

UTERINE PATTERNING, ENDOMETRIAL PROGRAMMING AND REPRODUCTIVE PERFORMANCE

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Abstract

Uterine functionality is central to reproductive success. Mechanisms that regulate uterine tissue patterning during pre- and perinatal life define a developmental program that must be optimized to insure reproductive health. Here, the biology of uterine tissue patterning and endometrial programming are reviewed and consequences of xenobiotically induced developmental disruption, as can occur with exposure to environmental endocrine disruptors, are discussed.

Introduction

The uterus is an essential part of the female reproductive tract (FRT). Embryologically, the uterus is a mesodermally derived specialization of the Müllerian (paramesonephric) ducts. These paired tubular structures arise from invaginations of coelomic epithelium on the lateral aspects of the embryonic urogenital ridges, grow caudally and begin to fuse. The degree of Müllerian duct fusion, which can be complete, partial or incomplete, is species-specific and defines gross (i.e. simplex, bicornuate, or duplex) morphological characteristics of adult uteri [1]. Patterning of the Müllerian ducts along their anteroposterior axes results in segmentation of these embryonic tubes into structurally and, ultimately, functionally unique segments of the FRT defined as the oviducts, uterus, cervix and anterior vagina [1]. Radial patterning, required for establishment of tissue-specific histoarchitecture, begins prenatally but is completed postnatally [1, 2]. This process establishes the three classic histological elements of the uterine wall, including the: (1) *endometrium*, a serous-type mucosa; (2) *myometrium*, which consists of an inner (adluminal) layer of circularly oriented and an outer layer of longitudinally oriented smooth muscle; and (3) *perimetrium*, the serous peritoneal coat of the uterus [1].

Functions of the uterus in eutherian mammals include: (1) transport, storage (some mammals) and maturation of spermatozoa; (2) spacing (in polytocous species), recognition and reception of embryos and provision of an embryotrophic environment for conceptus development; and (3) expulsion of the fetus(es) and placenta(e) at parturition. Additionally, while not the case in (ex.) primates, carnivores or marsupials, the uterine endometrium, is required for ovarian cyclicity in many animals, including the guinea pig, pig, cow, sheep, goat and horse, all of which depend upon endometrial prostaglandin production for luteolysis [1].

Uterine abnormalities and dysfunctionalities can compromise fertility, increase embryonic mortality, exacerbate conditions leading to intrauterine growth retardation (IUGR) and associated postnatal complications in offspring, and potentiate dysplasia and disease [3-12]. A growing body of data supports the idea that the integrity, stability and functional capacity of adult uterine tissues are defined, to a significant extent, by the course of events associated with developmental ‘programming’ of uterine tissues during pre- and perinatal life [3, 7, 8, 13].

Tissue Patterning – Primary Elements of the Organizational Program

Pattern formation (patterning) is a central feature of biological systems that occurs at every level of organization from molecular to organismal. In mammalian systems, as in other complex eukaryotic organisms, morphogenesis (structural patterning) and cytodifferentiation (functional patterning) are coupled processes. For epithelial-mesenchymal organs such as the uterus, genesis of form and function requires the progressive generation of increasingly complex and specific cellular relationships and interactions [3, 8, 12-15]. These interactions are accompanied by and, in fact, drive the evolution of organizationally critical, temporally and spatially unique morphoregulatory gene expression domains that direct and specify cell fate, define patterns of development and, ultimately, determine cell and tissue identity and functionality. Genes that are most centrally involved in tissue patterning and the regional specification of cell fate include those that encode: (1) transcription factors, and; (2) signaling ligands, their receptors and downstream elements of signaling pathways [16]. Elements of the primary organizational palette of factors governing formation and patterning of the uterus are described below.

Formation of Müllerian Ducts and Genesis of the FRT. Efforts to identify genes and gene networks required for development of the mammalian FRT and uterine tissue patterning have been aided by advances in molecular profiling techniques, combined with the power of murine genetics and inferences drawn from phenotypes produced in this animal model through studies involving targeted mutagenesis [8, 12, 17]. To date, such studies indicate that expression of genes encoding the homeodomain-containing transcription factors *Pax2*, *Lim1*, and *Emx2*, as well as *Wnt4*, a secreted morphoregulatory glycoprotein, is required for FRT formation [8]. Information about these and other genes associated with FRT formation and development can be found on the web by visiting “GeneCards” (See: <http://www.rzpd.de/cards/index.html>).

Pax2-null female mutants lack kidneys as well as reproductive tracts, owing to degeneration of the Müllerian ducts during embryogenesis. Normally, *Lim1* is expressed in developing Müllerian epithelium, as well as in the mesonephros, metanephros and fetal gonads [17]. Female *Lim1*-null mutants have morphologically normal ovaries, but lack all Müllerian derivatives. In addition, *Lim1*-negative Müllerian epithelial cells do not contribute to the uterine epithelium, but can contribute to developing uterine stroma [17]. This indicates a requirement for “cell-autonomous” *Lim1* expression in developing Müllerian epithelium as a prerequisite to successful uterine patterning [8, 17]. *Emx2*-null mutants lack kidneys, gonads and reproductive tracts [8]. *Wnt4* is one of several Wnt gene family members, including *Wnt5a* and *Wnt7a*, implicated in patterning and function of the FRT [13, 18, 19]. *Wnt4*-null mutant female mice lack a female reproductive tract, but contain a normal male reproductive tract [18]. This condition is due to the presence of Leydig cells in ovaries of *Wnt4*-null females and their production of androgens, which support development of mesonephric duct derivatives [8]. Müllerian ducts fail to form in either male or female *Wnt4*-null mutants [18]. Thus, *Wnt4* is required to initiate Müllerian duct formation [8, 18]. The fact that female mice with compound (but not single) mutations for retinoic acid receptor (RAR) genes can lack either all or caudal portions of the FRT [20, 21] indicates that complex RAR signaling is also required for the formation of these tissues [8].

Uterine Patterning. Patterning events required for differentiation of the uterine segment of the FRT from Müllerian derivatives occur in both anteroposterior and radial axes. Anteroposterior patterning establishes histologically distinct anterior boundaries between the oviducts and the

uterine body (corpus; in simplex uteri) or uterine horns (cornua; in duplex and bicornuate uteri), as well as the posterior boundary between uterus and cervix. Radial patterning establishes uterine histoarchitecture. Temporospatial coordination of these basic developmental processes is governed locally by a group of highly conserved morphoregulatory gene products, including those coded for by members of both the *Hox* and *Wnt* gene families. Evidence for involvement of these genes in FRT patterning and uterine programming is reviewed below. To learn more about these important morphoregulatory gene families, readers are encouraged to visit the “Hox-Pro Database” (See: <http://www.iephb.nw.ru/hoxpro>) and the “Wnt Gene Homepage” (See: <http://www.stanford.edu/~rnusse/wntwindow.html>).

Homeobox genes encode nuclear proteins that act as transcription factors [22, 23]. These genes contain a common DNA sequence, the 183 bp ‘homeobox’, that codes for a 61-amino acid ‘homeodomain’. It is the homeodomain that recognizes and binds to specific DNA motifs. This binding allows the transcription factors to activate or repress target gene expression events required to establish positional identity of cells along anteroposterior axes in developing tissues [23]. In mice and humans, the 39 Class I homeobox or *Hox* genes (*Hox* genes in mice/non-human species vs *HOX* genes in human) are organized in four genomic clusters on different chromosomes. During development, *Hox* gene expression occurs in a manner that can be related to the genomic organization of these genes, both temporally and spatially. This colinear mode of expression means that 3’ *Hox* genes are expressed early in development and control anterior patterning events, while 5’ *Hox* genes are expressed later in development and control more posterior patterning events. The 5’ vertebrate *Hox* genes control development of the lumbosacral region, including the genitalia [22-24].

Prior to differentiation, *Hox* genes in the abdominal-B *Hoxa* cluster, including *Hoxa9*, -10, -11 and -13, are expressed uniformly along the anteroposterior axis of the Müllerian duct [12, 24]. Segmentation of the Müllerian duct along this axis is associated with restricted, overlapping expression of these genes such that *Hoxa9* is expressed in oviduct, *Hoxa10* and -11 in the uterine segment, and *Hoxa11* and -13 in cervix and anterior vagina, respectively [12, 24]. Results of targeted mutagenesis studies indicate that expression of *Hox* genes in their respective segments of the FRT is required to define and stabilize tissue boundaries along the anteroposterior axis of the tract. This segment-specific ‘Hox code’ [25] must be established and maintained to insure FRT patterning success. Thus, uterine segmentation requires stable expression of *Hoxa10* and -11, which define the uterine ‘Hox code’ [22]. Both functional redundancies and interactions were described for these two uterine *Hox* genes [26]. 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 *Hoxa10* expression produced a FRT phenotype in the mouse characterized by dissolution of histological and functional boundaries between the oviducts and uterus [27]. In contrast, disruption of *Hoxa11* expression affects radial patterning in the uterine segment, possibly associated with dysregulation of a critical *Wnt/Hox* axis (see below) [12, 28]. Functional redundancies and overlapping expression domains described for *Hoxa* genes governing FRT patterning may explain how mutations in genes not ultimately associated with the uterine ‘Hox code’ can affect uterine phenotype. The hand-foot-genital (HFG) syndrome is an autosomal dominant disorder in which a nonsense mutation of *HOXA13* truncates the homeodomain and inhibits DNA binding by this transcription factor [29]. Uterine anomalies in human females with HFG are common

and involve defects in Müllerian duct fusion. Consequently, the normal simplex uterus is absent in most HFG patients and, instead, partially (i.e. bicornuate) or completely (i.e. didelphic) divided uterine morphologies are seen [29]. Observations reinforce the importance of Hox gene expression in uterine patterning and indicate a role for *Hoxa13* in orchestration of Müllerian duct fusion, a process central to the genesis of diversity in uterine forms found in nature [1].

Wnt genes encode secreted, cysteine-rich glycoproteins that are related to *wingless*, the segment polarity gene in *Drosophila* [30]. Mammalian Wnt gene products initiate or regulate patterning events associated with establishment of cell boundaries and act as mediators of cell-cell interactions that can determine cell fate [19]. The vertebrate Wnt gene family may have as many as 21 members. Among these, *Wnt4*, *-5a*, and *-7a* are expressed in a coordinately regulated fashion during the course of FRT development, as well as in adult uterine tissues where they may regulate endometrial patterning cyclically [13, 31]. Wnt proteins act on target cells via seven-pass transmembrane receptors belonging to the Frizzled family [30, 32]. A Wnt-Frizzled co-receptor system related to the low-density lipoprotein (LDL) receptor and designated LRP5/6 (for LDL receptor-related proteins 5 and 6), as well as negative regulators of this system that include the secreted Dickkopf (Dkk) and Frizzled-related (FRP) proteins, Wnt inhibitory factor (WIF), and Cerberus, have also been described [31, 33, 34].

Disruption of Wnt gene expression has significant consequences for FRT patterning and uterine development. *Wnt4* expression, as indicated above, is required to initiate Müllerian duct formation. Epithelial expression of *Wnt7a* is required to maintain, but not to induce uterine stromal expression of *Hoxa10* and *Hoxa11* [35]. Overall, however, *Wnt7a* is thought to mediate organizationally critical stromal-epithelial interactions in the developing uterine wall by responding to and enforcing positional signals dictated by the *Hoxa* genes [35]. Loss of *Wnt7a* expression results in loss of uterine *Hoxa10* and *Hoxa11* expression in adult mice and, as might be expected, produces a homeotic transformation characterized by posteriorization of the uterus at gross, cellular and molecular levels. Compared to the wild-type control, *Wnt7a*-null murine uteri are hypoplastic and display vaginal-like histoarchitecture with stratified epithelium, thin stroma, no endometrial glands and overgrown, disorganized smooth muscle layers [12, 35]. Loss or suppression of uterine *Hoxa10* and *Hoxa11* expression in *Wnt7a*-null mice precedes loss of expression of other uterine genes such as *Wnt4* and *Wnt5a*, both of which are normally expressed by endometrial stroma [12, 35, 36]. Collectively, data can be interpreted to suggest a mechanism whereby epithelial *Wnt7a* acts to regulate and stabilize stromal expression of *Wnt4* and *Wnt5a*, which act in concert to affect stromal expression of *Hoxa10* and *Hoxa11*. To the extent that these combinatorial relationships are conserved in eutheria, the evolution and stability of uterine phenotype in both anteroposterior and radial axes can be said to depend upon the genesis and stabilization of a temporally and spatially appropriate, tissue-specific *Hox/Wnt* expression domain in the uterine segment of the developing FRT. Evidence that a *Hox/Wnt* expression domain develops in neonatal porcine uterine tissues [36-38] supports this concept.

Tissue Programming and Consequences of Developmental Disruption

It has long been recognized that aberrant stimuli encountered during ‘critical periods’ of development can affect organizational programs and induce permanent changes in the structure and function of tissues, organs, and even entire organisms [39]. In recent years, the term *programming* has been adopted to describe long-term effects of exposure to environmental,

nutritional or xenobiotic factors that affect the course of pre- or perinatal development, with lasting consequences for both reproductive performance and health [40, 41]. All components of the reproductive system, including the FRT, are potential targets for such factors [7, 12, 13, 42-44].

Studies catalyzed by the observation that prenatal exposure of human fetuses to the synthetic xenoestrogen diethylstilbestrol (DES) [45] can alter the organizational program of FRT tissues, thereby setting the stage for cervicovaginal cancer and other complications [42, 44-46], provided important insights into the roles of the steroid hormone superfamily of receptors and related ligands in normal and aberrant programming of the FRT. Loss-of-function studies showed that estrogen receptor- α (ER) expression is required for normal uterine growth, while both the progesterone receptor (PR) and ER are required for normal uterine (and general reproductive) function [47-49]. Data show that neither ER nor PR expression is necessary to support primary uterine patterning events in pre- and/or early perinatal life. However, aberrant activation of these and related receptor systems during critical organizational periods can have significant consequences for uterine function and reproductive health [3, 7, 42-46, 50].

Risks of exposure to compounds with the potential to disrupt steroid-sensitive uterine programming events are real. Categories of developmentally disruptive environmental xenochemicals likely to be encountered by animals were recently defined [3, 7] to include: (1) pharmaceuticals designed as endocrine modulators for therapeutic purposes, such as growth promotants or agents used to control timing of ovulation; (2) natural endocrine modulating chemicals found in feedstuffs, such as phytoestrogens or mycotoxins; and (3) a host of industrial xenochemicals that can act as hormonal mimics or 'selective steroid receptor modulators' (SSRMs).

In laboratory animals, perinatal exposure to estrogen or related xenobiotics produced lesions in adult uteri that included: altered steroid receptor concentration and responsiveness; changes in estrogen 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 [3, 42-44, 50]. Complementary data generated in ungulate models, including the pig, sheep and cow, indicate clearly that adult uterine phenotype can be programmed by targeted disruption of hormone-sensitive postnatal organizational events [3-6, 51, 52].

In the pig, as in other mammals, radial patterning of the uterine wall is incomplete at birth [2, 53, 54]. Uterine morphogenetic events characteristic of the first 60 days of postnatal life in the pig, including appearance and proliferation of endometrial glands, development of endometrial folds, and differentiation and growth of myometrial smooth muscle layers, occur normally following bilateral ovariectomy at birth (postnatal day = PND 0), whereas ovaries are required for normal uterine growth past PND 60 [53]. Thus, as reported for other species [2], early postnatal events associated with radial patterning of the porcine uterine wall are ovary- and, most likely, steroid hormone-independent. Consistently, estrogen and progestin sensitivities develop postnatally in porcine uterine tissues [55-58].

The porcine uterus is ER-negative at birth. Onset of ER expression, documented between PND 0 and 15 in uterine stroma and glandular epithelium (GE), but not in luminal epithelium (LE), is associated temporally with appearance and proliferation of nascent endometrial glands [55, 56]. Moreover, the ER antagonist ICI 182,780 retards endometrial development and inhibits gland genesis during this period when administered from birth [56]. Thus, the ER is both a marker and mediator of endometrial maturation and radial patterning in the neonatal porcine uterus. Aberrant hyperactivation of this uterine ER system by administration of estradiol valerate (EV) from PND 0-13, while acutely uterotrophic [59], is ultimately both anti-uterotrophic and anti-embryotrophic [6, 53]. The hypoplastic, neonatally EV-exposed, adult porcine uterus does not accommodate pregnancy with increases in weight, size or luminal protein content typical of normal embryotrophic responses to periattachment stage (day 12 post-mating) maternal and conceptus signals. Additionally, patterns of endometrial gene expression are altered in adult EV-exposed gilts [6]. Furthermore, embryo survival in adults exposed to EV from PND 0-13 was reduced 22% by gestational day 45 [53]. Thus, PND 0-13 represents a critical period for ER-dependent, estrogen-sensitive programming of porcine uterine tissues. Recent data for the pig can be interpreted to suggest that effects of transient neonatal EV exposure on adult uterine morphology and functional capacity may reflect dysregulation of morphoregulatory gene expression in developing endometrial tissues similar to those proposed for murine model systems.

New data [36-38], involving *in situ* hybridization studies, show that a *Hoxa/Wnt* axis develops in the porcine uterine wall between birth and PND 14. Normally, stromal expression of *Hoxa10*, *Hoxa11*, *Wnt4* and *Wnt5a* is detectable at birth and increases to PND 14. Epithelial expression of *Wnt7a*, undetectable at birth, is evident on PND 7 and strong on PND 14 in LE. Up regulation of *Wnt7a* expression in LE coincides with differentiation of GE, which remains *Wnt7a*-negative. Thus, *Wnt7a* expression marks LE differentiation as ER expression marks GE differentiation. Treatment of gilts with EV from birth changes temporospatial *Hoxa/Wnt* expression patterns characteristic of the PND 14 uterine wall. Exposure to EV from birth reduced signal for *Wnt7a* in LE and *Wnt4* in stroma, altered spatial patterns of stromal *Wnt5a* expression, and increased stromal *Hoxa10* signal on PND 14. Taken together with studies reviewed above, data for the pig provide strong support for the idea that EV-induced, ER-mediated dysregulation of the neonatal uterine *Hoxa/Wnt* expression axis between birth and PND 14 contributes to programming events leading to adult uterine hypoplasia, impaired uterine function, and embryo mortality in a large domestic animal.

Transient exposure to SSRMs from birth can have dramatic effects on uterine phenotype in other large domestic animals. The ‘uterine gland knock-out’ (UGKO) phenotype, characterized in both sheep and cattle, provides a definitive example. Adult UGKO ewes and cows possess hypoplastic uteri that lack endometrial glands [3]. This extreme, stable phenotype was induced by exposure of animals to a progestin (sheep), or to a progestin in combination with an estrogen (cattle) from birth [3, 52]. While UGKO adults may cycle normally and can conceive, reproductive efficiency is severely depressed in these animals, owing largely to impaired endometrial function [3, 4, 52, 60]. Detailed investigations in sheep indicate that effects of progestin (norgestomet) exposure on neonatal uterine programming events responsible for induction of the UGKO phenotype include ablation of uterine epithelial ER expression and changes in expression patterns for a number of paracrine-acting growth factors and/or their receptors implicated in early postnatal uterine patterning events [61, 62], including uterine

hepatocyte growth factor and fibroblast growth factor receptor 2IIIb [61, 62]. Data for domestic ungulates support the concept that temporally aberrant activation of steroid receptor systems in developing tissues during ‘critical periods’ change the uterine organizational program by altering primary and secondary (i.e. downstream) morphoregulatory gene expression domains that affect development of microenvironmental conditions which ultimately determine cell and tissue identities and define both uterine morphology and functionality.

Uterine patterning and tissue programming can also be affected directly by peptide hormone signaling cascades, as well as indirectly by *crosstalk* between these cascades and steroid hormone signal transduction systems [51, 63-65]. Consider relaxin (Rlx), a member of the insulin-like growth factor family that is uterotrophic in the pig. New data [66, 67] show that porcine uterine expression of transcripts for the relaxin/insulin-like peptide-3 receptors, LGR7 and LGR8 (LGR7/8) [68], increases from birth to PND 14, a critical period for uterine development in this species. Recombinant porcine Rlx, administered for two days from birth, prior to onset of uterine ER expression, is not uterotrophic in the LGR7/8-positive porcine uterus by PND 3. However, Rlx is grossly uterotrophic [66] and stimulates endometrial development (Bartol, Wiley and Bagnell, unpublished observations) by PND 14 when administered on PND 12-13, after overt expression of ER in the endometrium [56]. These uterotrophic effects of Rlx were inhibited with ICI 182,780 [66], indicating that effects of Rlx are mediated, in part, via *crosstalk* between activated LGR7/8 and ER signal transduction systems. Because LGR7/8, but not ER, is detectable in the porcine uterus at birth, while all of these transcripts are detectable by PND 7 and later, uterine morphoregulatory effects observed for Rlx administered at birth might be expected to be ligand-specific and more subtle, while the more pronounced effects of Rlx given later in the neonatal period could reflect amplification of the Rlx signal through crosstalk with the developing ER system. The fact that Rlx can augment estrogen-induced up-regulation of uterine stromal *Hoxa10* expression *in vitro* [69], indicates that such amplification may work both ways. Observations also reinforce the idea that aberrant activation of the LGR7/8 system in developing uterine tissues could affect primary morphoregulatory gene expression patterns and alter critical tissue programming events with long-term consequences. Data for the ewe, indicating that uterine gland genesis can be inhibited with bromocryptine and stimulated with prolactin [51], implicates yet another peptide growth factor signaling system in ungulate uterine patterning with potential for programming effects via crosstalk with other signaling systems [70].

Summary and Conclusions

The uterus, an epithelial-mesenchymal organ, develops from the Müllerian ducts through a process involving increasingly complex and specific cellular interactions that are accompanied by and support the evolution of organizationally critical, temporally and spatially unique patterns of primary and secondary (i.e. downstream) morphoregulatory gene expression. Structural and functional patterning of uterine tissues, which begin prenatally but are completed postnatally, are coupled processes. Aberrant exposure of developing tissues to endogenous or exogenous agents that affect the state of activation of signal transduction systems in uterine tissues during critical developmental periods and alter primary morphoregulatory gene expression patterns, can change the normal organizational program with lasting and often negative consequences for uterine phenotype and functionality. Agents with potential to affect uterine programming during critical organizational periods include SSRMs, as well as peptides and their mimics that may affect intra- and intercellular signaling events through their own, as well as through crosstalk with other

receptor signaling systems. Critical periods of uterine organization, when tissues are uniquely sensitive to developmental disruption, are likely to be species-specific and have yet to be defined for the range of bioactive factors with potential to affect uterine programming. This is particularly true for economically important domestic animals. Application of genomic and proteomic screening procedures to define ‘molecular phenotypes’ characteristic of normal as compared to organizationally or programmatically ‘lesioned’ uterine tissues should provide important information that can be used to determine the developmental history and functional potential of the adult uterus.

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