell Biology* **162** 899-908.

Tulac S, Nayak NR, Kao LC, Van Waes M, Huang J, Lobo S, Germeyer A, Lessey BA, Taylor RN, Suchanek E and Guidice LC (2003) Identification, characterization, and regulation of the canonical *Wnt* signaling pathway in human endometrium *Journal of Clinical Endocrinology and Metabolism* **88** 3860-3866.

Vainio S, Heikkila M, Kispert A, Chin N and McMahon AP (1999) Female development in mammals is regulated by *Wnt*-4 signalling *Nature* **397** 405-409.

Vallet JL, Christenson RK, Bartol FF and Wiley AA (1995) Effect of Treatment with Retinyl Palmitate, Progesterone, Estradiol and Tamoxifen on Secretion of a Protein Similar to Retinol-Binding Protein During Uterine Gland Development in Neonatal Pigs *Journal of Reproduction and Fertility* **103** 189-197.

Veeman MT, Axelrod JD and Moon RT (2003) A second canon. Functions and mechanisms of beta-catenin-independent *Wnt* signaling *Developmental Cell* **5** 367-377.

Walterhouse DO, Lamm ML, Villavicencio E and Iannaccone PM (2003) Emerging roles for hedgehog-patched-Gli signal transduction in reproduction *Biology of Reproduction* **69** 8-14.

Wang J and Wynshaw-Boris A (2004) The canonical *Wnt* pathway in early mammalian embryogenesis and stem cell maintenance/differentiation *Current Opinion in Genetics and Development* **14** 533-539.

Warot X, Fromental-Ramain C, Fraulob V, Chambon P and Dolle P (1997) Gene dosage-dependent effects of the *Hoxa*-13 and *Hoxd*-13 mutations on morphogenesis of the terminal parts of the digestive and urogenital tracts *Development* **124** 4781-4791.

Wodarz A and Nusse R (1998) Mechanisms of *Wnt* signaling in development. *Annual Review of Cell and Developmental Biology* **14** 59-88.

Yan W, Wiley AA, Bartol FF and Bagnell CA (2005a) Tissue-specific effects of relaxin on the reproductive tract of neonatal gilts *Annals of the New York Academy of Sciences* **1041** 132-135.

Yan W, Lasano S, Steinetz BG, Bartol FF and Bagnell CA (2005b) Presence of relaxin in the milk of lactating sows and its transmission to neonatal pigs via suckling. *Biology of Reproduction* **Special Issue** 94.