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THE ROLES OF WNT GENES IN THE DEVELOPMENT OF THE MOUSE FEMALE REPRODUCTIVE TRACT.

by

Elizabeth Cary Miller

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1999

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This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

THE ROLES OF WNT GENES IN THE DEVELOPMENT OF THE MOUSE FEMALE REPRODUCTIVE TRACT.

By Elizabeth Cary Miller

Thesis Advisor: David A. Sassoon, Ph.D.

The murine female reproductive tract differentiates during postnatal development in response to specific mesenchymal-epithelial interactions and changing levels of circulating steroid hormones. The expression of members of the Wnt family of signaling molecules has been recently identified in this system. The expression patterns of Wnt genes in the developing and adult female reproductive tracts are described. The Wnt gene expression patterns in the female reproductive tract suggest that Wnt genes may indeed play roles in the mesenchymal-epithelial interactions critical for female reproductive tract development and function.

Gain-of-function and loss-of-function experiments were performed to examine the roles of Wnt genes in the development and adult function of the female reproductive tract. Retrovirally-mediated ectopic expression of Wnt-1 re-directs cytodifferentiation in tissue grafts. Additionally, the roles of Wnt-7a in the female reproductive tract were evaluated in Wnt-7a null mice. A number of defects were observed which suggest that Wnt-7a functions in anterio-posterior patterning in the developing female reproductive tract. In addition to a global posterior shift in the female reproductive tract, the organization of the uterine smooth muscle is affected, indicating that development along the radial axis has also been altered. These results suggest that a mechanism whereby Wnt-7a signaling from the epithelium maintains the molecular and morphological

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boundaries of distinct cellular populations along the anterior-posterior and radial axes of the female reproductive tract.

The observed phenotype of the Wnt-7a null mice closely resembles the reproductive tract morphologies observed in female mice exposed prenatally to DES. This observation raises the possibility that *Wnt*-7a plays a role in the response to DES. A specific molecular response to DES has not been identified that fully accounts for the DES syndrome. I propose that the observed transient down-regulation of *Wnt*-7a is sufficient to account for the murine DES syndrome. DES-like substances such as Tamoxifen have recently been shown to be effective in decreasing the incidence of breast cancer in premenopausal women, although uterine cancer increases by 2-fold. A better understanding of the molecular responses in breast and uterine tissues to steroidal pharmacological agents will be of high importance should their use increase in clinical applications.

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Acknowledgments

As any graduate student knows, this work was not completed alone. Without the support and guidance I received from my mentor and advisor, Dr. David Sassoon, this work could not even have been started. My thesis stands testimony to David's belief in me, and to his willingness to let me study and explore on my own. Not many advisors offer that opportunity. Of course, had it not been for his time, advice, and suggestions, I would not be here. Thank you.

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Chapter 1: Introduction and Background

Development of the Mouse Female Reproductive Tract

The formation of the mouse urogenital tracts has been studied primarily at the morphological and tissue levels. The development of the genital tract appears to depend on the formation of the kidney. There are three stages of kidney development: the pronephros, the mesonephros and the metanephros. The pronephric duct is recognizable at 9 d.p.c. in the caudal part of the trunk. By 9.5 d.p.c., the pronephric ducts have canalized and pronephric tubules are also visible. At this time the gonadal ridges are also evident in the mid-trunk region. By 10 d.p.c., mesonephric vesicles are visible along the lateral gonadal ridge. The vesicles join and become canalized forming the mesonephric or Wolffian ducts. The Wolffian ducts elongate caudally, and by 10.5 d.p.c., they have reached the urogenital sinus and drain into it. The mesonephros is not likely functional in mice and is believed to represent a holdover from a more primitive body-plan. The metanephros constitutes the third stage of kidney development and are the functional kidneys in mice. Mice which have impaired mesonephric kidney development generally have defective genital systems. Pax-2 deficient mice, which lack kidneys and ureters, also lack the genital ducts [1].

In chick embryos, the presence of the Wolffian duct has been noted to induce a thickening in the gonadal ridge which develops into the paramesonephric or Müllerian ducts [2]. The Müllerian ducts descend caudally through the genital ridge, guided by the Wolffian ducts [2, 3]. Mice that are lacking Wolffian ducts or that show premature degeneration of the Wolffian ducts also lack the paramesonephric ducts. For instance, Pax-2 mutant mice show degeneration of the Wolffian ducts and completely lack Müllerian duct development [1], while Emx2 mutant mice show degeneration of both the Wolffian and Müllerian ducts [4, 5]. The Müllerian ducts descend laterally to the Wolffian ducts, but in the lower one third of the abdominal cavity, the Müllerian ducts cross over the Wolffian

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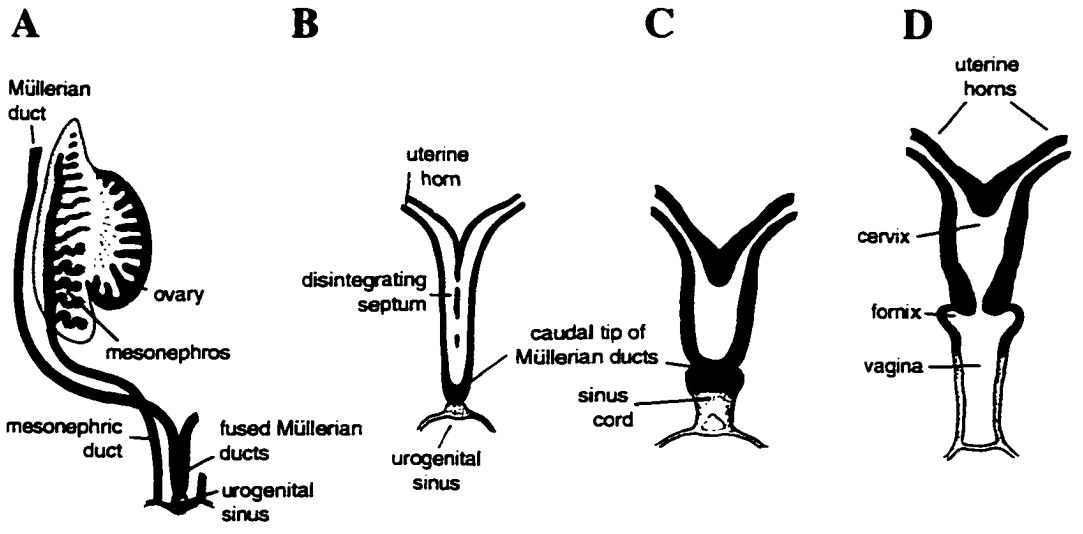

Figure 1.1 The Müllerian ducts and the urogenital sinus form the female reproductive tract. A. The Mullerian ducts descend caudally lateral to the mesonephric ducts. The Müllerian ducts cross the mesonephric ducts and fuse at the midline. The Müllerian ducts then fuse with the urogenital sinus. B. Canalization occurs between the Müllerian ducts in the fused region, forming the common cervix. C. Cells in the region of the future vagina proliferate, forming the sinus vagina. At this point the sinus vagina consists of a solid cord of cells. The anterior vagina derives from the caudal Müllerian ducts, while the posterior vagina derives from the urogenital sinus. D. Cells in the lumen of the vagina die, and the vaginal lumen becomes apparent. (Adapted from Sadler, 1990 [6])

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ducts and fuse together (see Figure 1.1). The two fused ducts descend caudally, join and fuse with the urogenital sinus at 14.5 d.p.c. Subsequent to this fusion, canalization occurs between the Müllerian ducts and the urogenital sinus.

The Müllerian ducts disintegrate in males starting at 12.5 d.p.c. in response to Müllerian Inhibiting Substance or Anti-Müllerian Hormone (MIS; AMH) which is secreted by the testis. AMH is a member of the TGF-ß family of molecules [7, 8], and acts on its receptor in the mesenchyme of the Müllerian ducts [9]. Males express the receptor for AMH in the stroma of the Müllerian ducts, and therefore these ducts regress [9-11]. Although females express the AMH receptor in the mesenchyme around the Müllerian ducts, they do not produce AMH and therefore the Müllerian ducts do not regress [9]. While the Wolffian ducts remain intact and develop in response to testosterone in males, they disintegrate in females due to a lack of androgens [12]. The end result is that males have Wolffian duct derivatives while females have Müllerian duct derivatives. The Müllerian ducts contribute to the oviducts, the bicornuate uterine horns, the cervix, and the vagina. The point at which the ovarian ligament, the gubernaculum, crosses the Müllerian duct determines the utero-tubal junction in all mammals. The portion of the Müllerian ducts cephalic to the gubernaculum become the oviducts, while the remainder of the Müllerian ducts becomes the uterine horns, cervix and anterior vagina [2, 13-15].

While the Müllerian ducts contribute to the anterior vagina, the endodermally derived urogenital sinus is thought to contribute to the posterior vagina [14, 16]. Evidence for this comes from the testicular feminization (TmY) mouse which contains a mutation in the androgen receptor. In TmY males, the Müllerian ducts regress as they should, however, the Wolffian ducts are androgen insensitive so they also regress [17]. The male mice therefore have no inner urogenital duct system, but only have a short, blind-ending vaginal pouch [16]. Since the Müllerian ducts have degenerated, the vaginal pouch must have derived from the urogenital sinus. It is possible that the epithelium from the anterior Müllerian-derived vagina migrates and replaces the posterior vaginal epithelium, so that

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while the stroma of the posterior vagina derives from the urogenital sinus, the epithelium derives from the Müllerian ducts [18].

A wide variety of female reproductive tract malformations have been reported in humans. The nature of the malformations indicate the precise patterning involved in the development of the female reproductive tract. Uterine problems vary from unicornuate uteri (lacking one uterine horn), to complete uterine atresia, to the presence of a longitudinal septum between the uterine horns. The presence of a septum can influence the shape and position of the cervix. If no canalization occurs between the two fused Müllerian horns, duplication of the cervix can occur. Some common vaginal abnormalities include the presence of longitudinal and transverse septums. Transverse septums have been observed in women exposed prenatally to diethylstilbestrol (DES) [19]. A longitudinal septum can result if there is incomplete fusion between the two Müllerian ducts in the region that forms the future vagina. It may appear to be a continuation of the cervix, and essentially results in a double vagina.

Cytodifferentiation of the Female Reproductive Tract

The murine female reproductive tract is relatively undifferentiated prior to birth, and consists of simple columnar epithelial ducts surrounded by the mesenchyme of the urogenital ridge [20]. At birth, the epithelium of the vagina is pseudostratified columnar while that of the uterus is simple columnar [21, 22]. The mesenchyme of the uterus starts to differentiate into the smooth muscle and stromal layers between 0 and 3 days postnatally [20]. The origin of the uterine smooth muscle cells is unknown (see Figure 1.2). For instance, mesenchymal cells may be pluripotent, and specific cells may differentiate into smooth muscle cells in response to an inductive signal. Alternatively, a subset of mesenchymal cells may be programmed to differentiate into smooth muscle regardless of post-natal signaling events. Differentiated smooth muscle cells are detectable by 3-5 days post-natally. The inner layer of smooth muscle is detectable using antibodies to smooth

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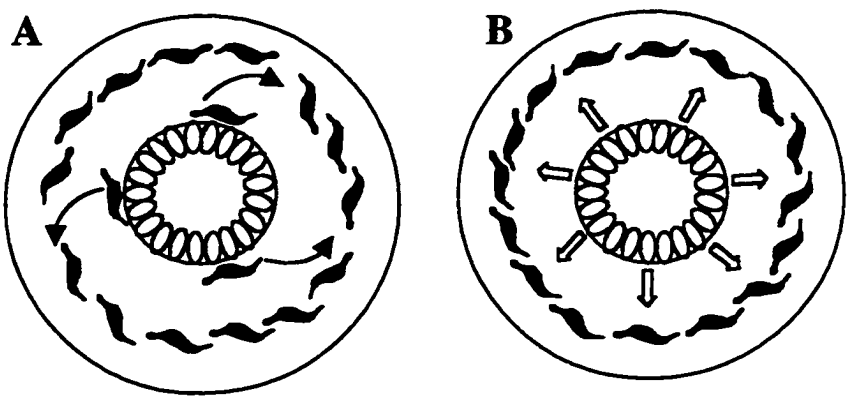

Figure 1.2 The origin of smooth muscle cells during uterine cytodifferentiation is unknown. A schema of a cross-section through a newborn uterine horn is shown. The simple columnar epithelium lines the lumen. Although the epithelium is surrounded by mesenchymal cells, only the differentiating smooth muscle cells are depicted. Two potential models are shown. **A.** A limited number of mesenchymal cells may be capable of differentiating into smooth muscle cells. Smooth muscle cells may originate near the epithelium in response to an epithelial signal, and then migrate to the outside of the uterus. **B.** Conversely, all mesenchymal cells may be capable of differentiating into smooth muscle. In the schema shown, some cells differentiate in response to a signal secreted from the epithelium. This factor may repress smooth muscle formation in regions where it is concentrated, allowing smooth muscle to form only in the periphery of the tissue.

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muscle actin by 5 days, however, the outer layer of smooth muscle is not distinguishable until 10 days after birth [20]. The two smooth muscle layers that make up the myometrium are fully developed by two weeks after birth [20]. Although much is known about the formation of skeletal muscle, very little is understood at a molecular level regarding visceral smooth muscle formation, thus an elucidation of the signaling events underlying smooth muscle induction is of high interest.

Uterine glands form through invagination of the luminal epithelium. The first sign of rudimentary uterine gland formation can be observed by 10 days after birth [22]. Gland formation is influenced by exposure to steroid hormones. Gland formation is reduced in rodents treated prenatally or neonatally with various estrogens [23-27]. Chronic progestin treatment of sheep from the time of birth inhibits gland formation [28]. Disruption of the estrogen receptor results in glandular hypoplasia [29], while the progesterone receptor mutant mouse shows glandular hyperplasia [30]. The only molecule known to be involved in gland formation other than steroid hormones and their receptors is Hoxa-11, since the Hoxa-11 mutant mice have a reduced number of glands [31].

Vaginal epithelium differentiates from pseudostratified in the newborn to stratified squamous in the adult. The adult epithelium can appear cornified or mucified depending on the stage of the estrous cycle. The stage of the estrous cycle can be determined by histological examination of the vaginal epithelium [32]. In diestrus, the epithelium is only 5-6 layers thick and lacks a basement membrane. In proestrus, there are 12-13 layers of epithelium, and the top layers stain very lightly with eosin. During estrous, the epithelium has an intact, superficial cornified layer whereas during metestrus, the cornified layer is shed and the epithelium is invaded by large numbers of leukocytes, which are often in lacunae. Additionally, noticeable changes in the epithelium of the uterine horns and the oviducts can be noted throughout the estrous cycle [32]. These morphogenetic changes occur in response to circulating steroid hormones and are dependent upon mesenchymal-epithelial interactions [33, 34].

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Mesenchymal-Epithelial Interactions

Mesenchyme and epithelium of different tissues have been demonstrated to possess inductive capacities, and the ability to respond to inductive signals. Mesenchymal-epithelial interactions have been shown to be critical in the formation of many organs including lung [35, 36], mammary gland [37-39] and both the male and female reproductive tracts (see [40] for review). The contributions of the mesenchymal and epithelial components can be evaluated through tissue recombinants prepared from the same (homotypic) or different (heterotypic) tissue sources.

Tissue recombinant experiments have been performed for both the uterus and the vagina. The epithelium of the reproductive tract is quite plastic and undifferentiated until about five days after birth [22, 40]. Prior to this time, the epithelium is capable of responding to inductive signals from either uterine or vaginal mesenchyme. For example, when uterine mesenchyme is recombined with vaginal epithelium, the mesenchyme induces the vaginal epithelium along a uterine cytodifferentiation pathway (Fig. 1.3 and [22]). The resultant graft has simple columnar epithelium that is characteristic of the uterus, rather than the stratified squamous morphology that is normally seen in the adult vagina. Similarly, vaginal mesenchyme can induce uterine epithelium to form vaginal-like stratified epithelium [22]. These morphogenetic changes are accompanied by the expected changes in gene expression [41]. Msx 1 is normally expressed in the epithelium of the uterus. It is expressed in tissue recombinants that contain uterine stroma regardless of the source of the epithelium. Conversely, Msx 1 is not expressed in recombinants of vaginal stroma and uterine epithelium [41]. Uterine epithelium loses the capacity to respond to inductive signals from the vaginal mesenchyme between 5 and 7 days after birth [22, 40]. The nature of the inductive signals and the transient capacity to respond to these signals is not understood in molecular terms.

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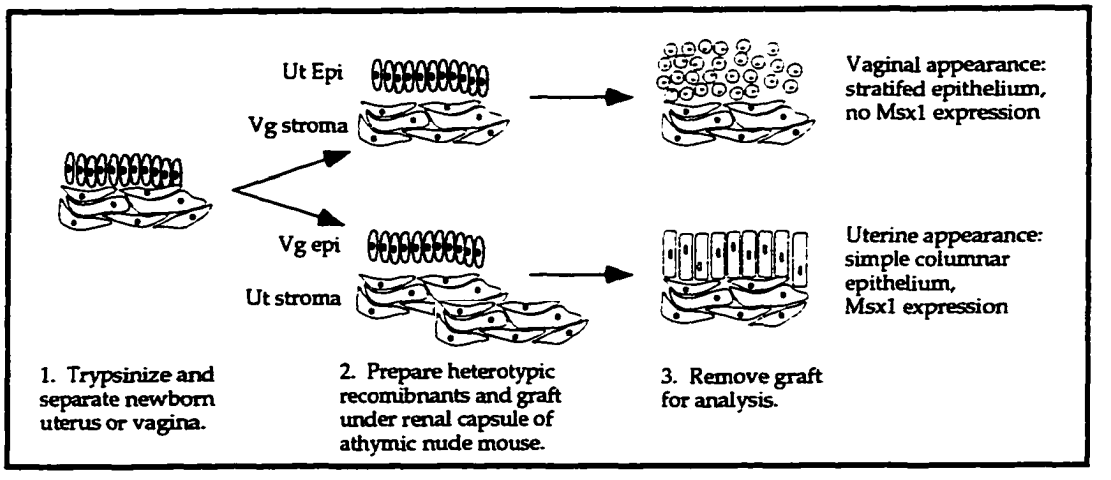

Figure 1.3 Tissue recombinants allow for investigation of the inductive processes which occur between mesenchyme and epithelium during the development of the female reproductive tract. Epithelium from neonatal female reproductive tracts can follow the cytodifferentiation pathway induced by the stroma. Vaginal stroma induces newborn uterine epithelium to become stratified, express vaginal specific genes, and lose expression of uterine specific genes (Msx1). Conversely, uterine stroma induces newborn vaginal epithelium to become simple columnar, to lose vaginal specific gene expression, and to express uterine specific genes (Msx1).

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Steroid Hormones and Their Roles in the Female Reproductive Tract

Steroid hormones are critical for the function of the adult female reproductive tract. Estrogens, especially estradiol, are important for growth of the uterus and vagina, for the initial preparation of the uterus for implantation, and for regulating the cervical mucus to allow sperm passage [42]. Progesterone is important in maintaining pregnancy and also is critical for implantation [42]. The actions of estrogen and progesterone are mediated through their respective steroid hormone receptors (see Figure 1.4). Steroid hormones are secreted by endocrine organs and are carried in the blood to their target organs or tissues. Since they are hydrophobic, they are bound to specific carrier proteins while in the blood [43]. Once reaching their target cells, the steroid hormones carrier proteins are thought to dock at specific receptors in the cell membrane (see [44] for review). The sex hormones are then released from their carrier proteins, diffuse into the cell, and bind to their specific receptor in the nucleus. This causes a conformational change in the receptor. The hormone-receptor complex can then dimerize with another hormone receptor, and bind to specific hormone-response elements [45]. These elements can be either in the promoter or enhancer of the target genes. Once bound to DNA, the hormone-receptor complex transactivates the specific hormone response genes. The roles of estrogen and progesterone in the development and function of the female reproductive tract have only recently been elucidated through the disruption of the estrogen receptor and progesterone receptor genes [29, 30].

(i) The Estrogen Receptor

The estrogen receptor (ERα) has been detected in the Müllerian ducts as early as 13 d.p.c. using immunocytochemistry, or autoradiography with [^{3}H]estradiol or [^{3}H]diethylstilbestrol [46-49]. A certain amount of inconsistency has been observed during the characterization of estrogen receptor ontogeny in the female reproductive tract which is summarized in Table 1. [^{3}H]diethylstilbestrol has been shown to bind to the

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estrogen receptor in the developing female reproductive tract as early as 13 d.p.c. [49]. Higher levels of binding were detected by 15 d.p.c. in the mesenchymal cells [49], but at this same point, estrogen receptors can be detected by immunostaining in the nuclei of both the mesenchymal and epithelial cells [46]. A similar conflict exists in data at 17 d.p.c.. However, at birth, there is overall agreement that estrogen receptors are present only in the mesenchyme of the developing uterine horn, and not in the epithelium. The post-natal ontogeny of the estrogen receptor in the epithelium of the uterine horn is dependent on the strain of mouse: estrogen receptors are detectable at least 2 days earlier in outbred CD-1 mice than in the inbred BALB/c strain [47].

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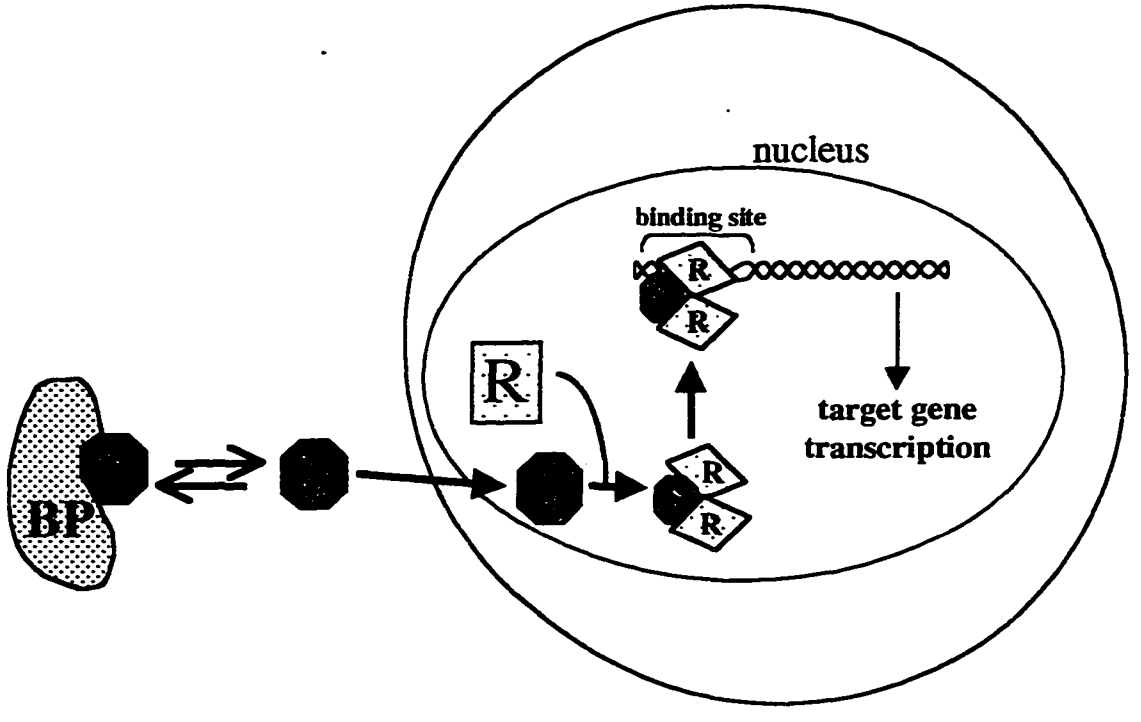

Figure 1.4 Activation of steroid hormone responsive genes. Steroids (S) travel through the bloodstream coupled to binding proteins (BP). They are released from the BP at the target cell. Since they are hydrophobic, steroids can diffuse into the nucleus of the target cell. Once in the nucleus, they bind to the appropriate steroid hormone receptor (R). The bound receptor changes conformation. The dimerized receptor-steroid complex can bind to specific steroid hormone binding sites in the promoter or enhancer of target genes. The binding of the steroid hormone and its receptor results in transactivation of the target gene.

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	[^{3}H]diethyl-stilbestrol [49]	immunocyto-chemistry [50]	immunocyto-chemistry and [^{3}H]estradiol [47]	immunocyto-chemistry [46]
13 d.p.c.	mesenchyme (slight)			
15 d.p.c.	mesenchyme (more cells and staining)			epithelium and mesenchyme
16 d.p.c.	mesenchyme [48]			
17 d.p.c.	mesenchyme			mesenchyme (staining varied in epithelium)
birth	mesenchyme	mesenchyme		mesenchyme
3 days				
4 days		mesenchyme and 25-30% of epithelial cells	epithelium and mesenchyme (CD-1)	mesenchyme and some epithelium
6-7 days		mesenchyme and more epithelial cells	epithelium and mesenchyme (BALB/c)	
10-19 days		mesenchyme and luminal and glandular epithelial cells		epithelium and mesenchyme
adult	epithelium and mesenchyme			

Table 1.1 Ontogeny of the estrogen receptor during the development of the mouse uterus. Although there is general agreement that estrogen receptors are only in the uterine mesenchyme at birth, some controversy exists over the distribution of uterine estrogen receptors prior to birth.

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The estrogen receptor can be detected in embryos as early as the blastocyst stage [51]. Its additional detection in the embryonic and perinatal female reproductive tract suggests that estrogen and its receptor may play roles in implantation and development [51]. However, ERα mutant (ERKO) mice are perfectly viable [29]. Male and female ERKO mice are infertile, but their reproductive tracts develop normally until the time of puberty, implying that estrogen is not needed for implantation or the development of the female reproductive tract [29]. Additionally, ovariectomy and adrenalectomy of newborn mice has no significant effect on neonatal uterine growth until 25 days after birth [52]. However, the ER is needed for the maturation and function of the female reproductive tract. ERKO mice have hypoplastic uteri with decreased myometrial, stromal, and glandular compartments [29]. Although a second estrogen receptor has been cloned (ERß) [53], it is likely that it plays no significant role in the reproductive tract of the ERKO (ERα) mouse since the vagina and uterus do not display any signs of estrogen stimulation even though the levels of circulating estradiol are 10 times higher than normal [54].

Estradiol treatment stimulates uterine epithelial proliferation and DNA synthesis. The effect of estradiol on mitogenesis had been thought to be mediated through stromal estrogen receptors [55]. Recent definitive evidence came through studies with ERKO mice. When tissue recombinants are prepared between the stromal and epithelial components of ERKO and wild-type uteri, stromal estrogen receptors are required for mitogenesis [56]. Estrogen stimulation also influences the formation of uterine glands. It remains to be seen whether stromal or epithelial estrogen receptors are necessary or sufficient for gland formation.

(ii) The Progesterone Receptor

Progesterone exerts its actions through two forms of the progesterone receptor that arise from the same gene [57]. Immunocytochemical analysis of the perinatal female reproductive tract has determined the pattern of progesterone receptor expression [58].

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Immuno-staining for the progesterone receptor is present in weak amounts in the epithelial, muscle and stromal cells of the newborn uterus. The staining is more intense in all components from day 7, and this pattern is maintained into adulthood.

The expression of the progesterone receptor in the uterus is regulated largely by estrogen treatment [59]. Estrogen and its agonists induce expression of the progesterone receptor. It is therefore difficult to evaluate the roles of progesterone and its receptor in the uterus without considering the functions of estrogen. The progesterone receptor gene was therefore disrupted in mice in order to determine precisely the roles of progesterone [30]. Like the estrogen receptor, the progesterone receptor is expressed from the blastocyst stage [51], therefore it was a surprise that progesterone was not required for implantation and development [30]. Male and female progesterone receptor mutant mice (PRKO) are fully viable but like the ERKO mice, the females are infertile. The infertility could be explained by a lack of ovulation, and by an observed deficit in mating behavior [30, 60]. PRKO uteri show excessive glandular proliferation when treated with estradiol and progesterone. This demonstrates the role of estrogen in glandular proliferation, since the hormone induced hypertrophy of the PRKO uterus must be due to estrogen stimulation [30]. Progesterone plays an inhibitory role in the formation and proliferation of glands. This was clearly shown by Bartol *et al.* who treated newborn pigs with a progestin from the time of birth, inducing a glandless uterus [28]. The use of PRKO mice will allow for functional examination of the roles of progesterone, and the functions of estrogens in the absence of an induced progesterone response.

(iii) Mediators of Steroid Hormone Action

The ERKO and PRKO mice allow for an investigation of the roles of estrogen and progesterone on the adult uterus, however, the actions of estrogen and progesterone are mediated through downstream genes. EGF may mediate the growth stimulatory effects of estradiol in the uterus and vagina [61]. In the neonate, EGF is localized to the stroma and

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epithelium of the vagina, but is detectable only in the stroma of the uterus. Neonatal estrogen treatment increases levels of EGF and EGF receptor in adult animals [61, 62]. EGF requires the estrogen receptor to exert its estrogen-like effects in the uterus [63]. Although EGF-R is expressed in the ERKO mice, EGF treatment of ERKO mice does not induce DNA synthesis or cause an increase in the progesterone receptor [63]. Finally, EGF signaling was shown to be important in the estrogen mediated growth of the uterus and vagina [64]. Tissue grafts between the epithelial and stromal compartments of EGF-R mutant and wild-type mice showed that EGF-R expression in the stroma has an impact on the stromal labeling index. If the stroma does not express EGF-R, regardless of whether the epithelium expresses EGF-R, stromal cell mitogenesis will not increase in response to estrogen.

TGFα acts through the receptor for EGF. Similar to EGF, its expression in the uterus is enhanced by estrogen treatment. Activation of a TGFα transgene in the ovary leads to the inhibition of the gonadotropin-stimulated production of ovarian hormones [65]. While TGFα clearly plays a role in mediating the effect of gonadotropins on the ovary, the disruption of the TGFα gene does not affect the reproductive tract [66, 67]. While mice that express a TGFα transgene in the reproductive tract show no neoplastic alterations, they do exhibit an increase in diethylstilbestrol induced changes in the reproductive tract [68, 69]. These results suggest that while TGFα may not be involved in the development of the mouse uterus, it does function in the response to gonadotropins and steroid hormones.

TGFß1, ß2, and ß3 are expressed primarily in the epithelium of the uterus and vagina, and their expression is up-regulated by estrogen treatment [69, 70]. The transient stimulation of TGFß expression occurs significantly before estrogen induced DNA synthesis, suggesting that the TGFß family could be a mediator of estrogen -induced mitogenesis [70].

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The Wnt Family of Signaling Molecules: Molecular Candidates for Signaling Events in the Female Reproductive Tract

The *Drosophila* segment polarity gene *wingless* encodes a putative secreted signaling molecule [71]. Wingless has been implicated in patterning and the establishment of cell boundaries during embryogenesis (see [72] for review). Int-1, later renamed Wnt-1, was determined to be the vertebrate homolog of *wingless* [73]. It was initially identified as a gene frequently activated by mouse mammary tumor virus insertion, resulting in breast tumors in mice [74, 75]. Expression of an ectopic Wnt-1 transgene in the mammary gland also leads to mammary tumors, suggesting Wnt-1 is a proto-oncogene which likely functions as a secreted growth factor [76]. The vertebrate Wnt family is comprised of at least 16 members whose expression patterns throughout embryogenesis and in the adult suggest that Wnts are involved in regional specification of cell fates and/or cell-cell communication [41, 72, 77-80].

Targeted deletions of several members of the Wnt family provide evidence for roles in patterning and cell-cell communication. Wnt-4 expression is necessary for the formation of tubules in the developing kidney [81]. The mesenchymal to epithelial transformation that must occur for the kidney tubules to form does not occur in the Wnt-4 mutant mice [81]. Wnt-7a is required for normal dorsal-ventral and antero-posterior polarity in the forming limb [82]. Wnt-7a is expressed in the dorsal limb ectoderm and it acts as a dorsalizing molecule since ventralization of the limb occurs in its absence [82]. Additionally, Wnt-7a mutant mice have a variable loss of posterior digits reflecting interactions between Wnt-7a and Sonic hedgehog during limb development [82]. Lmx1, a homeogene, has been suggested to be responsible for limb dorsalization in the chick [83]. Wnt-7a is required to maintain expression of Lmx-1b in the distal limb mesenchyme of the mouse [84, 85]. When Wnt-7a is misexpressed in the ventral limb ectoderm of mice and chicks, it can induce Lmx1 in the ventral limb mesenchyme [83, 85]. Similar interactions

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between Wnt and homeogenes have been reported in the development of different organisms and organ systems [86, 87].

The Wnt Signaling Pathway

The *Drosophila frizzled* gene and its vertebrate homologs have been suggested to be receptors for *wingless* and the Wnt family [83, 88]. Although the specificity of receptor-ligand interaction is not understood, we now have a tentative understanding of the Wnt signaling pathway (see Fig. 1.5). This simple model involves the binding of the secreted Wnt molecule to its receptor, frizzled (fz). Multiple frizzled homologs have been cloned from a variety of organisms. They consist of serpentine 7-transmembrane proteins, with a structure similar to that of G-protein-coupled receptors. Activation of frizzled through Wnt binding activates the cytoplasmic protein dishevelled (dsh). Dsh inactivates glycogen synthase kinase (GSK-3), a serine/threonine kinase. It is thought that GSK-3 phosphorylates either ß-catenin or another protein, adenomatous polyposis coli (APC), when it is coupled to ß-catenin. This phosphorylation targets ß-catenin for degradation through the ubiquitin pathway. If GSK-3 is inactivated, there is a higher concentration of ß-catenin in the cytoplasm. ß-catenin couples with homologs of the TCF/LEF-1 family (T-cell factor/lymphocyte enhancer factor-1) of high mobility group transcription factors. The ß-catenin/TCF/LEF-1 complex enters the nucleus and then binds to and activates transcription of Wnt-responsive genes. Several lines of evidence indicate that this model is incomplete, and does not take into account recent discoveries in different organisms which suggest that not only may the described components have alternate functions, but that there are components which have yet to be identified. For instance, although multiple vertebrate frizzled members have been cloned, the specificity of Wnt/receptor interaction for the most part has not been elucidated. It has been suggested that Wnt-5a can signal specifically through human Fz-5 (hFz5) to mediate axis induction in Xenopus [89] but other specific interactions have not yet been reported.

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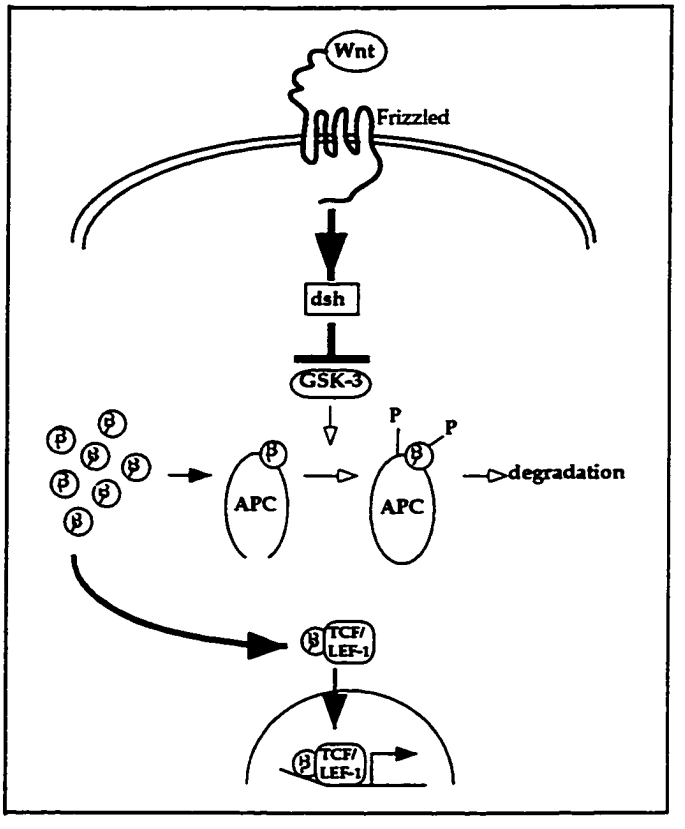

Figure 1.5 One pathway for Wnt gene signaling. Receptors for Wnt genes mediate the intracellular signalling cascade in response to the extracellular Wnt signal, ultimately transmitting the signal to the nucleus. Wnts bind to the serpentine frizzled molecules in the plasma membrane. This signal is somehow transmitted to dishevelled (dsh), which blocks the activity of glycogen synthase kinase (GSK-3). GSK-3 normally phosphorylates either ß-catenin or adenomatous polyposis coli (APC) when the two are complexed. This targets the ß-catenin/APC complex for degradation by the ubiquitin pathway. When GSK-3 is inhibited, there is an increase in the levels of cytosolic ß-catenin. Free ß-catenin complexes with homologs of the TCF/LEF-1 family and enters the nucleus. This complex acts as a transcriptional activator for Wnt-responsive genes.

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Some Fz members seem to activate alternative pathways to the one shown in Figure 1.5. For instance, Wnt-5a can stimulate intracellular Ca^{2+} release through the G-protein-linked phosphatidylinosital pathway in Xenopus [90]. Additionally, APC has been seen to be a positive regulator of Wnt signaling in *C.elegans* and a negative regulator of Wnt signaling in tumors and tissue culture [91, 92]. The roles of APC in Wnt signaling and also in cell migration remain to be determined. Another controversial issue is the nature of the GSK-3 substrate. GSK-3 can phosphorylate both APC and ß-catenin in vitro [93, 94]. The phosphorylation sites in the N-terminus of ß-catenin are required for its degradation, however, kinases other than GSK-3 can phosphorylate ß-catenin [95].

ß-catenin is thought to function in the nucleus in the Wnt signaling pathway. When plasma-membrane tethered ß-catenin is expressed in Xenopus, it activates Wnt signaling even though it cannot enter the nucleus [96]. It is possible that TCF/LEFs repress Wnt-responsive genes and ß-catenin alleviates the repression by sequestering TCF/LEFs [97]. It is also possible that the specificity of the Wnt response may be due to activation of specific members of the TCF/LEF family. However, 4 members of this family are expressed in largely overlapping patterns during embryogenesis and are not widely expressed in adult tissues [98, 99]. Recent data suggest that although ß-catenin does not have a classic nuclear localization signal, its entry into the nucleus is not dependent on TCF/LEF. ß-catenin binds directly to the nuclear pore machinery and its translocation into the nucleus is energy dependent [100].

Although ß-catenin is known to function in Wnt signaling, it also mediates cell-adhesion in the adherens junctions, where it is complexed with cadherins (see [101] for review). ß-catenin, along with plakoglobin, links the transmembrane cadherin molecules with α-catenin, and thereby indirectly links cadherins to the actin cytoskeleton. It has been argued that the roles of ß-catenin in patterning cell-fate determination may occur through cadherin-mediated cell adhesion (reviewed in [102]). Tyrosine phosphorylation of catenins in response to extracellular signaling leads to a dissociation of the cadherin-catenin

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complex, and a decrease in cell adhesion [103]. Wnts may act to change cell-adhesion, thus increasing the levels of cytosolic ß-catenin. Recently, it was shown that the signaling and adhesion functions of ß-catenin can be separated, suggesting that ß-catenin plays an active, rather than passive, role in Wnt signaling [104, 105]. However, there may still be cross-talk between the Wnt signaling and cell-adhesion pathways.

Wnt Genes in the Mammary Gland

The morphological changes which occur in the adult mammary gland have been attributed to both hormonal fluctuations and mesenchymal-epithelial interactions [80]. Wnt genes have been suggested to play a role in the developmental changes in the perinatal mouse mammary gland [79, 80, 106]. At least 7 members of the Wnt family are differentially expressed in the murine mammary gland after birth [79, 80, 107]. The expression of individual genes is primarily restricted either to stroma or to epithelium ([80] and see Table 2). The expression levels of the Wnt genes in the mammary gland change with pregnancy and lactation, and in some cases, transcripts are down-regulated in response to ovariectomy [80].

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Wnt gene	Cell type specificity	Response to hormones	Reference
Wnt-2	- myoepithelial compartment of mammary ducts	- strong in development - undetectable by 17.5 d.p.c. - decreases with ovariectomy	[80, 108]
Wnt-4	- epithelium	- expressed during pregnancy - undetectable by 17.5 d.p.c. - decreases with ovariectomy	[79, 80]
Wnt-5a	- stroma	- strong in development - undetectable by 17.5 d.p.c.	[79, 80]
Wnt-5b	- epithelium	- expressed during pregnancy - decreases with ovariectomy	[79, 80]
Wnt-6	- epithelium and stroma	- expressed during pregnancy - turns off during lactation	[79, 80]
Wnt-7b	- epithelium	- strong in development - undetectable by 12.5 d.p.c.	[79, 80]
Wnt-10b	- epithelium	- expressed during development - decreases in early pregnancy - undetectable in lactation	[107]

Table 1.2 Wnt gene expression in the mouse mammary gland. At least 7 members of the Wnt family are expressed in the mammary gland during development and/or during pregnancy. Many of these genes show regulation by steroid hormones during pregnancy, lactation, or in response to ovariectomy.

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The expression levels of Wnt-2 and -5b have been extensively investigated in the PRKO mouse [109]. Combined estrogen and progesterone treatment of control mice resulted in a decrease in Wnt-2 expression but an increase in Wnt-5b expression. The change in Wnt-2 expression was still observed in hormonally treated PRKO mice, however, no changes were seen in the levels of Wnt-5b. This implies that estrogen induces the decrease in Wnt-2 expression. The estrogen induced decrease in Wnt-2 expression correlates with the development of lobuloalveolar structures. Further transplant studies between wild-type and PRKO mice showed that the increase in Wnt-5b expression is dependent on epithelial progesterone receptors.

Wnt-1 is normally not expressed in the neonatal or adult mammary gland. When Wnt-1 is ectopically expressed in mammary gland epithelium of transgenic mice, the glands of virgin mice resemble those of pregnant mice [76] . It has been hypothesized that, in this situation, Wnt-1 activates a proliferative response normally controlled by other Wnt proteins [79]. As Wnt-4, -5b, and -6 are expressed in ductal epithelium and are strongly up-regulated during pregnancy [80] , it is tempting to speculate that Wnt-1 may be constitutively activating receptor(s) for one or all of these molecules [106]. Wnt-10b is expressed in the developing mammary glands. When Wnt-10b is over-expressed in transgenic mice, both male and female adult mice demonstrated excessive ductal growth, and de-regulation from hormonal control [107]. In an elegant transplant experiment, Bradbury *et al.* constitutively expressed Wnt-4 in epithelium of mammary glands using a retroviral vector [106] . The glands of virgin mice underwent premature branching that resembled tissue normally seen during pregnancy and resembled the mammary glands of the transgenic Wnt-1 line [106] . These experiments demonstrate that the Wnt family likely plays a role in directing the morphological changes that occur in the adult mammary gland in response to changes in the levels of circulating steroid hormones.

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Homeobox Genes in the Female Reproductive Tract

Although Wnt genes are important in morphological events, they are an extracellular signaling molecule and ultimately must involve nuclear signals. Homeobox genes, which are nuclear transcription factors, are attractive candidates for the regulation of pattern formation during embryogenesis. Gene disruption and gain-of-function studies have correlated homeobox gene expression with developmental defects [110-112]. Both clustered and non-clustered homeobox containing genes are expressed in the mouse female reproductive tract.

The vertebrate Msx genes are homologs of the *Drosophila* muscle segment homeobox (msh) gene (see [113] for review). Msx 1 is expressed in specific positions in the developing embryo [114] that generally are sites of mesenchymal-epithelial interactions, such as the tooth bud [115], limb [114, 116], mammary gland [117, 118] and female reproductive tract [41]. The expression patterns suggest that Msx 1 may play a role in tissue-specific cell differentiation [114]. Msx 1 is expressed in the epithelium of the immature and adult female reproductive tract [41]. Adult uterine epithelium is remains plastic since it must be able to respond to changing levels of hormones during the estrous cycle and during early pregnancy. It also responds to the signals for implantation. Msx 1 deficient mice have been generated [119]. Although the mice have bone and tooth malformations [119], the female reproductive tract is morphologically and molecularly normal at 18.5 d.p.c. (Miller and Sassoon, data not shown). Msx 1 mutant mice die within 24 hours of birth [119], therefore adult uterine morphology cannot be studied. Since the perinatal $Msx^{-/-}$ reproductive tract appears normal, Msx 1 may not play a critical role in the formation of the Müllerian tract, however, Msx1 may still play a role in the mesenchymal-epithelial interactions which regulate adult tissue morphology [33]. Epithelial expression of Msx 1 is dependent upon uterine mesenchyme [41] therefore, Msx 1 serves as a potential marker of correct uterine cytodifferentiation and morphogenesis.

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The clustered Hox genes expressed in the female reproductive tract include Hoxa-10, a-11, a-13, Hoxd-10, d-11, d-12, d-13, and Hoxb-7 [120-125]. The Hoxa and Hoxd clusters are expressed in overlapping patterns along the developing female reproductive tract [123, 126]. After birth, Hoxa-9 is expressed in the oviduct, Hoxa-10 in the uterus, Hoxa-11 in the uterus and cervix, and Hoxa-13 in the vagina [126]. Similarly, in late gestation, Hoxd-10 and -11 are expressed in the oviduct, Hoxd-12 is expressed in the uterus, and Hoxd-13 is expressed in the posterior uterus and in the vagina [123].

Disruption of Hoxa-10 in female mice results in infertility due to an intrinsic defect in the uterus during implantation [121, 127]. Hoxa-10 expression is responsive to levels of steroid hormones in both mice and humans [128, 129]. Expression is strongly stimulated by progesterone and is repressed by estrogen. The responsiveness of Hoxa-10 to hormones may support a role for the gene in adult life during embryo implantation. Additionally, Hoxa-10 mutant females display a homeotic transformation of the uterine horns so that the anterior 25% of the uterine horn has the cellular and molecular characteristics of oviduct [127]. The mutant males display cryptorchidism [121]. The descent of the testes is dependent on the correct formation and position of the gubernaculum. The point of the utero-tubal junction in females is also determined by the gubernaculum. It seems likely that Hoxa-10 expression is important in determining the correct location of this ligament, and when Hoxa-10 is lost, the gubernaculum crosses the urogenital ducts in an abnormal position causing the structural defects in the male and female genital tracts. Hoxa-10 expression in the reproductive tract would therefore be important for determining the positional identity of structures outside the reproductive tracts.

Hoxa-11 mutant females are sterile as a result of a maternal defect during implantation [31, 120]. Levels of Hoxa-11 vary during early gestation. In the absence of Hoxa-11, no burst of LIF expression is observed. It has been noted that in the absence of LIF, implantation does not occur [130, 131]. Additionally, the Hoxa-11 mutant females

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have thinner, shorter uterine horns that contain fewer glands than wild-type uterine horns [31].

Malformations of the posterior female reproductive tract and infertility were evident in a naturally occurring mouse mutation in Hoxa-13 [122]. A dominant human mutation in HOXA-13 results in hand-foot-genital syndrome where common reproductive tract malformations include defects in Müllerian duct fusion [[75]71]. Hoxa-13 disrupted mice show agenesis of the posterior Müllerian ducts [132]. Disruption of Hoxd-13 does not result in any alteration in female fertility [133], however, Hoxa-13 $^{+/-}$/Hoxd-13 $^{-/-}$ compound mutant females display Müllerian duct agenesis, and multiple vaginal anomalies [132]. The vaginal defects include improper separation of the vagina from the anus and urethra, as well as the presence of a vaginal septum. These defects implicate Hoxa-13 and Hoxd-13 in the division of the cloaca, and in Müllerian duct fusion, suggesting that the Hox genes are important for defining the different compartments of the developing female reproductive tract.

Specific Background

At the time this project began, there was little understanding of the molecular signals which underlie the development and function of the female reproductive tract. Although we knew that Wnt-5a and Msx1 were expressed in the uterus, we could only postulate functions for these molecules [41]. Through the use of a number of techniques, we sought to elucidate the functions of the Wnt family of signaling molecules in the female reproductive tract. Using RT-PCR, we determined which of the Wnt molecules were expressed in the female reproductive tract. In situ hybridization analysis of the expressed Wnt genes allowed us to determine patterns of expression. The Wnt genes are expressed primarily in either the mesenchymal or epithelial tissues of the reproductive tract. We then performed a gain-of-function study using retroviruses to ectopically deliver genes to the mesenchyme of the reproductive tract. Loss-of-function analysis was also performed. The

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roles of Wnt-7a in the epithelium of the female reproductive tract were determined using the Wnt-7a mutant mouse. Finally, a relationship between Wnt-7a expression and the effects of prenatal exposure to DES was established. The relevance of Wnt-7a signaling and the effects of estrogen analogs as well as the importance of Wnt signaling and its relationship to human pathologies is discussed in this thesis.

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Chapter 2 Methods and Materials

Mice

For in situ hybridization analysis, CD-1 mice were used (Charles River). At least 2 samples were used for each experimental time point. Neonatal tissues were isolated following timed breedings with the morning of the vaginal plug counted as 0.5 days post-coitum (d.p.c.). For post-natal tissues, the day of birth is counted as day 0. The stage of the estrous cycle was determined by histological examination of the vaginal epithelium [32] . In diestrus, the epithelium is only 5-6 layers thick and it lacks a basement membrane. In proestrus, we see 12-13 layers of epithelium, and the top layers stain very lightly with eosin. During estrous, the epithelium has an intact, superficial cornified layer whereas during metestrus, the cornified layer is shed and the epithelium is invaded by large numbers of leukocytes, often in lacunae.

RT-PCR

Total RNA was isolated from the adult CD-1 female reproductive tract using the TRIZOL method (GIBCO). First-strand cDNAs were synthesized using random hexamers as primers with MMLV reverse transcriptase (Boehringer-Mannheim). PCR was performed using *Taq* polymerase (Promega) with 1.5 mM $MgCl_2$, under the following conditions: 5 min of denaturation at 94°C, 29 cycles of denaturation (30 seconds, 94°C), annealing (30 seconds, 65°C), and extension (45 seconds, 72°C). For each PCR reaction we used control RNA that had not been reacted with reverse transcriptase (-RT). The different primers used were: for Wnt-1: 5'-GTGGCCGATGGTGGGGCATCGTGAA-3', and 5'-TTTGCACTCTTGGCGC-3'; for Wnt-2: 5'-CTGGCTCACCCCTGAGGTACATGAGA-3', and 5'CAAGAAGCGCTTTACAGCCTTCCTTCCA-3'; for Wnt-3a: 5'-TACCCGA-TCTGGTGGTCCTTGGCTG-3' and 5'TGACTGGCGATGGCCTGGCGCCC-3'; for Wnt-4: 5'-CCGCGAGCAATTGGCTGTACCTGGC-3' and 5'-CATGTGTGTCAAGA-

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TGGCCTTCCTGC-3'; for Wnt-5a: 5'-GAAGCTAATTCTTGGTGGTCTCTAGGTATG-3' and 5'-GCCAGGTTGTATACTGTCCTACGGCC-3'; for Wnt-5b: 5'-GCCAACTCC-TGGTGGTCACTAGCTC-3' and 5'-CATCTTATACACGGCCCGGCGGCCA-3'; for Wnt-6: 5'-ATGGACTGTGGTGGGCCGTGGGCA-3' and 5'-TGACTCCGCACCGC-CAGCCTGCC-3'; and for Wnt-7a: 5'-GGCATAGTCTACCTCCGGATCGGTG-3' and 5'-GTTCTCCTCCAGGATCTTCCGACCC-3'. The primers used for the tubulin reactions were 5'-TGGCCAGATCTTCAGACCAG-3' and 5'-GTAAGTTCAGGCACAGTGAG-3'. RT-PCR was performed twice, using different RNA samples.

Histology and in situ Hybridization

Tissues were fixed overnight in 4% PBS-buffered paraformaldehyde. Paraffin embedded tissues were sectioned at 5-6 μm. For histological examination, sections were stained with hematoxylin and eosin. Techniques for in situ hybridization were performed as previously described [134]. Antisense riboprobes were generated for Msx 1 [135] , Wnt-4 and -7a [77] , Wnt-5a [79], smooth muscle myosin heavy chain (SMMHC) [73], Hoxa-10 [121], and Hoxa-11 [120]. Antisense riboprobes were generated under identical reaction conditions and were used at a final concentration of 105,000 dpm/μl hybridization buffer. Emulsion coated slides (NTB-2, Kodak) were allowed to expose for 1 or 2 weeks at 4°C. In situ analysis was performed at least twice per tissue sample with each probe and at least 2 different samples were used per data point.

Tissue Recombinants

Recombinants were prepared using techniques described previously [136] . Briefly, intact reproductive tracts were isolated from neonatal (0-2 days post-partum) *Wnt-7a* mutant and wild-type mice and maintained in calcium-magnesium-free Hank's buffer (CMF-HBSS, GIBCO) at 4°C until use. Samples were incubated at 4°C in 1% trypsin (Difco 1:250) in CMF-HBSS for 1-1.5 hours and were rinsed three times with CMF-HBSS supplemented

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with 10% FCS; the first rinse in the presence of 0.1% deoxyribonuclease 1 (Sigma). The tissues were separated into mesenchymal or epithelial components by gentle teasing with forceps or by drawing into a flame-blunted drawn Pasteur pipette [136]. The mesenchymal and epithelial fragments were made into recombinants on solidified agar medium [41] . Tissues were allowed to re-adhere overnight before grafting under the renal capsule of Avertin (Aldrich) anaesthetized female athymic nude mice (Taconic NCI).Control and experimental tissues were grafted to opposite kidneys in the same host for 3-4 weeks. Tissues were subsequently isolated and processed for histological examination and in situ hybridization.

Retroviral Infection of Dissociated Mesenchymal Cells and Tissue Fragments

Retroviruses were obtained through transfection of Bosc 23 cells with the desired construct as described by Pear *et al.* [137]. The constructs and retroviruses were prepared and obtained from the laboratory of Dr. Jan Kitajewski (Columbia). Briefly, the cells were transfected with 20 μg of DNA using the calcium phosphate technique. The media containing retroviruses was collected 2 days after transfection. Control plates which had been transfected with a LacZ containing construct were stained for X-gal activity to ensure high levels of transfection. The retroviral supernatant was either frozen immediately, or used within 1 hour.

Uterine and vaginal mesenchyme were isolated as described above. Fragments of uterine or vaginal mesenchyme were dissociated into single cells by digesting in collagenase (Gibco). The cells were counted and $5x10^5$-$1x10^6$ were plated onto 6 cm dishes. 24 hours after plating, the cells were incubated with 4 ml of retroviral supernatant for 4-6 hours. 2 days after the retroviral incubation, the cells were stained for LacZ activity. Mesenchymal fragments were incubated with retroviral supernatant in a similar manner. The fragments from 4-6 uteri or vagina were incubated with ~1.5 ml of retroviral

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supernatant at 37°C for 20-24 hours. After incubation with the retroviral supernatant, the mesenchyme was made into recombinants with freshly obtained neonatal epithelium as described above. 1-2 days after recombination, grafts were stained for X-gal activity or inserted under the kidney capsule of athymic nude mice.

Wnt-7a Mutant Mice

Wnt-7a mutant mice were generated by homologous recombination in ES cells as described previously by Parr *et al.* [82] . The targeting strategy inserted a neomycin-resistance gene into the second exon of Wnt-7a. 129/Sv sibling or age-matched females were used for control tissues in the described experiments. Neonatal tissues were isolated following timed breedings with the morning of the vaginal plug counted as 0.5 days post-coitum (d.p.c.). For post-natal tissues, the day of birth is counted as day 0. Genotype was determined by the appearance of the limbs and confirmed by Southern blot analysis [82] . At least 2 animals were examined for each time point. 10 mutant mice were examined for oviduct morphology and 12 were examined for the presence of a vaginal septum.

DES Exposure

DES treatment was performed as described by Iguchi *et al.* [26, 138]. Briefly, diethylstilbestrol (Sigma) was resuspended at a concentration of 2 mg/ml in sesame oil (Sigma). Timed pregnant CD-1 mice (Charles River) were injected subcutaneously with 0.1 ml of DES (200 μg) or 0.1 ml of oil alone on days 15-18 of pregnancy. The morning of the vaginal plug counted as 0.5 d.p.c.. On day 19, the pups were delivered by cesarean section and were fostered by untreated CD-1 females. Oil and DES treated females were analyzed at 0, 5, 10, 15, 20, 25, and 30 days after birth. The day of delivery was considered to be day 0. The reproductive tracts were isolated and processed for histological examination and in situ hybridization. At least 2 samples were analyzed per time-point for each treatment.

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Chapter 3 Wnt Gene Expression Patterns during the Development of the Female Reproductive Tract and during the Estrous Cycle

The work described in this chapter is based on the paper "Wnt gene expression patterns during the development of the female reproductive tract and during the estrous cycle" by Cary Miller, Anna Pavlova, and David Sassoon (Mechanisms of Development, 1998, 76, pp.90-99). Anna Pavlova was a former student in the laboratory. She determined which Wnt genes were expressed in the female reproductive tract using RT-PCR. Shortly after I entered the laboratory and started working on this project, Anna left to continue her post-doctoral studies. I generated all of the figures shown in this chapter.

Introduction

The murine female reproductive tract is one of several organ systems that is relatively undifferentiated and rudimentary at birth [20]. The post-natal events which result in the maturation and differentiation of the female reproductive tract occur in response to circulating steroid hormones, and are dependent upon specific mesenchymal-epithelial interactions [40]. The nature of the inductive signals involved in the development of the female reproductive tract and the transient capacity to respond to these signals is not understood at the molecular level.

Members of the Wnt family of signaling molecules have been identified in the female reproductive tract [41, 139, 140]. In this chapter, I describe the expression patterns of three Wnt genes in the mouse female reproductive tract. Wnt-4, -5a, and -7a are expressed in specific mesenchymal-epithelial patterns during post-natal development and in the adult reproductive tract. The expression patterns of Wnt-4 and -5a are responsive to changes in the circulating levels of steroid hormones during the murine estrous cycle. The changes in expression patterns during the estrous cycle correspond to the changes seen in

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hormone treated tissues. The changes in Wnt gene expression patterns during development are consistent with a role in cytodifferentiation. The presence of epithelium influences the expression patterns of these three Wnt genes. I propose that Wnt genes play a key role in the mesenchymal-epithelial interactions which are important both in the development and in the adult function of the murine female reproductive tract.

Results

Wnt genes Are Expressed in the Murine Female Reproductive Tract

A preliminary screen was performed on the murine Wnt genes -1, -2, -3a, -4, -5a, -5b, -6, and -7a using RT-PCR and RNA isolated from adult uteri and vaginas. This survey revealed that Wnt-4, -5a, and -7a transcripts are present in the female reproductive tract (Fig. 3.1) whereas other members of the Wnt gene family were not detected (data not shown). Tubulin was detected in both the vagina and uterus (Fig. 3.1, lanes 2 and 4). Wnt-4 was also detected in both the vagina and the uterus (Fig. 3.1, lanes 7 and 9). Wnt-5a and Wnt-7a were both detected only in the uterus (Fig. 3.1, lanes 15, and 23 respectively). Positive controls for the PCR (cDNA plasmids) were included and specific bands were detected for Wnt-4, -5a, and -7a (Fig. 3.1, lanes 11, 17, and 23). No PCR products were detected in lanes without the reverse transcriptase reaction (-RT).

In situ hybridization was performed on adult female reproductive tracts using probes corresponding to Wnt-4, -5a and -7a. In the adult, these three genes are expressed in specific mesenchymal-epithelial patterns in the uterus and vagina. Wnt-4 is expressed strongly in the stroma subjacent to the luminal epithelium of the uterus (Fig. 3.2B). Wnt-5a is also expressed in the stroma of the uterus. It is restricted from the epithelium and the uterine myometrium (Fig. 3.2C). Wnt-7a is expressed only within the luminal epithelium of the uterus and not within the glandular epithelium (Fig. 3.2D). In the vagina, Wnt-4 expression is detected in the vaginal epithelium (Fig. 3.2F) while levels of Wnt-5a and -7a are undetectable (Fig. 3.2G,H).

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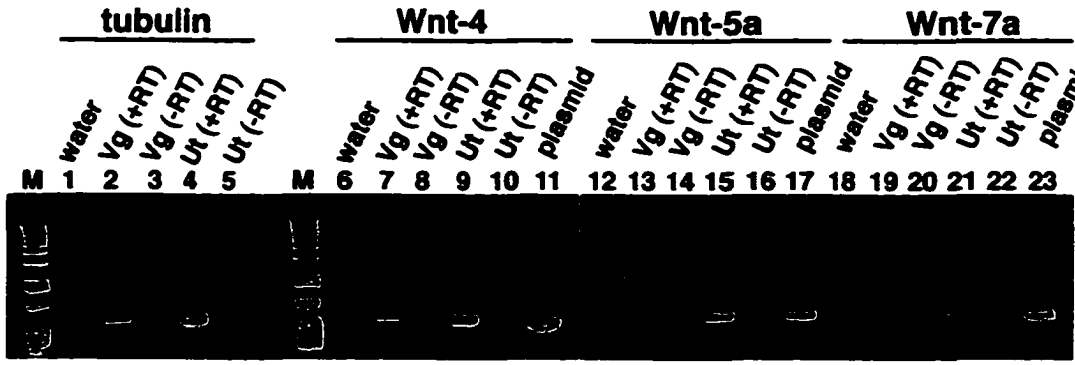

Figure 3.1 Specific Wnt genes are expressed in the adult female reproductive tract as shown by RT-PCR. We do not detect PCR products in the reactions that were not reverse transcribed (lanes 3, 5, 8, 10, 14, 16, 20, and 22). Tubulin is expressed in both the uterus and vagina (lanes 2 and 4). A Wnt-4 specific band is detected in the uterus and vaginal samples (lanes 7 and 9), and in the plasmid control (lane 11). Wnt-5a and Wnt-7a are expressed is detected only in the uterine sample (lanes 15, and 21, respectively) and in the plasmid controls (lanes 17, and 23, respectively). Note: expression of Wnt-5a can be observed in the vagina and is dependent on the stage of the estrous cycle (see Fig. 3.3). Abbreviations: Vg (vagina), Ut (uterus).

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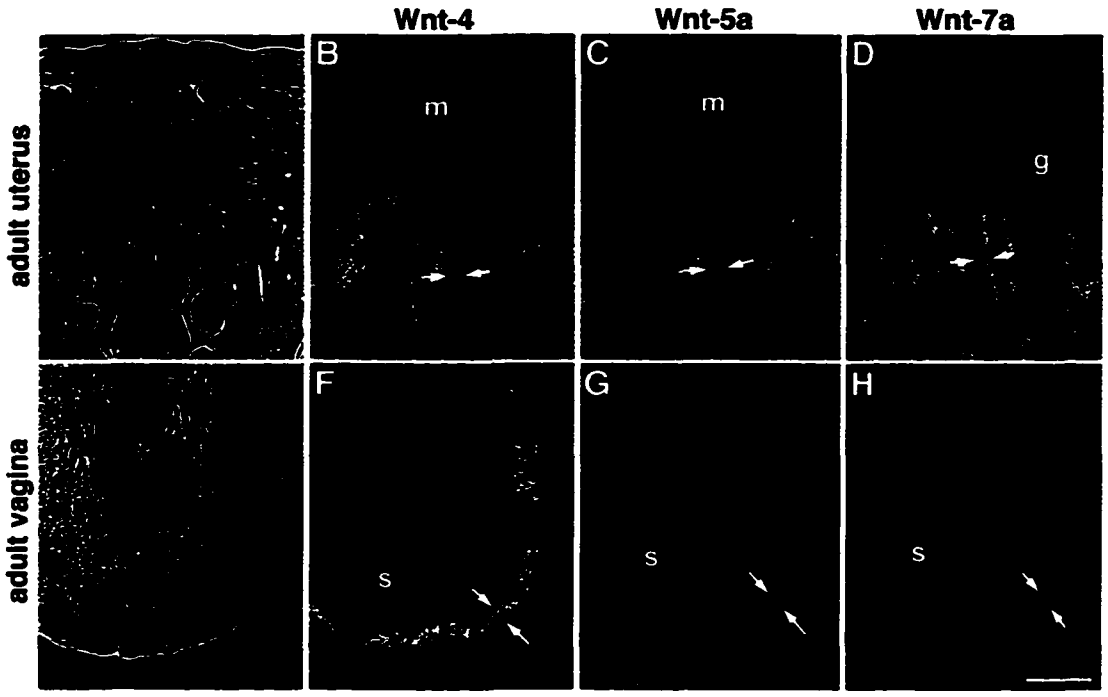

Figure 3.2 Wnt gene expression patterns in the adult female reproductive tract are restricted to the mesenchyme or epithelium. A-D show neighboring sections of an adult uterus. A shows a bright -field photomicrograph of adult uterus demonstrating the different tissue types: smooth muscle, stroma which contains uterine glands, and luminal epithelium (double arrows). B shows a dark-field image of the section shown in A for Wnt-4. Wnt-4 expression is detected primarily in the mesenchyme subjacent to the luminal epithelium and not in the epithelium itself. C shows a neighboring section hybridized for Wnt-5a; transcripts are detected primarily in the uterine stroma. Note that expression is restricted from the smooth muscle cells and the luminal epithelium. D shows a neighboring section hybridized for Wnt-7a. Wnt-7a is expressed solely in the luminal epithelium of the uterus and not within the glandular epithelium. E-H show neighboring sections of adult vagina. E shows a bright-field photomicrograph of adult vagina demonstrating the stroma and the stratified epithelium (double arrows). F shows the section from E hybridized for Wnt-4. Transcripts are detected in the stratified epithelium. G and H show neighboring sections hybridized for Wnt-5a and Wnt-7a respectively. No vaginal expression is detectable with either probe. Scale bar, 200 μm. Abbreviations: m (muscle), s (stroma), and g (uterine glands).

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Wnt Gene Expression Patterns are Responsive to Steroid Hormones Levels

We noted that the levels and precise localization of Wnt-4 and -5a varied among individual samples, raising the possibility that their expression may be regulated during the estrous cycle. We therefore performed a detailed study of Wnt gene expression during the estrous cycle. The stage of estrous cycle was determined by the appearance and cell morphology of the vaginal epithelium [32]. The pattern of Wnt-4 expression changes dramatically during the estrous cycle. During diestrus, Wnt-4 is expressed at very low levels (Fig. 3.3E). By early proestrus, transcripts are detectable primarily in the uterine epithelium (Fig. 3.3F). During oestrus, strong expression is observed both in the stroma and the epithelium (Fig. 3.3G) and by metestrus, expression is restricted to the stroma subjacent to the luminal epithelium (Fig. 3.3H). Uterine Wnt-5a expression also changes during the estrous cycle. During diestrus, Wnt-5a transcripts are detected in both the stroma and the epithelium of the uterus (Fig. 3.3I). By proestrus, transcripts are detected primarily in the uterine stroma (Fig. 3.3J) but by oestrus, transcripts are once again observed in the stroma and the epithelium (Fig. 3.3K). During metestrus, Wnt-5a is primarily in the uterine stroma although low levels of expression are detected in the epithelium (Fig. 3.3L). These results suggest that high levels of circulating estradiol may repress Wnt-5a expression in the uterine epithelium. While the pattern of Wnt-7a expression in the luminal epithelium during the estrous cycle remains constant (Fig. 3.3M-P), there may be variations in the level of expression. Expression levels appear higher during diestrus and metestrus (Fig. 3.3M,P) than during proestrus and oestrus (Fig. 3.3N,O). Wnt-5a expression levels change in the vagina during the estrous cycle. During diestrus, Wnt-5a is expressed at low levels in the stroma and epithelium of the vagina (Fig. 3.3Q,R). Wnt-5a is expressed at higher levels in proestrus although it is still in the stroma and epithelium (Fig. 3.3S,T). During oestrus, expression is lower than in proestrus and is restricted to the vaginal stroma (Fig. 3.3U,V). By metestrus, Wnt-5a expression is not detectable in the vagina (Fig. 3.3W,X).

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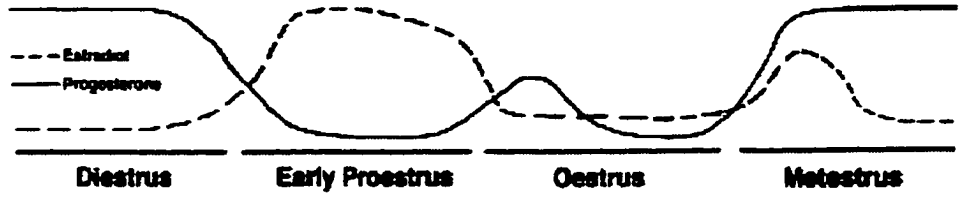
	Diestrus	Proestrus	Oestrus	Metestrus
Wnt-4	Stroma and epithelium (low)	Epithelium (high)	Stroma and epithelium (high)	Stroma (high)
Wnt-5a (uterus)	Stroma and epithelium	Stroma	Stroma and epithelium	Stroma
Wnt-5a (vagina)	Stroma and epithelium (low)	Stroma and epithelium (high)	Stroma (low)	Not detectable
Wnt-7a	Epithelium (high)	Epithelium (low)	Epithelium (low)	Epithelium (high)



From [141]

Table 3.1 Wnt gene expression in the female reproductive tract during the estrous cycle. The levels and patterns of Wnt-4, -5a, and -7a expression are responsive to the changing levels of estradiol and progesterone during the mouse estrous cycle. This table summarizes the data depicted in Figure 3.3.

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Figure 3.3 Wnt gene expression patterns change during the mouse estrous cycle. In situ hybridization was performed on neighboring sections of mouse uterus during the various stages of the estrous cycle. The approximate levels of circulating steroid hormones are shown in the panel below (see (Walmer, et al., 1992)). A-D show bright-field images of the tissues indicating luminal epithelium (double arrows), stroma, smooth muscle, and glands. E-H show sections hybridized with a probe corresponding the Wnt-4. During diestrus (E), Wnt-4 expression is undetectable. Expression increases in proestrus (F) when transcripts are detected primarily in the uterine epithelium, although low levels are detectable in the stroma. During oestrus (G), transcripts are detectable at approximately equal levels in the uterine stroma and epithelium. Wnt-4 expression declines in the uterine epithelium so that in metestrus (H), transcripts are only detectable in the uterine stroma. I-L show sections hybridized with a probe corresponding to Wnt-5a. During diestrus (I), Wnt-5a transcripts are detected in the uterine stroma and epithelium. By proestrus (J), Wnt-5a is expressed primarily in the uterine stroma while little expression is detected in the epithelium. During oestrus (K), we detect transcripts in both the uterine stroma and epithelium, but expression is restricted to the stroma during metestrus (L). M-P show the pattern of Wnt-7a during the estrous cycle. Wnt-7a expression remains in the luminal epithelium of the uterine horn during the various stages of the estrous cycle, although levels of Wnt-7a may fluctuate slightly during the different stages. Q-X show sections of adult vagina hybridized with a probe corresponding to Wnt-5a. Q, and R show the bright-field and dark-field images of a vagina during diestrus. Low levels of Wnt-5a transcripts are detected in the stroma and epithelium. S, and T show bright-field and dark-field images of a vagina during proestrus. Wnt-5a expression is detected in both the stroma and epithelium at higher levels than seen in diestrus. U, and V show sections during the oestrus phase where Wnt-5a expression is detected at low levels in the vaginal stroma, but not the epithelium. W, and X show sections during metestrus where Wnt-5a expression is not detectable in either the stroma or epithelium. Scale bar, 200 μm.

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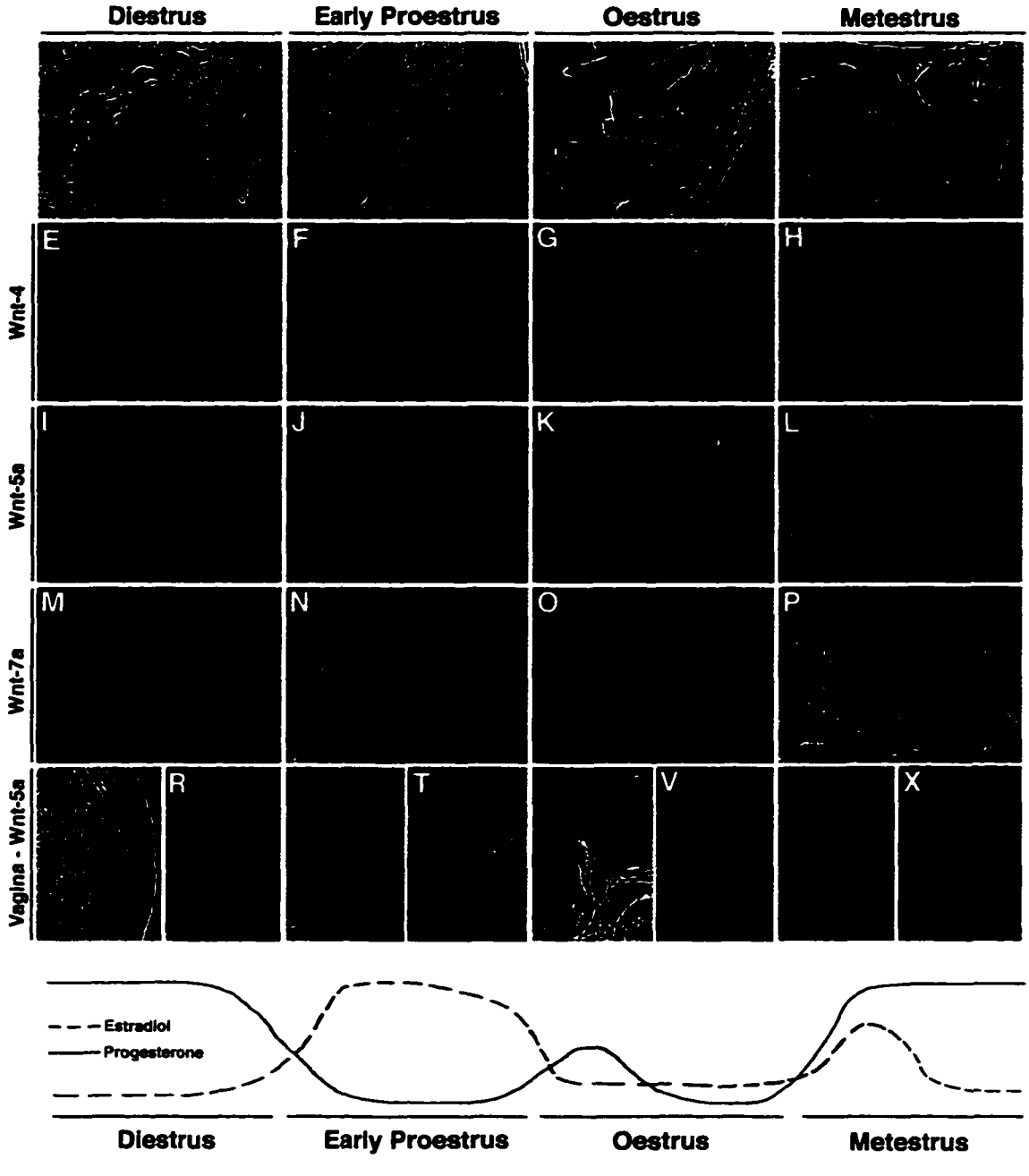

Figure 3.3 Wnt gene expression patterns change during the mouse estrous cycle.

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Wnt Gene Expression Patterns Are Dynamic during the Development of the Female Reproductive Tract

Wnt genes have been postulated to play a role in the functioning of the adult mammary gland [79, 80] and female reproductive tract [41, 142]. Furthermore, Wnt genes have a demonstrated role in developmental processes [81, 82, 85]. We therefore examined the expression patterns of Wnt-4, -5a and -7a during the development of the female reproductive tract.

At 17 d.p.c., Wnt-4 expression is detected in the mesenchyme of the future uterine horn and in the epithelium of the sinus vagina (Fig. 3.4B) but is not detected in the epithelium of the Müllerian vagina. At birth, transcripts remain undetectable in the vagina and cervix (Fig. 3.4F) but are seen in the mesenchyme of the uterine horn (Fig. 3.4J). By 6 days after birth, Wnt-4 expression is restricted to the mesenchyme near the luminal epithelium in the uterine horn, and to the mesenchyme of the oviduct (Fig. 3.4N). By this time, we also detect Wnt-4 transcripts in the epithelium of the vagina (Fig. 3.4R). Wnt-5a expression is also regulated during development. At 17 d.p.c., Wnt-5a is expression is restricted to the mesenchyme of the genital tract (Fig. 3.4C). At birth, Wnt-5a remains restricted to the mesenchyme of the female reproductive tract (Fig. 3.4G,K and see [41]). At 6 days post-natal, Wnt-5a expression in the uterine stroma becomes excluded from the region of forming smooth muscle (Fig. 3.4O). It is expressed by the mesenchyme and epithelium of the oviduct (Fig. 3.4O). By 6 days, Wnt-5a expression declines in the vaginal stroma although low levels are still detectable (Fig. 3.4S). Wnt-7a is initially expressed throughout the epithelium of the Müllerian duct at 17 d.p.c. (Fig. 3.4D). Strong expression is detected in the epithelium of the cervix, vagina, and uterine horn at birth (Fig. 3.4 H,L). 6 days after birth, transcripts are seen in the luminal epithelium of the uterine horn (Fig. 3.4P). Wnt-7a is detected in the forming glands (Fig. 3.4P), although it is not expressed in the glands of the adult uterus (Fig. 3.2D). Wnt-7a is also expressed in the epithelium of the oviduct (Fig. 3.4P). By 6 days after birth, Wnt-7a expression is declines

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in the vaginal epithelium (Fig. 3.4T). Although transcripts are still detectable in the anterior vagina, they are undetectable in the posterior vagina. By 10 days after birth, Wnt-7a transcripts are undetectable in the vaginal epithelium (data not shown). The timing of the loss of Wnt-7a from the vagina corresponds to the timing of vaginal cytodifferentiation [22], implying that Wnt-7a may confer the ability to respond to the uterine inductive signals.

	Wnt-4	Wnt-5a	Wnt-7a
Tissue type	-Primarily mesenchyme	-Primarily mesenchyme	-Epithelial
17 d.p.c.	-Mesenchyme of uterine horns	-Mesenchyme of uterine horns, cervix, and vagina	-Epithelia of uterine horns, cervix, and vagina
Birth	-Mesenchyme of uterine horns	-Mesenchyme of uterine horns, cervix, and vagina	-Epithelia of uterine horns, cervix, and vagina
4 days	-Mesenchyme of uterine horns -Epithelium of vagina (low)	-Mesenchyme of uterine horns -lower levels of expression in posterior vagina	-Epithelia of uterine horns, cervix - decreased expression in posterior vagina
10 days	-Mesenchyme of uterine horns -Epithelium of vagina (high)	-Mesenchyme of uterine horns -not detectable in vagina	-Epithelia of uterine horns -not detectable in vagina

Table 3.2 The expression patterns of the Wnt genes in the developing female reproductive tract. The expression patterns of Wnt-4, Wnt-5a, and Wnt-7a change during the post-natal development. This table provides a summary of the expression patterns demonstrated in Figure 3.4.

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Figure 3.4 Wnt gene expression patterns are dynamic during the development of the female reproductive tract. A-D show neighboring longitudinal sections through a 17 d.p.c. female reproductive tract. A shows a bright-field image demonstrating the simple columnar epithelium of the future uterine horns (double arrows), and the Müllerian vagina (single arrow). B shows the section in A hybridized with a probe corresponding to Wnt-4. Transcripts are localized to the mesenchyme of the future uterine horn and the ureter. Although Wnt-4 is not expressed in the Müllerian vagina, it is expressed in the sinus vagina. C shows that Wnt-5a is expressed in the mesenchyme of the entire reproductive tract. D shows Wnt-7a expression is restricted to the epithelium of the reproductive tract; transcripts are detected within the epithelium of the Müllerian vagina and the uterine horns. E-L show neighboring sections through a 0 day reproductive tract. E-H show the vagina and cervix. The anterior-posterior (A-P) axis is indicated (the cervix is to the right). In F, Wnt-4 expression is undetectable in the vagina and cervix, while in G, Wnt-5a expression is restricted to the vaginal and cervical stroma. H shows that Wnt-7a transcripts are detectable in the epithelium of the cervix and the vagina. I-L show neighboring sections through a newborn uterine horn. I is a bright-field image the uterine horn showing stroma, and luminal epithelium (double arrows). Note that no smooth muscle is apparent at this stage. J. and K show that Wnt-4 and -5a transcripts, respectively, are expressed primarily in the stroma. L depicts Wnt-7a expression which is detected solely in the luminal epithelium of the uterus. M-P show neighboring sections through a 4 day uterus and oviduct. M shows a bright-field image demonstrating the oviduct, uterine horn, and forming smooth muscle layers. N shows the section from M hybridized for Wnt-4 expression; transcripts are detected primarily in the mesenchyme of the uterine horn. Note that expression is restricted from uterine epithelium and from the forming smooth muscle. Weak expression is detected in the mesenchyme and epithelium of the oviduct. O shows that Wnt-5a expression is also restricted to the uterine mesenchyme and that like Wnt-4, expression is lower in the uterine epithelium and smooth muscle. Wnt-5a transcripts are

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detected throughout the thickness of the oviduct. P shows Wnt-7a expression is restricted to the epithelium of the uterine horn and oviduct. Wnt-7a transcripts are detected throughout the luminal epithelium, even in the invaginations which will become glands. Q-T show neighboring longitudinal sections through a 6 day vagina. Q shows a bright-field image with the anterior-posterior axis indicated; the cervix is to the left. The stratified epithelium is denoted (double arrows). R shows the section in P hybridized for Wnt-4. Transcripts are detected throughout the vaginal epithelium. S shows that Wnt 5a is still expressed in the vaginal stroma, although expression is lower in the posterior vagina than it is near the cervix. T shows that although Wnt-7a transcripts are not detectable in the epithelium of the posterior vagina, we do detect expression in the anterior vaginal epithelium. The schemas shown below summarize the panels. The smaller image represents the situation at birth whereas the larger figure represents the adult expression patterns. U shows that Wnt-4 is initially expressed in the stroma of the uterine horns but later is expressed both in the uterine horns and in the epithelium of the vagina. V shows that Wnt-5a is initially expressed throughout the stroma of the reproductive tract but becomes post-natally restricted to the uterine horns. X shows that Wnt-7a is initially expressed throughout the epithelium of the reproductive tract and, like Wnt-5a, its expression becomes restricted to the uterine horns. Scale bar, 200 μm, 100μm (I-L). Abbreviations; g (glands), m (muscle), s (stroma), sv (sinus vagina), ovi (oviduct), u (ureter), and ut (uterine horn).

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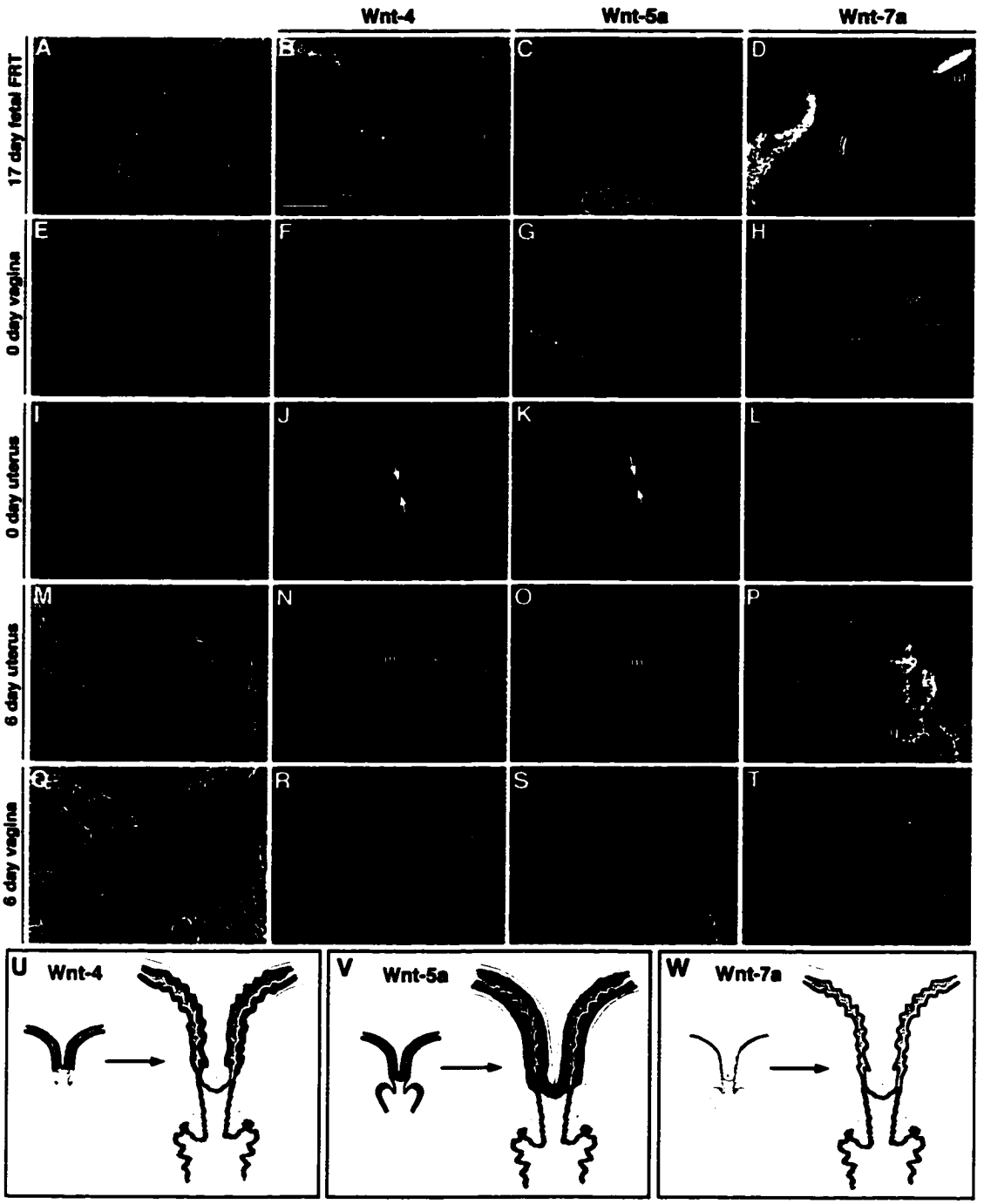

Figure 3.4 Wnt gene expression patterns are dynamic during the development of the female reproductive tract. Wnt-4 is primarily in the mesenchyme of the uterine horns, and in the epithelium of the vagina. Wnt-5a is mainly expressed in the mesnchyme of the reproductive tract, while Wnt-7a is restricted to the epithelium.

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Epithelial Regulation of Wnt Gene Expression in the Uterine Stroma

Inductive mesenchymal-epithelial signaling events are responsible for the morphogenesis and cytodifferentiation of the female reproductive tract [22]. The specific expression patterns of the Wnt genes may reflect such tissue-type specific signaling and may change in the presence or absence of epithelium. Uterine stroma in the presence or absence of epithelium can be grafted under the kidney capsule of nude mice, and allowed to grow in an in vivo situation [20, 22]. When newborn uterine mesenchyme is recombined with newborn uterine epithelium, the resultant graft has simple columnar epithelium, a stromal layer that contains no glands, and a layer of smooth muscle (Fig. 3.5A). The smooth muscle is arranged in a layer around the outside of the graft in a circular distribution (Fig. 3.5B). We also detect Wnt-4 and Wnt-5a in the stroma (Fig. 3.5C,D). The patterns of Wnt-4 and -5a expression in the grafts shift to reflect the stage of estrous in the host mouse (data not shown). Additionally, Wnt-7a is expressed in the simple columnar epithelium (Fig. 3.5E). When newborn uterine stroma without epithelium is grown in a nude mouse host for 3 weeks, much of the resulting tissue is positive for smooth muscle myosin heavy chain (SMMHC) by in situ hybridization (Fig. 3.5G). Wnt-4 and -5a transcripts are detectable in the stromal grafts, however, they are expressed at lower levels than in the intact uterus or the grafts containing epithelium. Wnt-4 and Wnt-5a expression are most localized to regions with little smooth muscle, although Wnt-5a expression is more restricted than Wnt-4 expression (Fig. 3.5I). We do not observe expression of Wnt-7a in the absence of epithelium, thus unlike Wnt-4 and -5a, Wnt-7a is completely restricted to the epithelium, even under these experimental conditions (Fig. 3.5J).

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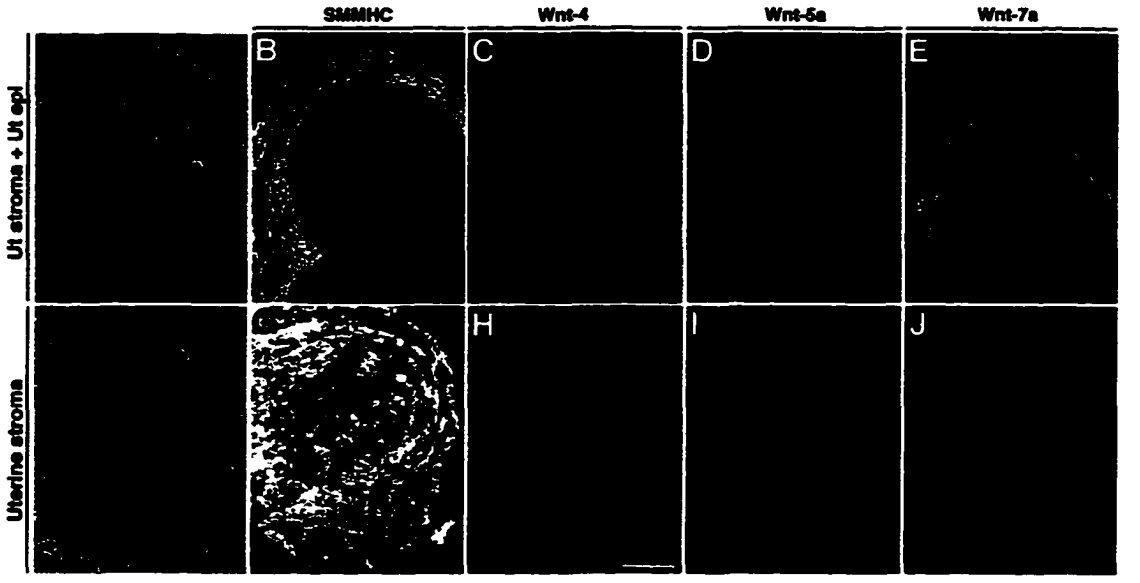

Figure 3.5 Wnt gene expression in the uterine stroma is influenced by the presence of epithelium. Wnt gene expression was examined in stromal tissue which was grown in nude mice in the presence and absence of epithelium. Uterine mesenchyme recombined with uterine epithelium forms a ball-like structure. It is lined by simple columnar epithelium, and contains distinct layers of stroma and smooth muscle (A). Using a probe corresponding to smooth muscle myosin heavy chain (SMMHC), the exterior of the graft can be shown to be composed of smooth muscle bundles (B). Wnt-4 and -5a are expressed in the stroma of the graft (C,D) and Wnt-7a is expressed in the simple columnar epithelium (E). When stroma is grown in the absence of epithelium much of the tissue is composed of disorganized bundles of smooth muscle indicated by the detection of abundant SMMHC transcripts (G). Although Wnt-4 expression is detected in the grafts, it is strongest in the regions which have little smooth muscle (H). Wnt-5a expression is similar to Wnt-4, except the distribution is even more restricted from the smooth muscle positive areas (I). Wnt-7a expression is not detectable, showing that its expression is dependent on the presence of epithelium (J). Scale bar, 200 μm.

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Discussion

Wnt Gene Expression Patterns Suggest Roles in Patterning and in Adult Function

We report here that Wnt-4, -5a, and -7a are expressed in the female reproductive tract. The tissue specificity and the dynamic patterns of expression during development suggest that Wnt gene signaling plays a role in the morphogenesis and cytodifferentiation of the female reproductive tract. During early female reproductive tract development (prior to 6 days post-natal), Wnt-5a and -7a are expressed throughout the mesenchyme and epithelium, respectively, of the female genital tract. After birth, expression becomes restricted primarily to the uterine horns. The loss of expression from the vagina is coincident with vaginal cytodifferentiation. The epithelium of the entire Müllerian tract remains plastic and undifferentiated until approximately five to seven days after birth [22, 40]. During this period, the epithelium can respond to inductive signals from either uterine or vaginal mesenchyme. Wnt-4 is initially restricted to the mesenchyme of the uterine horns and the sinus vagina. The later expression of Wnt-4 in the vaginal epithelium coincides with the decline in Wnt-5a and -7a expression in the vagina. The down-regulation of Wnt-5a and/or Wnt-7a and the up-regulation of Wnt-4 in the vagina may be may be required for subsequent tissue differentiation and morphogenesis. Data presented in Chapter 5 support the hypothesis that Wnt genes are critical for patterning and cytodifferention in the female reproductive tract.

Wnt Gene Regulation of Smooth Muscle Formation

In the newborn uterine horn, Wnt-4 and -5a are expressed throughout the width of the mesenchyme. During smooth muscle formation, Wnt-4 and -5a are down-regulated and eventually excluded from regions of smooth muscle differentiation. In the adult uterus, smooth muscle is devoid of Wnt gene expression. Others have shown that smooth muscle differentiation is dependent on the presence of epithelium [143]. In contrast to these earlier

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studies, we observed that in the absence of epithelium, stromal grafts contain abundant smooth muscle positive cells. The highest levels of Wnt-4 and -5a expression are in regions that contain few differentiated smooth muscle cells. Therefore, the observation that Wnt-4 and -5a expression is mutually exclusive from smooth muscle differentiation is not dependent upon the presence of epithelium. However, epithelium is required to organize the grafts. Wnt-7a is restricted to the epithelium and never expressed in the stroma (even in the absence of epithelium), suggesting that Wnt-7a may be involved in this organizational process. We observe that loss of Wnt-7a leads to a marked disorganization of uterine smooth muscle, supporting a role for Wnt-7a in epithelial-mesenchymal signaling (Chapter 5 and [142]).

Wnt Genes and Circulating Levels of Steroid Hormones

The changes in the patterns of Wnt gene expression observed during the estrous cycle suggests that Wnt signaling may underlie the cellular changes that occur in the female reproductive tract in response to hormonal fluctuations. In the adult, Wnt-4 is always expressed in the vaginal epithelium but its expression in the uterine horn is dependent on the stage of the estrous cycle. Changes in Wnt-4 expression in the uterine epithelium correlates with the timing of maximal growth and proliferation (proestrus and oestrus) [144]. Wnt-4 is expressed solely in the uterine epithelium during proestrus when the levels of estrogen are highest. This suggests that estrogen may regulate Wnt-4 expression in the uterus.

Wnt-5a expression changes in both the uterus and the vagina during the estrous cycle. In the uterus, it is expressed in the stroma and the epithelium during diestrus and oestrus and in the stroma alone during proestrus and metestrus. The restriction of Wnt-5a from the uterine epithelium corresponds to the stages of the estrous cycle that have the highest levels of estrogen. It is possible that estrogen represses the expression of Wnt-5a in the uterine epithelium. This may occur through mesenchymal-epithelial signaling.

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Estrogen receptor activation in the stroma may be required for the activation of a paracrine signal that acts on the epithelium to in turn cause the down-regulation of Wnt-5a. Alternatively, activation of the estrogen receptor in the uterine epithelium itself may be sufficient to down-regulate Wnt-5a in the epithelium.

The levels of Wnt-7a expression in the uterus vary during the estrous cycle. Levels are highest during diestrus and metestrus, and lower during proestrus and oestrus. The highest levels of expression correspond to high levels of circulating progesterone, suggesting that progesterone may induce expression of Wnt-7a in the uterine epithelium. Alternatively, estrogen may repress the expression of Wnt-7a.

One other hormonally responsive tissue is the female mammary gland. Expression of Wnt-2, -4, -5a, -5b, -6, and -7b has been detected in this tissue [79, 80, 108]. Although expression of these Wnt genes in the mammary gland has not been determined during the estrous cycle, levels of expression were seen to change during pregnancy and lactation [79, 80]. Additionally, Wnt-2, -4 and -5b levels were found to be responsive to ovariectomy [80]. Wnt gene responsiveness to ovarian hormones in the mammary gland and the uterus adds support to the hypothesis that Wnt genes may underlie the morphological changes which occur in response to circulating hormone levels in these two tissues.

Wnt Genes are Candidate Signaling Molecules Underlying Mesenchymal-Epithelial Interactions in the Female Reproductive Tract

The changes in Wnt gene expression levels that coincide with changes in levels of hormones (this chapter and [41]), coupled with the dynamic expression patterns of the Wnt genes in the female reproductive tract suggests that they play key roles in the development and adult function of the reproductive tract. Wnts are secreted signaling molecules that have been linked to morphogenetic events. As such, the localization of Wnt gene expression in the female reproductive tract suggests that Wnts likely function in the

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mesenchymal-epithelial interactions which govern cytodifferentiation and morphogenesis in the female reproductive tract [22].

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Chapter 4 Establishment of a Retroviral Based System to Introduce Ectopic Wnt Gene Expression

In the previous chapter, we show that Wnt genes are expressed specifically in the mesenchyme or the epithelium of the developing female reproductive tract. Wnt-4 and Wnt-5a expression in the adult female reproductive are not as precise as they are during development. However, their expression patterns change from mesenchymal to epithelial in various combinations during the murine estrous cycle. These observations suggest that Wnt genes function in the mesenchymal-epithelial interactions which govern both the development and the adult function of the female reproductive tract. We therefore wanted to study the roles of the various Wnt genes in either the mesenchyme or the epithelium in a gain-of-function system. One difficulty with transgenic mice is the challenge to deliver an ectopic gene to an isolated tissue type. If a cell-type specific enhancer is not used in a transgene, the transgene could result in global effects that could indirectly influence the system being studied. At the time of these experiments, we did not have access to an enhancer in order to express an ectopic gene specifically in the mesenchyme or epithelium of the female reproductive tract. The isolation of such an element would have been time-consuming. One way to avoid this problem is to transfer an ectopic gene into the desired cell-type in an ex-vivo approach. We employed retroviral vectors to deliver genes specifically to the mesenchymal components of the newborn female reproductive tract in order to study the effects of ectopic Wnt gene expression.

Selection of the Retroviral Construct Used to Infect Primary Tissues

Several retroviral constructs were derived from a Murine Leukemia Virus (MLV) ecotropic retrovirus by Dr. Jan Kitajewski (Columbia). The constructs were used to transfect Bosc23 cells, and the resulting retroviral supernatants were used to infect primary cells. The constructs contained different promoters (SV40, MMTV, HIV, and CMV). We

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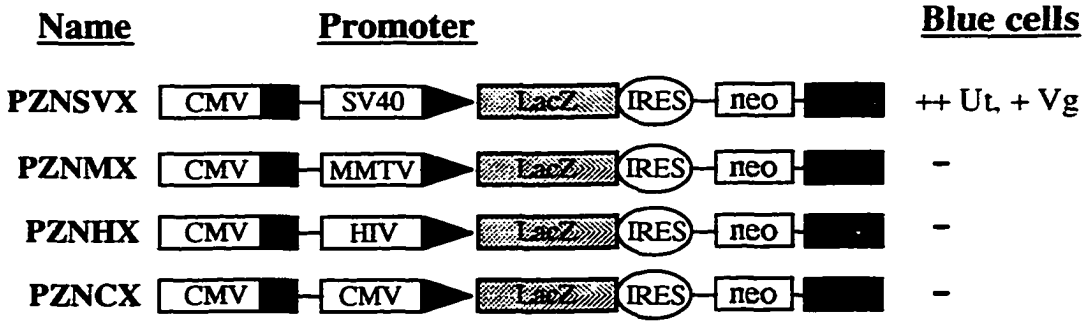

Figure 4.1 Retroviral vectors with various promoters were tested on dissociated uterine and vaginal mesenchymal cells. The dissociated cells were incubated with retroviral supernatant, and subsequently stained for LacZ activity. Only the cells incubated with PZNSVX had significant numbers of blue cells. Approximately 5% of the uterine cells stained blue, while only 1% of the vaginal cells stained blue. Only an occasional cell stained blue after exposure to the other retroviruses. All subsequent experiments were performed with the PZNSVX vector.

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needed to determine which of the promoters was most effective in driving gene expression in uterine and vaginal mesenchyme. Therefore, we tested each retroviral supernatant for its ability to infect primary uterine or vaginal mesenchymal cells in culture (see Figure 4.1). Primary cultures of uterine or vaginal mesenchymal cells were generated from newborn CD-1 female reproductive tracts. 24 hours after the cells were plated they were exposed to the various retroviral supernatants. Since retroviral infection should result in LacZ expression in the target cells, the primary cultures were stained for X-gal activity 2 days after infection. The only construct which infected uterine and vaginal mesenchymal primary cells contained an SV40 promoter (PZNSVX). This construct was used for all future studies. The retroviral constructs contained either a LacZ gene or the Wnt-1 gene. The SV40 promoter drives the transcription of a poly-cistronic message containing either LacZ or the Wnt gene along with a neomycin resistance (neo^r) gene. The internal ribosome entry site (IRES) allows for individual translation of both messages.

Retroviral Infection of Mesenchymal Cells is Effective for At Least 3 Weeks

Newborn uterine or vaginal mesenchyme was exposed to media containing LacZ carrying retroviruses for 20-24 hours at 37°C. The mesenchyme was then recombined with non-infected epithelium (see Figure 4.2). Recombinants were grown in vitro for 1-2 days after which they were either stained for X-gal activity, or grafted into host animals.

LacZ infected grafts were harvested after 3 weeks and were stained for X-gal activity (Figure 4.3). Prior to grafting into host animals, we observe blue cells in the recombinants that were exposed to the retrovirus (Figure 4.3B,D) while we observe no blue cells in the control grafts not exposed to the retrovirus (Figure 4.3A,C). Parallel recombinants were grafted into host mice and stained for X-gal activity after 3 weeks. Infected cells survived at least until this time, evidenced by the blue cells at these stages

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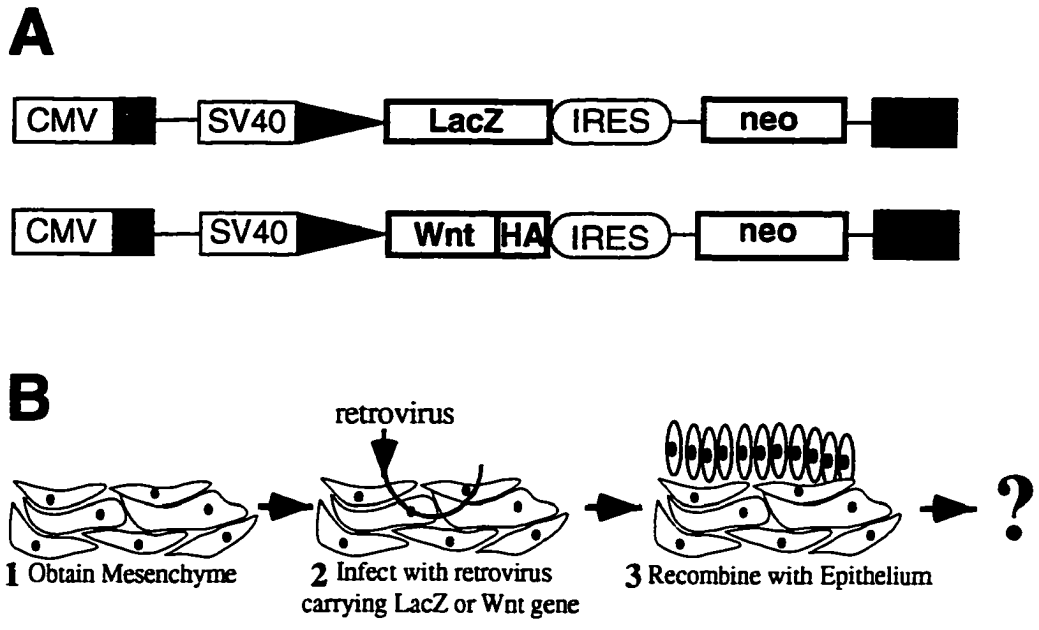

Figure 4.2 MLV derived retroviral constructs and a schema of the infection protocol

A. MLV derived retroviral constructs which were used to infect uterine or vaginal stromal cells and drive expression of LacZ or Wnt-1.

B. Schema of the retroviral treatment of vaginal or uterine stroma. Newborn uteri or vaginae were trypsinized, and the epithelium and stroma were separated manually. The stroma was incubated with media containing LacZ or Wnt carrying retrovirus for 20 hours. The stroma was then recombined with freshly obtained epithelium. The recombinants were allowed to re-adhere overnight on agar plates before they were surgically grafted underneath the kidney capsules of nude mice. After 3 weeks, the recombinants were removed for examination.

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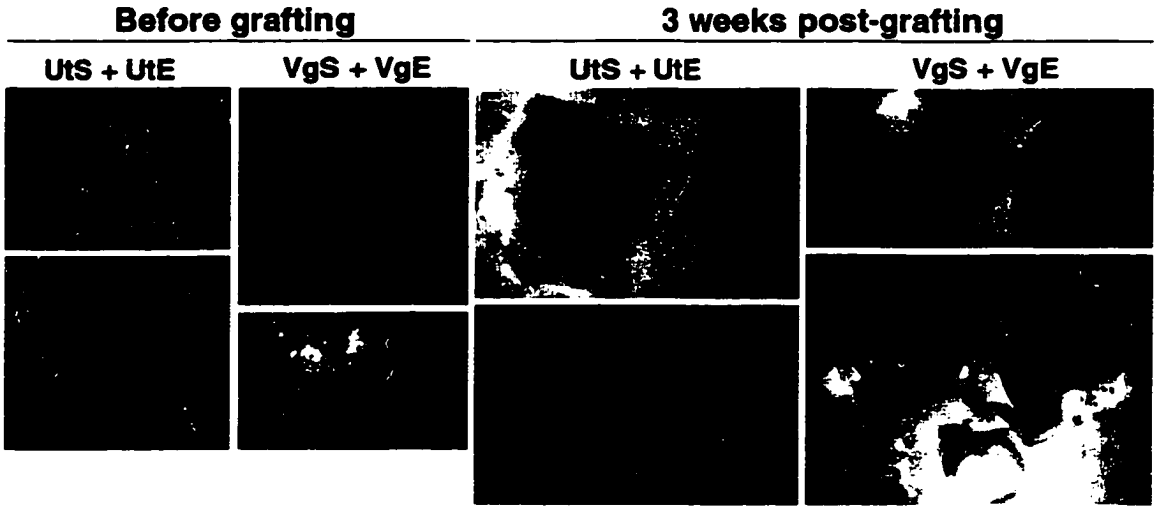

Figure 4.3 Retroviral infection of female reproductive tract mesenchyme is effective for at least 3 weeks in vivo. Uterine and vaginal mesenchymal tissues were infected with a LacZ carrying retrovirus and recombined with non-infected homotypic epithelium. The grafts were either stained for X-gal activity before grafting into a host, or after growing underneath the kidney capsule of a host mouse for 3 weeks. When the mesenchymal tissues were not incubated with the LacZ carrying retrovirus, no cells stained blue (A, C, E, G). When they had been incubated with the retrovirus (LacZ), blue cells were visible on all the grafts (B, D, F, H). Blue cells were not only apparent before grafting, they were also observed after growing in a host mouse for 3 weeks.

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(Figure 4.3F,H). No cells stained blue in control recombinants or grafts, indicating that the blue cells are specific for retroviral infection (Figure 4.3E,G).

Effects of Ectopic Wnt-1 Infection

In the mammary gland, limb bud, and kidney, ectopic Wnt-1 results in a hyper-proliferative response [76, 145, 146]. Wnt-1 results in the initiation of epithelial branching morphogenesis in both the kidney and the mammary gland [76, 146]. Wnt-1 is normally not expressed in these tissues or in the female reproductive tract. Since ectopically expressed Wnt-1 has been demonstrated to produce an obvious phenotype in at least three different systems, we chose to infect mesenchymal tissue with a Wnt-1 carrying retrovirus, believing that ectopic Wnt-1 expression would result in an observable change in morphology.

Uterine and vaginal mesenchymal fragments were infected by the Wnt-1 containing retrovirus using the schema shown in Figure 4.2. Recombinants prepared with non-infected epithelium were grafted into host mice. The grafts were examined after 3 weeks. The non-infected vaginal graft has the expected morphology. It has a layer of stroma, and has stratified epithelium that shows evidence of responding to the estrous cycle as demonstrated by the alternating layers of mucified and cornified cells in the lumen (Figure 4.4A). While the Wnt-1 infected grafts do not show any gross alterations in tissue morphology (Figure 4.4B,C), there are some subtle differences. The grafts shown in B, and C came from the same animal. The epithelium is noticeably different between the two grafts. Additionally, while cells have been shed into the lumen, we do not observe the layers characteristically seen in the vaginal grafts. In fact, the graft in Figure 4.4B appears to be shedding nucleated squamous epithelial cells into its lumen. A decrease in superficial cell differentiation, and an increase in the number of nucleated cells in Pap smears are hallmarks of cervical dysplasia in humans [146]. These results suggest that ectopic Wnt-1 expression in the vaginal mesenchyme is de-regulating the ability to respond to steroid

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hormones changes during the estrous cycle, and may also be inducing a pre-cancerous state in the infected tissues.

Non-infected uterine grafts have the expected tissue morphology. They have a simple columnar epithelium, a thin layer of stroma, and a layer of smooth muscle (Figure 4.4D). At 3 weeks, we observe a change in morphology in the Wnt-1 infected uterine grafts (Figure 4.4E,F). The Wnt-1 infected uterine grafts display significant and excessive gland formation (Figure 4.4E,F). The uterine Wnt-1 infected grafts also contain a region of stratified epithelium, which is normally not observed in uterine grafts (higher magnification, Figure 4.4F). Uterine grafts do not form extensive numbers of glands unless grown in a pregnant host. It has been assumed that an increase in the levels of circulating steroid hormones is required for the formation of glands in normal uterine grafts. Wnt-1 expression therefore seems to mimic the effects of steroid hormones in the infected grafts, suggesting that ectopic Wnt-1 expression allows the hormonal regulation of gland formation to be bypassed.

Wnt-1 expression was examined in the Wnt-1 infected uterine and vaginal grafts by in situ hybridization. Curiously, we were not able to detect Wnt-1, nor the neo^r gene which is present on the dicistronic Wnt-1 transcript. One possibility is that Wnt-1 expression is not compatible with long-term cell survival. In this case, infected cells express Wnt-1 and cause the observed changes in morphology, which are subsequently irreversible. This is similar to the situation in Wnt-1 transgenic mice in which cells in the limb bud ectopically expressing Wnt-1 during limb development are no longer present in the limb at birth [147]. These cells are initially hyper-proliferative but they do not appear able to undergo the mesenchymal condensation required for survival. The resultant limb morphology is stunted, clearly due to the effect of Wnt-1, even though no Wnt-1 expression is detectable by birth

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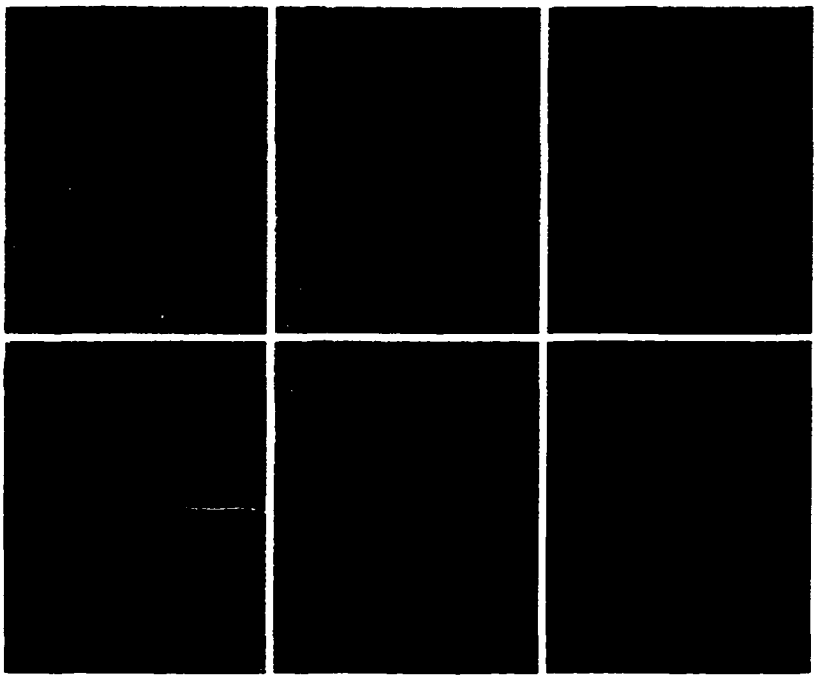

Figure 4.4 Wnt-1 infection of vaginal or uterine mesenchyme directs cytodifferentiation. Uterine or vaginal mesenchyme were infected with a Wnt-1 carrying retrovirus and recombined with non-infected homotypic epithelium. The grafts were grown underneath the kidney capsule of an athymic nude host mouse for 3 weeks. Representative hematoxylin and eosin stained sections of the various grafts are shown. The epithelium is denoted by the double arrows. Non-infected vaginal recombinants have stratified epithelium which is responsive to estrous cycle hormones (A). Alternating layers of cornified and mucified cells fill the lumen of the graft. Two Wnt-1 infected grafts from the same host animal are shown in B, and C. They clearly have different epithelia: B is mucified while C is cornified. Many of the cells in the lumen of B are nucleated, and there is an absence of alternating corny and mucous layers. Non-infected uterine recombinants have simple columnar epithelium (see inset), a layer of stroma, and a layer of smooth muscle surrounding the graft (D). There are no apparent glands. F is a higher magnification photomicro-graph of a region in E. The Wnt-1 infected uterine grafts display an abundance of glands (E, F). Although some of the epithelium is simple columnar, there is also a region of abnormal stratified epithelium visible (F). Abbreviations: VgS (vaginal stroma), VgE (vaginal epithelium), UtS (uterine stroma), UtE (uterine epithelium), c (cornified cells), m (mucified cells), epi (epithelium), and gl (endometrial glands). Scale bar, 50 μm (inset, F), 200 μm (A-E).

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Wnt Gene Expression re-Directs Cytodifferentiation in the Female Reproductive Tract

We conclude that ectopic Wnt gene expression (Wnt-1) can direct cytodifferentiation in the uterus and the vagina. In both cases, Wnt-1 expression in the mesenchyme results in a noticeable change in epithelial morphology, suggesting that the endogenously expressed Wnt genes may also signal from the uterine or vaginal mesenchyme to the epithelium. Additionally, Wnt-1 expression appears to supersede the regulation of mesenchymal-epithelial interactions dictated by steroid hormones in the reproductive tract. This is consistent with ectopic expression of Wnt-1 in the mammary gland, another hormonally responsive tissue whose development involves mesenchymal-epithelial interactions. These results reveal that Wnt gene can play a morphogenetic role for the Wnt family members in the development of the female reproductive tract.

The results of the ectopic Wnt-1 expression in the uterine and vaginal mesenchyme were exciting. However, at the same time I was performing the gain-of-function experiments, I was also investigating a loss of function situation in the Wnt-7a mutant mice. Loss of Wnt-7a in the female reproductive tract affected multiple morphogenetic events, and allowed us to determine the roles of at least one Wnt gene in its native setting. Although gain-of-function and loss-of-function studies can often be complementary, we chose to only pursue investigation of the Wnt-7a mutant mouse.

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Chapter 5 Wnt-7a Maintains Appropriate Patterning during the Development of the Mouse Female Reproductive Tract

The work in this chapter is based on the paper titled "Wnt-7a maintains appropriate patterning during the development of the mouse female reproductive tract" by Cary Miller and David Sassoon (Development, 1998, 125, pp. 3201-3211). Brian Parr and Andrew McMahon (Harvard) generated the Wnt-7a mutant mice and generously donated them to our laboratory. I performed all of the described experiments, and all the figures are based on my work.

Introduction

Three members of the Wnt family of signaling molecules are expressed in a dynamic pattern in the mouse female reproductive tract (Chapter 3 and [139]). Given these dynamic expression patterns, and the evidence that Wnt-1 can induce cytodifferentiation, the functions of Wnt-7a in the reproductive tract were investigated. Loss of Wnt-7a expression results in a partial posteriorization of the female reproductive tract at gross, cellular, and molecular levels. Specifically, the oviduct acquires characteristics of the uterus and the uterus acquires characteristics of the vagina, although both compartments also retain some characteristics of their own identity. The incomplete shift in the oviduct and the uterus may be due to a post-natal decline in the correct anterio-posterior expression of *Hox*a-10 and -11. Thus Wnt-7a is required to maintain but not induce *Hox*a-10 and -11 expression. Additionally, uterine development along the radial (luminal-adluminal) axis is altered. Aside from lacking uterine glands, we note that the smooth muscle layers in the mutant uterus are overgrown and poorly organized. Although the Müllerian ducts are essentially normal in overall morphology at birth in the mutant mice, marked differences in Wnt-5a and Wnt-4 expression can already be observed. We propose a mechanism

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whereby Wnt-7a acts to regulate the boundaries of expression of Wnt-5a and Wnt-4, which act in concert to establish the correct developmental axes of the uterus.

RESULTS

Wnt-7a Is Expressed in the Developing and Adult Female Reproductive Tracts

The expression pattern of Wnt-7a is dynamic during the development of the female reproductive tract (Fig. 5.1 and [139]). Some of the panels were also shown in Figure 3.4. Wnt-7a is expressed throughout the epithelium of the prenatal Müllerian tract (Fig. 5.1A,B) but becomes restricted to oviduct and uterine luminal epithelium after birth (Fig. 5.1C,D), and in the adult (Fig. 5.1E,F). It is not expressed in glandular epithelium in the adult uterus (Fig. 5.1E,F), or in the epithelium of the adult vagina (Fig. 5.1G,H).

(I) Gross and Cellular Morphology

Wnt-7a Mutant Female Reproductive Tracts Are Posteriorized

Previous studies revealed that Wnt-7a mutant female mice are sterile, however, the underlying causes were not examined [82]. Analysis of Wnt-7a mutant and heterozygous female reproductive tracts was undertaken at the gross morphological, cellular and molecular levels. Wnt-7a mutant uteri are smaller and thinner in diameter than wild-type or heterozygote counterparts at the same age (Fig. 5.2A-C). The oviducts in the mutant mouse are either reduced or absent, and there is a variable amount of oviduct coiling (out of 10 mice, 8 had no evident oviducts and 2 had loose oviduct-like coils on one uterine horn). The cell morphology of the wild-type and heterozygote oviducts are indistinguishable (Fig. 5.2D,E). The malformations in the mutant oviduct are accompanied by alterations in cell morphology. The cytoarchitecture of the mutant oviduct shows a high degree of variation: it can resemble uterine horn (Fig. 5.2F) or appear similar to normal oviduct and contain raised epithelial folds characteristic of this tissue (Fig. 5.2G).

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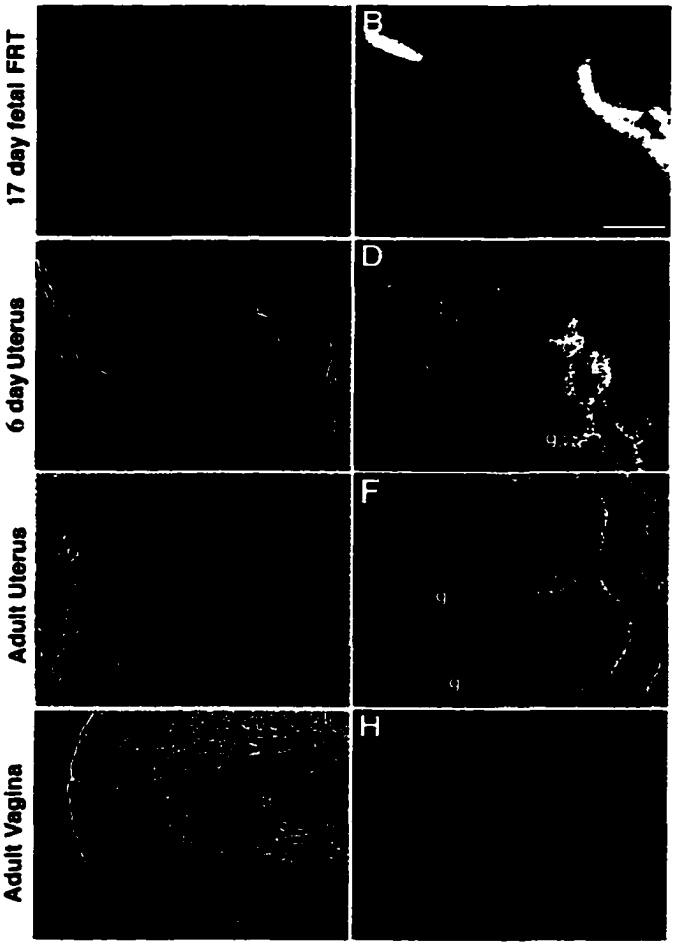

Fig. 5.1 Wnt-7a is expressed dynamically in the developing and adult female reproductive tract. The panels show the respective bright- and dark-field images of sections hybridized with a probe corresponding to Wnt-7a. (A) and (B) show longitudinal sections of a 17 d.p.c. female reproductive tract. Wnt-7a is expressed in the simple columnar epithelium of the Müllerian tract in both the future vagina (small arrow) and future uterine horns (ut; arrowhead). (C) and (D) show sections through a 6 day old anterior uterine horn (ut) and oviduct (ovi). Transcripts are detected in the epithelium of the uterine horn, including the forming glands (g) and in the epithelium of the oviduct. (E) and (F) show a section through an adult uterine horn during metestrus. Indicated are uterine glands (g), the simple columnar epithelium (double arrows), and the smooth muscle-stroma boundary (line). Wnt-7a is detected in the luminal epithelium of the uterine horns but not in the uterine glands. (G) and (H) show a section through an adult vagina. Wnt-7a is not detected in the vaginal epithelium (double arrows). Scale bar, 200 μm.

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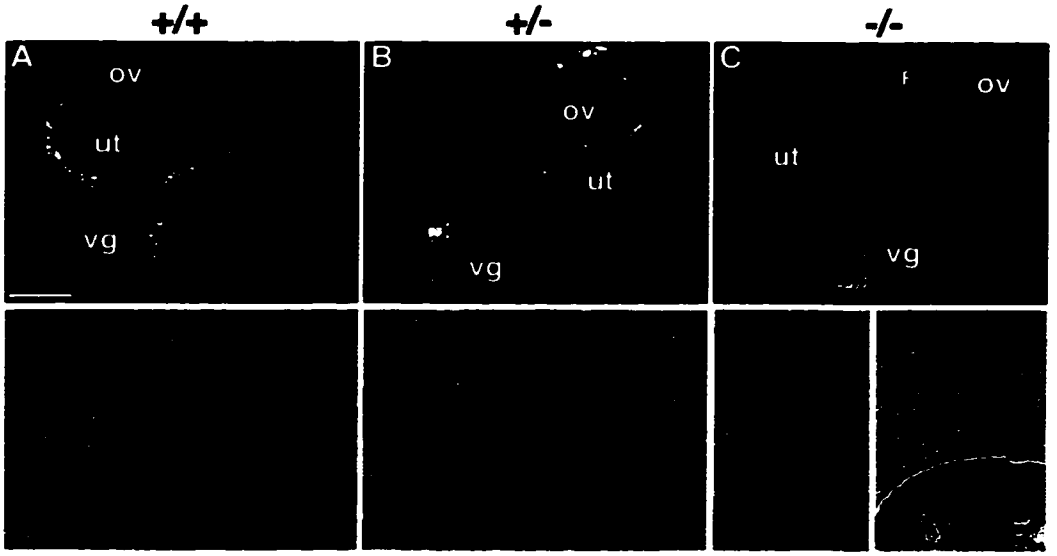

Fig. 5.2 The Wnt-7a mutant female reproductive tract shows changes in morphology. (A), (B), and (C) show adult wild-type, Wnt-7a heterozygous and mutant female reproductive tracts, respectively, during the proestrus stage of the estrous cycle. There are no obvious differences in morphology between the wild-type and heterozygous reproductive tracts. Note that the mutant reproductive tract is shorter than the wild-type tract and the mutant uterine horns (ut) are much thinner in diameter than the wild-type or heterozygous uteri. We note some variability in the degree of oviduct coiling between samples. The appearance and size of the ovary (ov) is normal. (D-G) are photomicro-graphs of hematoxylin and eosin stained sections through the oviduct regions of the respective mice. (D), and (E) show, respectively, the oviducts of the wild-type and heterozygous mice. The histology is indistinguishable. They contain highly convoluted epithelial folds, very little underlying connective tissue, and the loops are surrounded by a thin layer of smooth muscle. (F) shows a section through the anterior uterine horn of the reproductive tract from (C) as marked by the dashed line. The cytoarchitecture of this region is reminiscent of normal uterus and does not resemble the oviducts seen in (D) and (E). The epithelium is not highly convoluted, and there is a thick layer of underlying stroma and smooth muscle. Additionally, we observe numerous glands in the mutant oviduct while no glands are apparent in the wild-type or heterozygous oviducts. We observe significant variability in the degree of oviduct coiling and cell morphology in the mutant. (G) shows a mutant oviduct which has cytoarchitecture similar to that seen in the wild-type oviduct. Scale bar, 4 mm (A-C), 200 μm (D-G).

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We examined whether the differences in the mutant uterus are accompanied by alterations in cell morphology. Uterine horns, oviducts and cervix all derive from the Müllerian tract. The mesenchyme of the Müllerian tract differentiates into peripherally located smooth muscle cells, and an inner layer of stromal cells which is lined by epithelium [20]. Müllerian epithelium differentiates into both luminal and glandular epithelia post-natally. The epithelia of the oviduct, uterus and vagina are histologically distinct in normal female reproductive tracts. Wild-type uterine horn has simple columnar luminal epithelium, stroma containing endometrial glands, and two distinct layers of smooth muscle (Fig. 5.3A and inset). We readily detect perturbations in Wnt-7a heterozygous uterine cytoarchitecture implying a gene dosage effect, although no obvious change in fertility success is noted in these females. We note an excess of uterine glands in heterozygote uteri (Fig. 5.3B), which increases in severity as the animals age.

There are a number of differences between the wild-type and the Wnt-7a mutant uterine horns. The appearance of the mutant uterus combines features of the uterus and vagina. Normal vagina has stratified or multilayered epithelium, thin stroma and a layer of disorganized smooth muscle bundles (Fig. 5.3D). The mutant uterus has a multilayered epithelium, a relatively thin stroma, and no glands, but it does have two layers of smooth muscle like the wild-type uterus (Fig. 5.3C).

The diameter of the mutant uterine horns is generally smaller than that of wild-type or heterozygous uterine horns. Relative to the diameter of the uterus, the longitudinal and circular smooth muscle layers which surround the uterus are much thicker in the mutant uterus than in the wild-type uterus (Fig 5.3A,C). In order to verify the identity of the smooth muscle cell bundles and to define more clearly the location of smooth muscle cells, we performed in situ hybridization using a riboprobe corresponding to smooth muscle myosin heavy chain [SMMHC; (Fig. 5.4)]. We detect two layers of smooth muscle in both the wild-type and mutant uteri (Fig. 5.4A-D), however, there are changes in the distribution of smooth muscle in the mutant uterus. The inner circular layer of smooth

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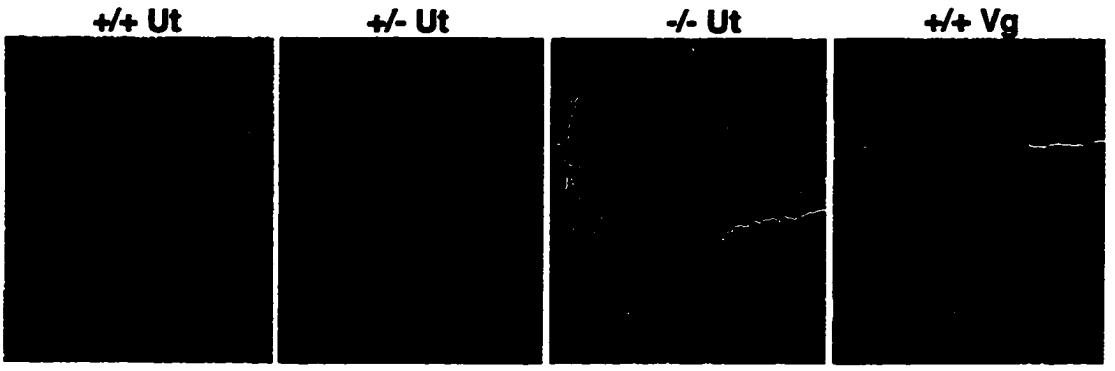

Fig. 5.3 Wnt-7a mutant uterine morphology acquires an intermediate cellular phenotype. (A) shows a cross-section through adult wild-type uterine horn showing 2 layers of smooth muscle (m), a thick stroma (s) populated by glands (g), and lined by simple columnar epithelium (double arrows). Inset is a high magnification of the luminal and glandular epithelium showing that the luminal epithelium is simple columnar. (B) depicts a section through a heterozygous uterine horn. The tissue cytoarchitecture is identical to that of the wild-type animal except there is an excess of uterine glands (g). (C) shows a cross-section through mutant uterine horn. Note that smooth muscle (m) is thicker in the mutant than in the wild-type uterus. The inner layer of smooth muscle is not only thicker, but has become highly disorganized and irregular (dm). In contrast to the wild-type uterus, there is not a sharp boundary between the stroma and smooth muscle. The thickness of the stroma (s) is greatly reduced in the mutant and is not populated by any discernible glands. The epithelium of the uterine horn is clearly stratified (double arrows). The inset shows that the epithelium consists of 6-7 layers of cells versus the 1 layer of cells seen in the wild-type uterus. (D) shows a transverse section through adult vagina. The stratified, keratinized epithelium is denoted by double arrows.
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muscle, which is normally compact, is composed of irregular and disorganized bundles in the mutant uterus (Fig. 5.4B,D). These bundles resemble those seen in the wild-type vagina (Fig. 5.4E,F). The distribution of smooth muscle cells in the heterozygous animals and in the mutant vagina is indistinguishable from wild-type mice (data not shown).

The Wnt-7a Mutant Female Reproductive Tract Phenotype Is Not Due to Extrinsic Factors

Since Wnt-7a is expressed in many tissues other than the uterus [77, 148], it could be argued that systemic changes in the mutant mouse, such as the levels of circulating steroid hormones, are responsible for the observed phenotypic differences. To distinguish between local and systemic effects, intact neonatal Wnt-7a mutant uterine horns were grafted under the kidney capsules of female athymic nude mice to assess development in an otherwise normal host environment [136]. The grafts were allowed to grow for 3 weeks in a non-pregnant or pregnant host. Gland formation in the wild-type uterus is only observed in grafts grown in a pregnant host. The phenotype seen in the mutant female reproductive tract is recapitulated in the mutant tissue grafts (Fig. 5.5C,G). We observe multilayered epithelium, little stroma, and an increased amount of smooth muscle in the mutant grafts (Fig. 5.5C,D,G,H). The smooth muscle is located much closer to the epithelium in the mutant grafts than in the control grafts (Fig. 5.5H vs. 5.5F). The smooth muscle surrounds the epithelium, and little or no stroma remains (Fig. 5.5H). We detect the formation of uterine glands (g) in the control graft from the pregnant host (Fig. 5.5E) whereas no glands are observed in the mutant tissue grafts from the same host (Fig. 5.5G).

Wnt-7a Regulates Uterine Smooth Muscle Development

The observation that Wnt-7a expression is restricted to the epithelium of the developing and adult uterus suggests that it participates in mesenchymal-epithelial interactions that guide post-natal development. Specifically, the phenotype of the Wnt-7a

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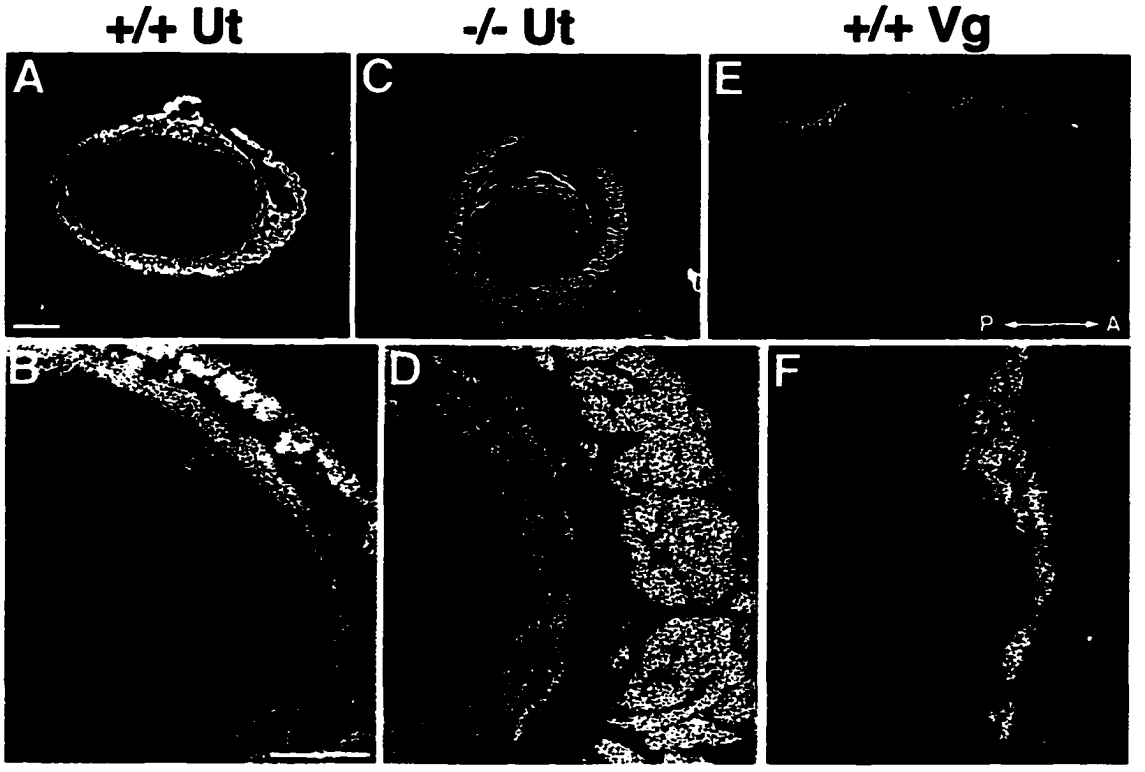

Fig. 5.4 The Wnt-7a mutant uterus has irregular bundles of smooth muscle. The panels show either wild-type uterus (A,B), mutant uterus (C,D) or wild-type vagina (E,F) hybridized with a cRNA probe corresponding to SMMHC. We detect 2 layers of smooth muscle in the wild-type uterine horn (A,B). Two layers of smooth muscle are also discernible in the mutant uterine horn, but the inner layer of smooth muscle has formed into irregular bundles interspersed by stromal cells (C,D). This contrasts with the compact circular layer of smooth muscle observed in the wild-type mouse. SMMHC transcripts are detected in irregular bundles on the periphery of the vagina. Note the similarity in appearance to the inner layer of smooth muscle bundles in the mutant uterine horn (E). The anterior-posterior axis is indicated.
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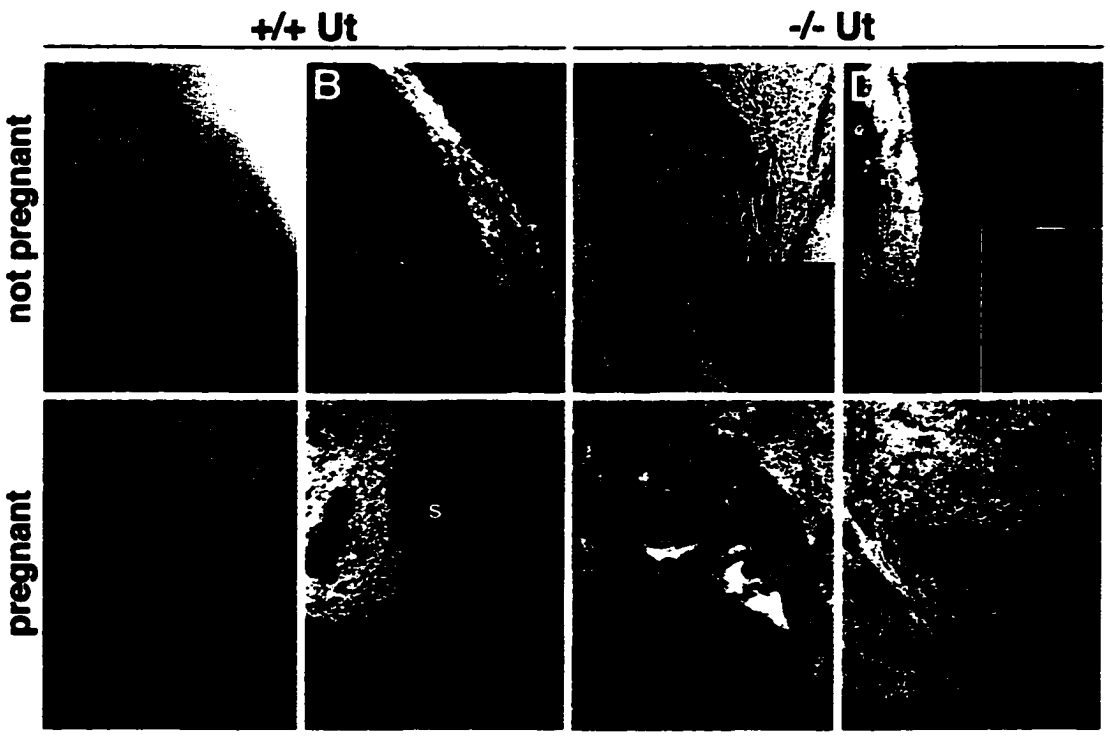

Fig. 5.5 Loss of Wnt-7a in the uterus is responsible for the mutant phenotype. Intact neonatal uterine horns from wild-type and Wnt-7a mutant mice were grafted underneath the kidney capsules of a pregnant or non-pregnant host for 3 weeks. Glands are only observed in the tissues grown in pregnant hosts. Control and mutant grafts, along with host reproductive tracts, were analyzed. H&E stained sections or nearby sections hybridized for SMMHC are shown. Wild-type tissue from non-pregnant hosts contains simple columnar epithelium, a layer of stroma and a layer of smooth muscle (A). The smooth muscle is not adjacent to the epithelium (B). Mutant tissues contain epithelium which varies from simple cuboidal to stratified, an abundance of smooth muscle (m), and little stroma (C). The smooth muscle is immediately adjacent to the epithelium (C,D). We observe the forma-tion of glands (g) in the wild-type tissue grown in a pregnant host (E). The tissue has simple columnar epithelium surrounded by a thick layer of stroma (s), and smooth muscle (E,F). The mutant graft from the pregnant host is comprised almost entirely of smooth muscle (G,H). There are only smooth muscle cells detectable adjacent to the epithelium. The epithelium is multilayered, disorganized and clearly abnormal (see inset), and no glands are detected. Scale bar, 200 μm (A-G), and 50 μm (insets).

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mutant uterus suggests Wnt-7a is secreted by the epithelium and provides a signal that maintains stromal-smooth muscle boundaries. The excess of smooth muscle in the Wnt-7a mutant mouse suggests Wnt-7a acts in the formation of smooth muscle. These properties were tested using tissue heterografts between Wnt-7a mutant and wild-type tissues. Normal recombinants (UtS + UtE, Fig. 6A) have the same characteristic structure as recombinants prepared with mutant mesenchyme (-/- UtS + UtE, Fig. 5.6C). They have an outer layer of smooth muscle, a distinct layer of stroma, and a simple columnar epithelium. In situ hybridization with the SMMHC probe on nearby sections show the presence of a stromal layer between the epithelium and the smooth muscle (Fig. 5.6B,D). Recombinants prepared with mutant epithelium mimic the mutant phenotype. They have an excess of smooth muscle, little stroma, and stratified epithelium (Fig. 5.6E). Additionally, the sharp boundary between stromal cells and smooth muscle cells easily noted in the control grafts is not apparent in the grafts containing mutant epithelium (see insets Fig. 5.6A,E). Smooth muscle cells are not separated from the epithelium by a distinguishable layer of stroma (Fig. 5.6F). Therefore, loss of Wnt-7a from the uterine epithelium is sufficient to account for changes in the uterine mesenchyme which differentiates into smooth muscle and stroma.

(II) Analysis of Gene Expression

Changes in Wnt Gene Expression Precede the Appearance of Morphological Perturbations

The expression patterns of Wnt-4, and -5a were examined in developing and adult wild-type, Wnt-7a heterozygous, and mutant female reproductive tracts by in situ hybridization. Prior to the emergence of overt phenotypic differences between mutant and wild-type female reproductive tracts (<5 days postnatally), differences already exist in the patterns of gene expression. In the wild-type neonate uterine horn, Wnt-5a is expressed primarily within the uterine mesenchyme (Fig. 5.7A). In the adult uterus, Wnt-5a

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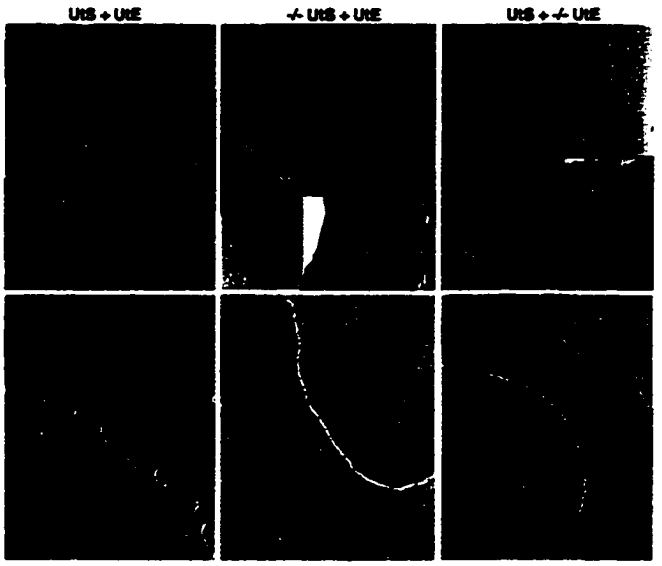

Fig. 5.6 Loss of Wnt-7a expression in the uterine epithelium is directly responsible for the phenotype observed in the Wnt-7a mutant mice. Recombinants between wild-type and mutant tissues are shown. The recombinants were grown under the kidney capsules of nude mice for 3 weeks. (B), (D), and (F) show neighboring sections to (A), (C), and (E) which were hybridized with a probe corresponding to SMMHC. The photomicrographs are composites of the phase images and dark-field silver grains (green) to allow for direct comparison of tissue identity and morphology. The epithelia in (B), (D), and (F) are denoted by the double arrows. (A) shows a recombinant composed of wild-type uterine stroma and epithelium. It contains simple columnar epithelium, a distinct layer of stroma (s), and a layer of smooth muscle (m). (B) shows that the smooth muscle in this graft is separated from the epithelium by an intervening layer of stroma (s). (C) shows the recombination of mutant uterine stroma (-/-UtS) and wild-type uterine epithelium. The high magnification inset shows that it has normal cytoarchitecture. The graft is surrounded by connective tissue (ct), presumably derived from the uterine mesothelium. (D) shows that a layer of stroma separates the epithelium and smooth muscle. (E) shows a recombinant composed of wild-type uterine stroma and mutant uterine epithelium (-/- UtE). The grafts contain excessive smooth muscle, little stroma (s), and stratified epithelium (double arrows). These features are shown at a higher magnification in the inset. (F) shows that there is no distinct layer of stroma separating the epithelium and smooth muscle; SMMHC is expressed in the tissue directly adjacent to the epithelium. Scale bar, 200 μm (A,C,E), 100 μm (B,D,F) and 50 μm (insets).

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expression is regulated by the estrous cycle [139] but its primary site of expression is the stroma (Fig. 5.7B). In the newborn Wnt-7a heterozygous and mutant uteri, Wnt-5a expression has shifted so that it is detected in both uterine mesenchyme and epithelium (Fig. 5.7C,E). During post-natal development, Wnt-5a expression is maintained in both the epithelium and stroma of the heterozygote uterine horn (Fig. 5.7D). Wnt-5a expression declines and becomes undetectable by 12-16 weeks in the mutant stroma (Fig. 5.7F). In addition, expression of Msx1, a marker of correct uterine cytodifferentiation, is not detectable in the epithelium of the adult Wnt-7a mutant uterus (data not shown).

Wnt-4 is normally expressed solely in the stroma of the neonatal wild-type uterus (Fig. 5.7G). Wnt-4 undergoes a dynamic pattern of expression in the uterus during the estrous cycle [139], thus we confined our study here to proestrus when expression is detected both within the stroma and epithelium (Fig. 5.7H). We note abnormal epithelial expression of Wnt-4 in the heterozygote and mutant uteri, both at birth and in the adult. At birth, Wnt-4 is expressed in both uterine mesenchyme and epithelium of the heterozygous and mutant animals (Fig. 5.7I,K). We observe little or no stromal expression of Wnt-4 in the adult heterozygote or mutant uterus at any stage of the estrous cycle (Fig. 5.7J,L). However, in contrast to wild-type mice, Wnt-4 is consistently expressed within the uterine epithelium regardless of the stage of the estrous cycle (Fig. 5.7J,L). Wnt-4 is expressed normally in the vaginal epithelium of both the mutant and heterozygote animals, and is expressed in the stroma of the mutant oviduct (data not shown).

Wnt-7a Maintains Expression of Hoxa Genes in the Uterine Horn

The observation that the changes in the mutant uterine radial axis resemble posterior homeotic transformations in the female reproductive tract led us to examine the expression of molecules previously implicated in anterio-posterior patterning: Hoxa-10 and -11. Hoxa-10 and -11 have similar patterns of expression in the neonatal uterine stroma (Fig. 5.8A, and [126]). Stromal expression is maintained throughout adult life (Fig. 5.8B).

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Fig. 5.7 Wnt-7a expression in the uterus sets up boundaries of expression for other Wnt genes and is important for maintenance of uterine genes. Neighboring sections of wild-type, heterozygous, and mutant uteri were hybridized for Wnt-5a and Wnt-4. The photomicrographs are composites of the phase images and dark-field silver grains (red) to allow for direct comparison of tissue and signal. The luminal epithelium is denoted by double arrows. (A-F) show sections hybridized for Wnt-5a. A neonatal wild-type uterus hybridized for Wnt-5a contains transcripts primarily in the mesenchyme (A). (B) A section of adult uterus has transcripts detectable primarily in the stroma with little expression in the epithelium or the smooth muscle layers (m). (C) A section of a neonatal heterozygous uterus has labeling seen both in the mesenchyme and in the epithelium, although little expression is detected in the forming smooth muscle (m). (D) shows Wnt-5a expression in adult heterozygous uterus with signal in the uterine stroma, and the luminal and glandular epithelium (g). Neonatal Wnt-7a mutant uteri have the same pattern of expression as the heterozygote: transcripts are detectable in both the mesenchyme and the epithelium of the uterine horn (E). In the adult mutant uterus, there is no detectable expression of Wnt-5a in the stroma (s) or the epithelium (F). (G-L) show sections hybridized for Wnt-4. Neonatal wild-type uteri show Wnt-4 transcripts which are restricted to the mesenchyme of the uterine horn (G). (H) An adult wild-type uterus during proestrus has labeling in both the epithelium and the stroma adjacent to the epithelium (s). Neonatal heterozygote uterus shows transcripts primarily in the mesenchyme but also in the epithelium at low levels (I). (J) An adult heterozygous uterus hybridized for Wnt-4 shows expression primarily in the uterine luminal epithelium. Null neonatal uterine horn shows Wnt-4 transcripts in the mesenchyme and the epithelium (K). The adult mutant uterus contains Wnt-4 transcripts only within the epithelium of the uterine horn (L). No detectable labeling is observed in the uterine stroma (s). (M) shows a schematic representation of the changes in Wnt gene expression in the heterozygote and mutant uteri compared to the wild-type uterus at both neonatal and adult timepoints. Scale bar, 100 μm (A,C,E,F,G,I,K,L), 200 μm (B,D,H,J).

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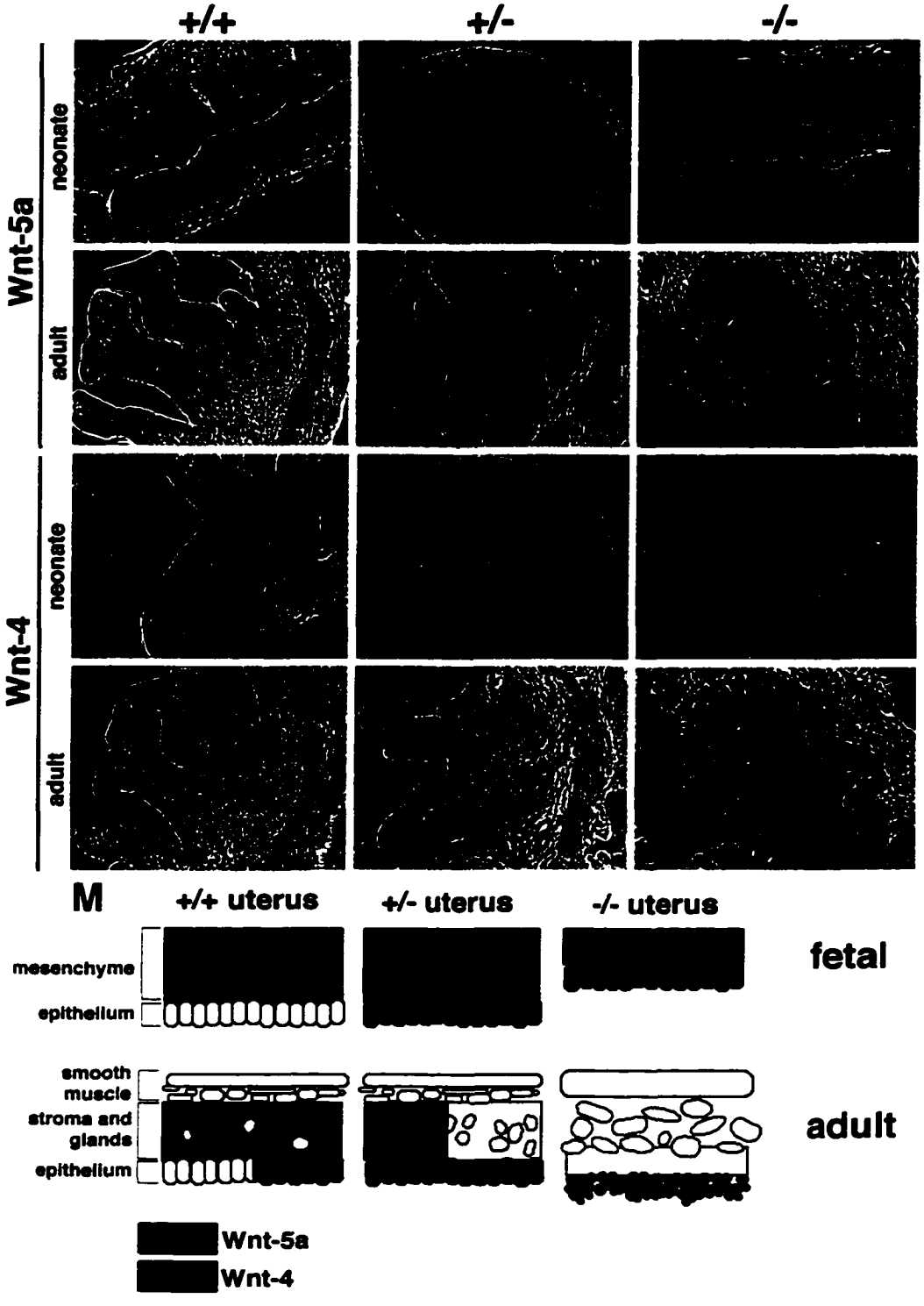

Fig. 5.7 Wnt-7a expression in the uterus sets up boundaries of expression for other Wnt genes and is important for maintenance of uterine genes.

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In the Wnt-7a mutant uterus, Hoxa-11 is expressed in the neonate stroma (Fig. 5.8C), but expression becomes undetectable as the animals age and the changes in uterine cytoarchitecture become apparent (Fig. 5.8D). Finally, the loss of Hoxa-10 and -11 expression precedes the loss of other uterine specific genes (Wnt-5a and Msx1).

Discussion

Wnt-7a Guides The Development of the Anterio-Posterior Axis in the Female Reproductive Tract

We report here that loss of Wnt-7a activity results in posteriorization of the reproductive tract at gross, cellular, and molecular levels. Evidence for posteriorization includes the lack of a discernible oviduct and changes in the uterine horn cytoarchitecture and gene expression patterns. Wnt-7a signaling in the uterus may act through a cascade which includes Wnt-5a (see Fig. 5.9). The adult mutant uterus exhibits a loss of Hoxa-10, and -11 gene expression, coupled with the appearance of stratified epithelium, and disorganized smooth muscle which are features typical of the vagina. Hoxa-10 and -11 have been implicated in anterio-posterior patterning in the female reproductive tract [31, 127]. The loss of Hoxa-10, and -11 expression from the stroma of the mutant uterus precedes the loss of Wnt-5a. The inability to maintain expression of uterine specific Hox genes may account for the intermediate appearance of the Wnt-7a mutant uterine horn. We propose that perinatal expression of Hoxa-10 and -11 help establish segmentation and anterior-posterior patterning. Thus the mutant female reproductive tract is compartmentalized along the anterio-posterior axis to a degree, and the uterine horns have some uterine characteristics. The postnatal loss of the uterine specific gene expression mimics the normal expression pattern in the vagina. This is accompanied by the development of features in the mutant uterus which are similar to the vagina. These results suggest that Wnt-7a directly or indirectly maintains the expression of uterine specific Hox

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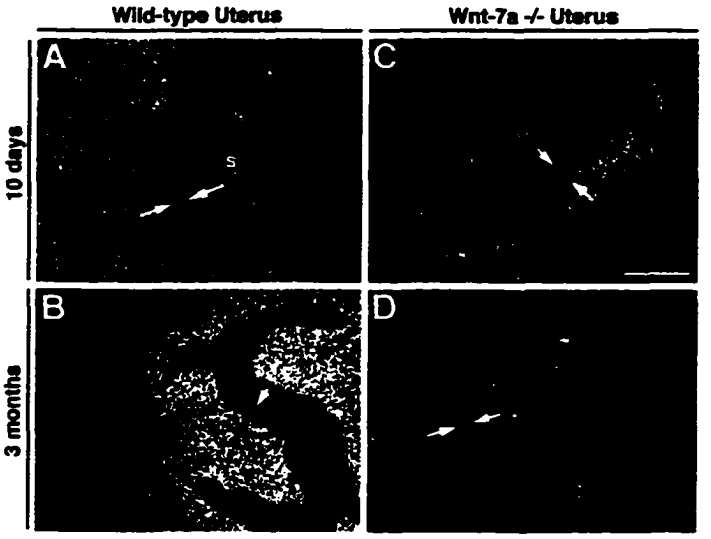

E

	Wild-type		Wnt-7a -/-		
	Early	Late	Early	Middle	Late
Wnt-5a	+	+	+	+	-
Wnt-7a	+	+	-	-	-
Hoxa-10	+	+	+	-	-
Hoxa-11	+	+	+	-	-

Fig. 5.8 Wnt-7a maintains the expression of uterine specific Hoxa genes. Dark-field sections of wild-type and Wnt-7a mutant uteri hybridized for Hoxa-11 are shown. The epithelium is denoted by the double arrows. In the presence of Wnt-7a, Hoxa-11 is expressed in the stroma both during neonatal uterine development (A) and during adult life (B). In the Wnt-7a mutant uterus, although we initially observe expression of Hoxa-11 in the stroma (C), we lose expression in the adult uterus (D). The expression of Wnt and Hoxa genes in the wild-type and Wnt-7a mutant uterus is summarized in (E). Initially in the mutant uterus, we observe expression of Wnt-5a, as well as Hoxa -10 and -11. We note the loss of Hoxa-10 and -11 from the uterine stroma (5-12 weeks) prior to noting changes in cell morphology. Additionally we note that loss of Wnt-5a expression in the mutant uterus follows the loss of the Hoxa genes (12-16 weeks). Scale bar, 100 μm (A,C), 200 μm (B,D).

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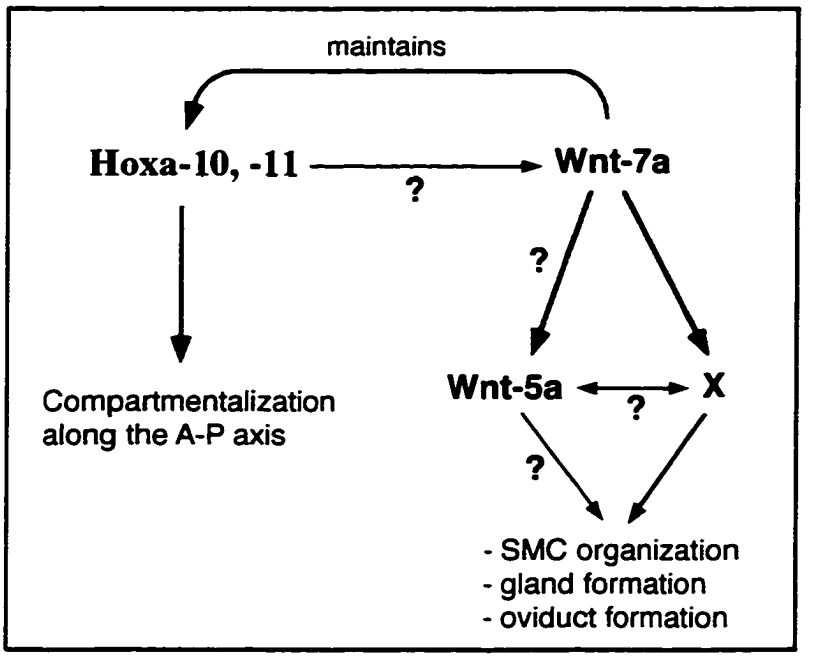

Fig. 5.9 Model showing interactions between Wnt-7a and Hoxa genes. Wnt-7a expression is required for gland formation, proper oviduct morphology, and smooth muscle organization. Wnt-7a expression is required to maintain expression of Hoxa-10 and -11 (this paper). Hoxa-10 and -11 expression have been implicated in the anterio-posterior segmentation of the FRT [31, 127] . Other interactions shown in light grey are tentative (see Discussion).

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genes, thus identifying a role for Wnt-7a in the anterio-posterior patterning of the female reproductive tract.

It seems likely that Wnt-7a may be responding to and enforcing positional signals dictated by clustered Hox genes in the female reproductive tract. The sharp boundaries between the different regions of the female reproductive tract are likely to be due to the boundaries of Hox gene expression. In combination with various members of the Hox families, Wnt-7a expression would dictate that a tissue be either uterine or oviduct in nature. When Wnt-7a is not expressed, a default pathway may exist in the oviduct so that it forms uterine-like structures, just as the default pathway in the uterine horns is to take on a vaginal-like cytoarchitecture.

Additionally, we note changes in the boundaries of expression of Wnt-4 and -5a prior to the appearance of abnormalities in the mutant or heterozygous uterine horns. Levels of Wnt-7a expression may therefore define the limits of expression of other Wnt genes in the uterus. It has been proposed that the *Drosophila* wingless gene may define its pattern of expression by a process termed self-refinement. In this model, Wingless protein mediates the transcriptional repression of wingless in neighboring cells [149]. Our results suggest that levels of Wnt-7a protein act to repress transcription of other Wnt genes in the same cells. There may be a threshold of Wnt-7a protein in the uterine epithelium which inhibits Wnt-4, and -5a expression. Wnt-4, and -5a expression shift to the epithelium in the homo- and heterozygous Wnt-7a mice. Thus, variations in the levels of Wnt-7a may modulate the dynamic expression pattern of Wnt-4 and -5a during the estrous cycle. Although Wnt-7a may define boundaries of Wnt gene expression by indirectly repressing transcription, in the absence of Wnt-7a expression, Wnt-5a expression in the uterus is not maintained. Thus, Wnt-7a is not required for the early expression of Wnt-5a in the uterus, but is required for its maintenance. However, Wnt-5a is expressed in the stroma of the mutant oviduct even though Wnt-7a expression is missing, indicating that Wnt-7a is not required for the induction and maintenance of Wnt-5a in the oviduct region. The

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differences between gene expression patterns in the different regions of the female reproductive tract may reflect the expression of other regulators that specify positional identity such as the HOM-C genes.

Wnt-7a is expressed throughout the epithelium at birth, but becomes restricted to the uterine luminal epithelium postnatally (Fig. 5.1, and [139]). The timing of loss of Wnt-7a from the vaginal epithelium corresponds to the onset of epithelial cytodifferentiation [22]. Vaginal epithelium becomes stratified and unresponsive to inductive signals from uterine mesenchyme. Wnt-7a appears necessary for epithelium to respond to uterine mesenchyme, thus loss of Wnt-7a may lead to vaginal cytodifferentiation. Loss of Wnt-7a expression in the epithelium of the mutant uterine horns results in the tissue mimicking a vaginal-like fate. The normal post-natal decline in vaginal Wnt-7a expression is accompanied by a decline in stromal Wnt-5a levels suggesting that down-regulation of both Wnt genes is required for vaginal development.

Wnt-7a Is Involved in Radial Axis Patterning in the Uterus

Wnt-7a is required for radial patterning in the uterus as well as for setting up the anterior-posterior axis. One major difference between wild-type and mutant uteri is the presence of endometrial glands which differentiate from luminal epithelium shortly after birth [22]. We do not observe glands in the mutant uterine horn. The lack of glands in the mutant mice may explain the observed infertility of mutant females [82]. The importance of uterine glands in fertility is demonstrated by the leukemia inhibitory factor (LIF) mutant mouse. LIF is expressed in uterine glands and is necessary for implantation [130]. Female mice with a targeted deletion of the LIF gene have phenotypically normal uteri but are infertile [131].

Uterine glands are induced in Wnt-7a heterozygous mice, however, higher levels of Wnt-7a expression are required to control glandular hyperplasia. Wnt-7a expression in the uterine epithelium likely stimulates the mesenchyme to induce uterine glands in the

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Müllerian epithelium (see Fig. 5.9). Wnt-7a could be signaling to Wnt-5a or a currently unidentified factor in the mesenchyme to promote the formation of glands. Data presented here do not rule out the possibility that glands may form in response to unidentified factors in the mesenchyme which may require stromal expression of Wnt-5a.

Wnt-7a Plays a Critical Role in Uterine Smooth Muscle Patterning

We observe smooth muscle disorganization in the Wnt-7a mutant uterine horn which becomes more pronounced during late post-natal development. Newborn uterine mesenchyme differentiates into smooth muscle and stroma. It has been noted previously that smooth muscle formation in uterine mesenchyme is dependent upon the presence of epithelium. Grafts of uterine mesenchyme alone showed little to no smooth muscle differentiation [143]. We have repeated these experiments, and in contrast, we observe many smooth muscle cells in these grafts using a probe to SMMHC, however, the cells are scattered throughout the mesenchyme and are not organized into layers (Figure 3.5 and [139]). We suggest that Wnt-7a maintains the organization of the smooth muscle in the uterus, and maintains the stroma-smooth muscle boundary. Whether the apparent increase in smooth muscle in the mutant uterus is due to stromal cells becoming smooth muscle cells or due to smooth muscle cell proliferation is unclear. Since Wnt-7a is expressed exclusively within the epithelium, its effects on smooth muscle are likely mediated through a molecule in the stroma. Wnt-5a may play a role in this process since its expression in the mutant stroma declines at a time coincident when the smooth muscle phenotype becomes evident. The roles of Wnt-5a in the uterus are currently being addressed utilizing Wnt-5a mutant mice.

It is interesting that in tissue recombinants and in intact tissues which are grown in a host animal, only one layer of smooth muscle forms. This suggests that positional cues may be missing which dictate the formation of the outer layer of smooth muscle. It is likely that these positional cues may be laid down by the expression of the Hox gene clusters.

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However, neither the Hoxa-10 nor Hoxa-11 mutant mice show defects in smooth muscle formation [31, 127]. Smooth muscle formation may be due to combinatorial gene expression, or may be influenced by HOM-C genes other than the Hoxa cluster. The signals which dictate the formation of the two layers of smooth muscle remain to be determined.

Wnt Gene Expression Directs Uterine Cytodifferentiation

We show that Wnt genes play a key role during post-natal female reproductive tract development, and in the maintenance of adult uterine function. It has been noted that morphogenesis and cytodifferentiation in the female reproductive tract occur in response to steroid hormones [40]. Wnt genes are responsive to changes in the levels of sex steroids both in the mouse mammary gland [79, 80] and the female reproductive tract (Figure 3.3 and [41, 139]). Wnt genes play roles in cell-cell communication, therefore, they may mediate the action of steroid hormones in these tissues. Although the Wnt-7a mutant uterine phenotype is more severely affected than the estrogen receptor knockout (ERKO) mouse uterus, the two have some similarities: both are hypoplastic with reduced amounts of stroma and endometrial glands [29]. It has been noted that prolonged exposure to estrogen results in endometrial glandular hyperplasia [150], which we observe in the Wnt-7a heterozygote uterus. It is possible that expression of steroid hormone receptors is altered in the Wnt-7a mutant uterus. Comparison of the effects of sex hormones in the uterus with the phenotypes observed in the Wnt-7a heterozygote and mutant uteri, along with the changes in Wnt-4 and -5a expression in the estrous cycle, suggests that Wnt gene activity may indeed mediate the effects of sex hormones in the uterus. Therefore, not only do the Wnt genes play a critical role maintaining the correct anterio-posterior and radial programs of the female reproductive tract, but likely participate in the hormonally mediated mesenchymal-epithelial signaling events that govern the adult uterine function.

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Chapter 6 The Wnt-7a Mutant Female Reproductive Tract Closely Resembles the Phenotype Observed in DES Exposed Mice

The work described in this chapter is based on a paper titled "Fetal exposure to DES results in de-regulation of Wnt-7a in the developing uterus" by Cary Miller, Karl Degenhardt, and David Sassoon (Nature Genetics, 20, pp. 228-230). Mice were injected with DES with the assistance of Karl Degenhardt. I performed the subsequent experiments, and generated the data shown in the figures.

Similarities Between the Female Reproductive Tracts of DES Exposed and Wnt-7a Mutant Mice

Diethylstilbestrol (DES) is an estrogen analog that was used in the United States from 1947-1971 to prevent miscarriage in humans. Approximately 1,000,000 women were exposed in utero to DES [151]. Prenatal DES exposure results in morphological changes of the uterus and vagina in both humans and mice [138, 152-154]. A specific molecular response to DES has not been identified that accounts for the DES syndrome. We have reported that Wnt-7a mutant mice have malformed female reproductive tracts [142]. The observed phenotype closely resembles the reproductive tract morphologies observed in female mice exposed prenatally to DES [138]. This observation raises the possibility that Wnt-7a plays a role in the DES response in the developing female reproductive tract. Although DES is no longer administered to pregnant women, DES-like substances such as Tamoxifen have recently been shown to be effective in decreasing the incidence of breast cancer in premenopausal women [155].

In order to directly compare the effects of loss of Wnt-7a to the effects of DES in the female reproductive tract, we generated DES mice following a previously established protocol [26, 138]. Control female reproductive tracts (oil treated) have normal

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morphology and tissue cytoarchitecture. DES treated and Wnt-7a mutant female reproductive tracts display stratified epithelium, reduced stroma and glands, and a disorganized inner layer of smooth muscle (Fig. 6.1). Women exposed prenatally to DES display epithelial or structural changes in the uterus, cervix, or vagina [152, 153, 156, 157]. More specifically, the vaginal malformations include vaginal adenosis or the appearance of epithelial structures in the vaginal stroma, vaginal adenocarcinoma, and fibrous structures or septums in the vagina . Also apparent were cervical malformations. The most common uterine malformation was a T-shaped uterus with a small cavity [153, 158]. The misshapen uterus with its constrictions has been attributed to smooth muscle hypertrophy [158] . This is especially interesting considering the smooth muscle phenotypes in Wnt-7a mutant and DES-exposed mice.

Wnt-7a Expression Declines in DES Exposed Mice

Although our initial observations led us to believe that vaginal morphology was unaffected in the Wnt-7a mutant mice, a close re-examination of this tissue reveals several abnormalities. Wild-type and heterozygous mice have two uterine horns which lead first into a Y-shaped cervix and then into a single vaginal lumen (Fig. 6.2A). While Wnt-7a mutant mice have 2 uterine horns which lead into a cervix, the septum which divides the anterior cervix continues into the vagina. The net result is that the anterior, Müllerian derived vagina is divided by a septum and therefore has 2 lumens (10 of 12 mice, Figure 6.2B,C). Wnt-7a seems to be important for canalization to occur between the fused Müllerian ducts in the region of the anterior vagina (see Figure 6.3). The longitudinal septum may prevent mating from occurring and is yet another possible reason for the infertility of the Wnt-7a mutant females. The presence of a longitudinal vaginal septum is a congenital abnormality that has been observed in humans. At least 300 cases of longitudinal vaginal septums in humans have been reported world-wide, although the authors estimate that many other cases exist but have not been detected [159-164].

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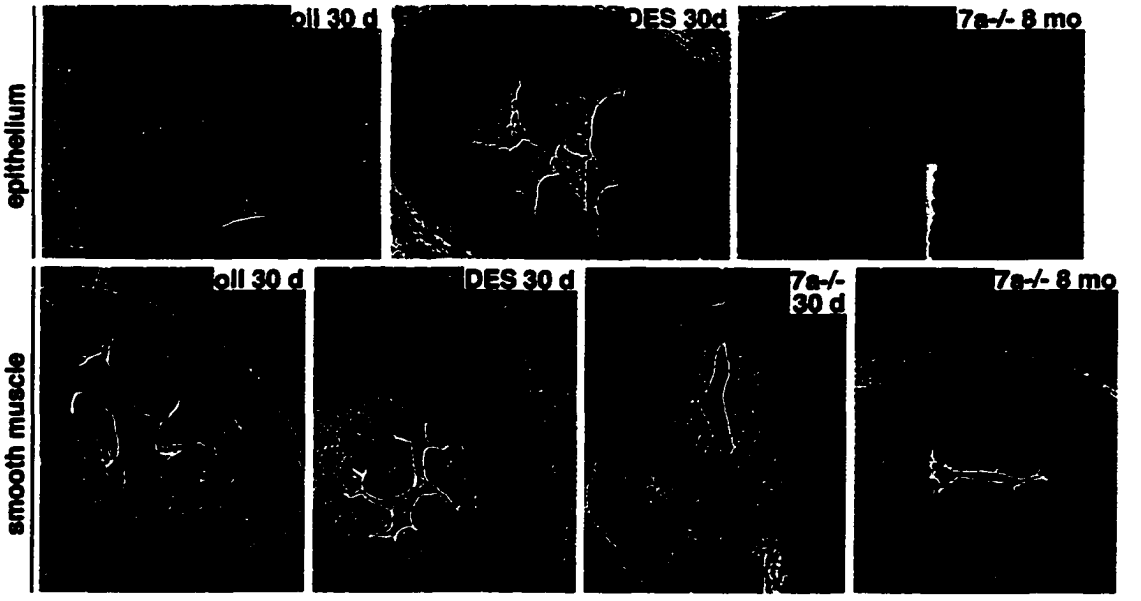

Figure 6.1 Prenatal DES treatment mimics the phenotype of the Wnt-7a mutant female reproductive tract. Pregnant CD-1 mice were treated with 200 μg/day of DES (Sigma) in sesame oil, or with oil alone, on days 15-18 of gestation 5, 7. The exposed female offspring were analyzed on the day of birth (day 0) and at subsequent timepoints. Hematoxylin and eosin stained sections show that control (oil treated) uteri have simple columnar epithelium, stroma (s) populated by glands and compact layers of smooth muscle (A). The uteri from DES treated mice have stratified epithelium, reduced stroma, a reduced number or lack of glands and a disorganized inner layer of smooth muscle (B) as do Wnt-7a mutant uteri (C). The organization of the smooth muscle layers is shown in panels D-G using a probe to SMMHC. The photomicrographs are composites of the phase images and dark-field silver grains (green) to allow direct comparison of tissue and signal. The inner smooth muscle layer (ism) is compact and organized in the control uterus (D), but disorganized in the DES treated uterus (E). The disorganization of the inner smooth muscle in the Wnt-7a mutant uterus is mild at one month (F) but pronounced at 8 months (G). The luminal epithelium is denoted by double arrows. Scale bar 200 μm (A-G).

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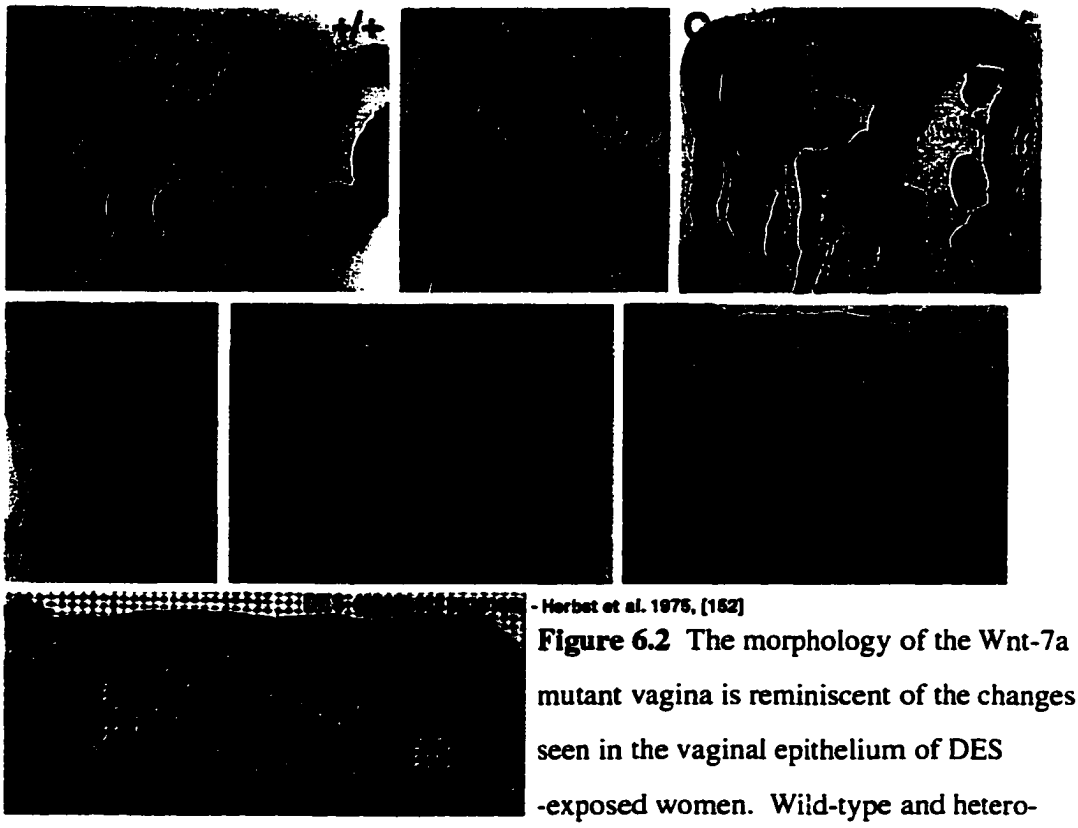

Figure 6.2 The morphology of the Wnt-7a mutant vagina is reminiscent of the changes seen in the vaginal epithelium of DES -exposed women. Wild-type and heterozygous mice have a single, common vaginal lumen, shown by a transverse section through a wild-type vagina (A). A high percentage of Wnt-7a mutant mice have double vaginas due to the presence of a septum from the cervix through the anterior, Mullerian derived vagina (B,C). Cross-sections (B), and longitudinal sections (C) through mutant vaginas also show this septum (s). We can also see the remnants of the Wolffian duct (w). (C) shows the presence of vaginal concretions (c). The appearance of the vaginal epithelium changes during the estrous cycle. The thickness of the epithelium in a wild-type mouse during pro-estrous is shown by the double arrows (D). Some regions of the vaginal epithelium in the Wnt-7a null mouse are not grossly different from the wild-type vaginal epithelium (E). Other regions are much thicker and have regions of proliferation which extend into the underlying stroma (F). The enclosed epithelium also has regions which appear gland-like (*). These changes are similar to vaginal adenosis, a change of the vaginal epithelium commonly seen in women exposed prenatally to DES (G, [152]). u (ureter), cvx (cervix).
Scale bar, 800 μm (A, B, C), 200 μm (D, E, F).

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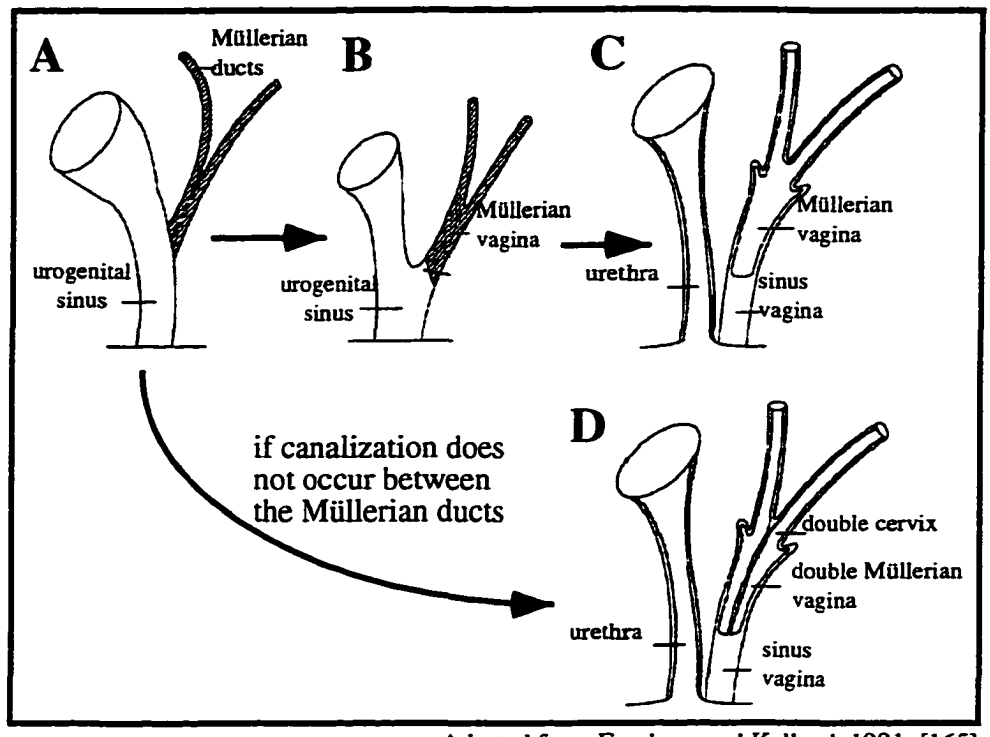

Adapted from Forsberg and Kalland, 1981, [165]

Figure 6.3 Schema demonstrating the normal development and duplication of the Müllerian vagina. **A.** The Müllerian ducts normally join together prior to fusing with the urogenital sinus. **B.** Canalization occurs so that the there is one common lumen (the Müllerian vagina)leading into the urogenital sinus. **C.** The urogenital sinus divides.The ventral portion becomes the urethra, and the dorsal portion becomes the sinus vagina. The Müllerian vagina is attached to the sinus vagina which consists of a solid cord of epithelial cells at thispoint. **D.** If canalization does not occur between the Müllerian ductsin A, the cells between the two ducts will persist and form a longitudinal septum. This septum divides the cervical region and the Müllerian vagina into two, and is observed in the Wnt-7a mutant female reproductive tract.

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The Wnt-7a mutant vagina contains remnants of the Wolffian ducts (Figure 6.2B), and has concretions forming in the vaginal fornices (Figure 6.2C). Retaining the Wolffian duct often leads to the formation of vaginal concretions. Wolffian duct remnants and vaginal concretions are commonly seen in mice treated with DES [138, 154]. The morphologies of wild-type and Wnt-7a mutant vaginal epithelium change during the estrous cycle [32, 142]. Figure 6.2D shows the thickness of the typical epithelium in a wild-type mouse during pro-estrus. In general, the vaginal epithelium of a Wnt-7a mutant mouse is much thicker than its wild-type counterpart (Figure 6.2E,F vs. D). The Wnt-7a mutant vagina has regions where the vaginal epithelium appears to have grown downward into the vaginal stroma, forming peg-like structures (Figure 6.2F). The vaginal stroma contains epithelial inclusions. The tissue morphology of the Wnt-7a mutant vagina at this late stage greatly resembles vaginal adenosis, a common finding in DES-exposed women (Figure 6.2G, from [152]).

We proceeded to examine the expression of Wnt-7a in the control and DES exposed uteri. Wnt-7a is normally expressed in the luminal epithelium of the perinatal uterus [142]. As expected, Wnt-7a is expressed in the epithelium of the wild-type uterus (Fig. 6.4B, D), however, in the DES treated uteri, low levels of Wnt-7a transcripts are detected at birth (Fig. 6.4F). Wnt-7a expression in the DES treated uteri returns to high levels by 5 days after birth (Fig. 6.4H), and is maintained at later stages (data not shown).

Hoxa Gene Expression and DES Exposure

Wnt-7a mutant mice and DES-exposed mice share many similarities, suggesting that the Wnt-7a mutant mouse is a good model for prenatal DES exposure. Recently, Ma *et al.* have reported that DES-exposure results in the down-regulation of Hoxa-10 expression in the Müllerian ducts [129]. We therefore examined Hoxa-10 expression levels in the control and DES-exposed uteri. No differences were noted in levels of Hoxa-10 expression in the DES-exposed mice (data not shown). The difference between our results

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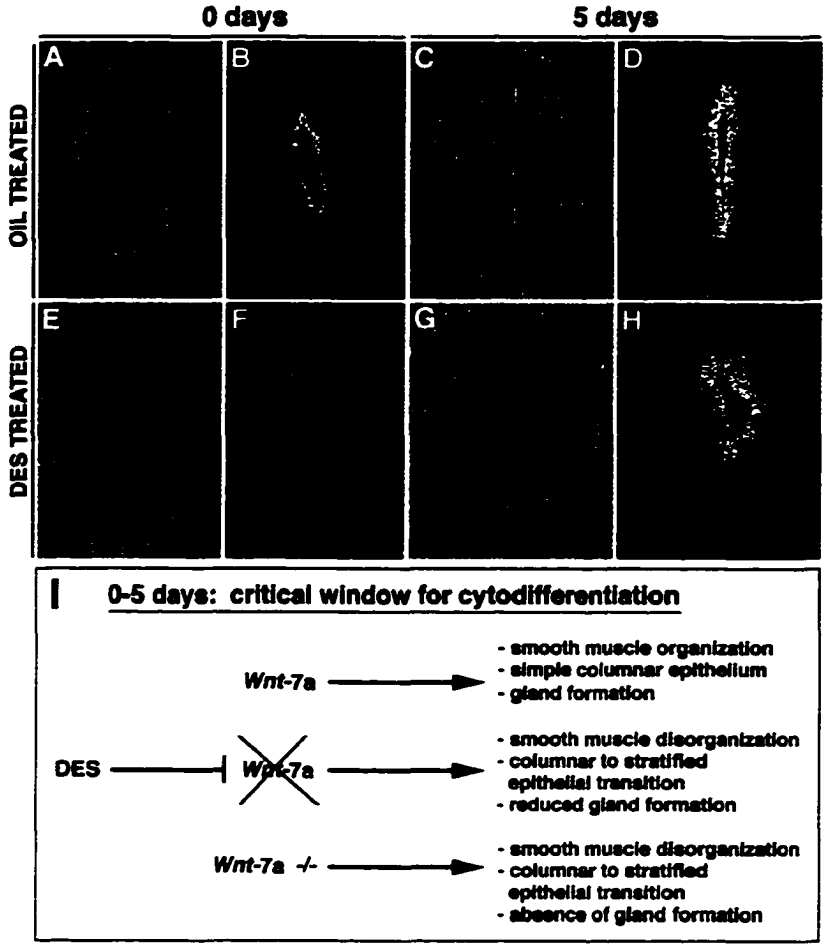

Figure 6.4 Wnt-7a expression is down-regulated in DES exposed mice at the time of birth. The photomicrographs show phase and dark -field images of control and DES treated uteri at birth and at 5 days post-natal examining Wnt-7a expression. Wnt-7a is expressed in the luminal epithelium of the control uterine horn at birth and at 5 days (B,D). Low levels of Wnt-7a are detected in the luminal epithelium of the DES treated uterus at birth (D), however, normal levels of expression are observed by 5 days after birth (H). (I) Schema depicting DES action through Wnt-7a. Wnt-7a expression is required for proper smooth muscle, epithelial and gland formation in the uterus. DES exposure results in a down-regulation of Wnt-7a during a critical postnatal period thereby interfering with proper uterine morphogenesis. DES exposure results in a phenocopy of the Wnt-7a null uterus. Scale bar 200 μm.

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and those of Ma *et al.* may be due to the differences in experimental approach. We used high levels of DES and administered the drug between 15-18 d.p.c. Ma *et al.* used much lower doses of DES and administered the drug between 9.5-15.5 d.p.c. Additionally, Ma *et al.* examined Hoxa-10 expression 6 hours after DES exposure whereas we examined expression levels starting 24 hours after exposure. It is possible that levels of Hoxa-10 expression recover 24 hours after DES exposure. It is noteworthy that many of the changes seen in the DES-exposed mice are not present in either the Hoxa-10 or Hoxa-11 mutant mice and are seen in the Wnt-7a mutant mouse [31, 127, 142]. For instance, neither the Hoxa-10 or the Hoxa-11 mutant mouse has stratified uterine epithelium or disorganized smooth muscle, which is seen in both the DES-exposed and Wnt-7a mutant uterus.

Early Expression of Wnt-7a Is Critical for Appropriate Cytodifferentiation of the Mouse Female Reproductive Tract

Previous studies have demonstrated that cytodifferentiation of the female reproductive tract occurs by 5-7 days after birth in mice [22, 40]. After this time, the uterine epithelium is no longer responsive to inductive signals from vaginal stroma [22]. Thus, the female reproductive tract has a critical temporal window to undergo key morphogenetic patterning events. Even though Wnt-7a expression returns to high levels by 5 days in DES exposed uteri, we propose that the down-regulation of Wnt-7a during this critical window is sufficient to provoke the DES syndrome. The effects of DES treatment on Wnt-7a expression is another molecular example of the importance of mesenchymal-epithelial signaling in uterine cytodifferentiation. Since the estrogen receptor is thought to be expressed only in the uterine mesenchyme at this stage of development [46-50], the effects of estrogen on Wnt-7a expression in the epithelium are likely indirect, and would involve a paracrine signaling pathway.

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Chapter 7 Potential Wnt Gene Roles in the Development of the Female Reproductive Tract: Relevance to Other Systems and to Human Pathologies

Compartmentalization of the Female Reproductive Tract

Classical studies of the female reproductive tract have focused on anatomical and cytological development and changes thereof during adult function. Structural malformations of the reproductive tract have been identified and categorized [166, 167]. Furthermore, the relationship between steroid hormone exposure and the development of endometrial cancer has been documented [150, 168]. Until recently, however, a satisfactory molecular basis underlying cell differentiation and morphogenesis in the female reproductive tract has been lacking. Advances within the last three years have identified roles for the Hox genes [31, 127, 132] and the Wnt genes [142, 169] in the development and adult function of the female reproductive tract.

Hoxa-10 expression has been demonstrated to establish the position of the utero-tubal junction [127]. However, there are conflicting data regarding the roles of the Hox genes in the prenatal establishment of the compartments of the female reproductive tract. Taylor *et al.* maintain that Hoxa-9, -10, -11, -12 and -13 are expressed throughout the length of the reproductive tract prior to birth, but their expression pattern becomes restricted to the various compartments (uterus, cervix, vagina) post-natally [126]. Our unpublished results show that differences exist in the anterior Hoxa gene expression boundaries prior to birth, but the differences in the posterior boundaries are determined post-natally (data not shown). Finally, Ma *et al.* have reported that the Hoxa genes have anterior and posterior boundaries along the Müllerian ducts at 16.5 d.p.c., that correspond to the different compartments of the reproductive tract [129]. Additionally, these boundaries are maintained into adult life [129]. These conflicting results will need to be resolved. Regardless, differences in physical compartmentalization are apparent prior to 16.5 d.p.c. since the Müllerian ducts fuse with each other at the level of the presumptive cervix, and

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fuse with the sinus vagina before this time. It seems likely that the Hoxa and Hoxd families participate in the early establishment of boundaries for compartmentalization. One possibility is that the Hoxd genes may set up the initial compartments of the reproductive tract, while the Hoxa family assumes this role at a later stage, or the two Hox families may simply be functionally redundant.

Data obtained in the studies of Wnt-7a suggest that Wnt genes function to maintain compartmentalization in the female reproductive tract. We observe that Wnt-7a maintains the expression of the Hoxa genes in the underlying mesenchyme [142], suggesting a mechanism whereby Wnt gene expression indirectly maintains compartmentalization. The role of Wnt-7a as revealed in the mutant mice, along with the specific expression patterns of Wnt-4 and Wnt-5a in the female reproductive tract, suggests that all three Wnt genes participate in compartmentalization. In the adult Wnt-7a mutant uterus, loss of Hoxa-10/-11 expression precedes the loss of Wnt-5a expression (data not shown and [41, 139, 142]). Coincident with the decline of Wnt-5a expression in the Wnt-7a mutant uterus, the tissue loses radial organization and assumes a vaginal cytoarchitecture [142]. Similarly, normal vaginal development involves a decline in Hoxa-10/-11 expression followed by a loss of Wnt-5a expression and the appearance of a characteristic vaginal cytoarchitecture. Wnt-4 is the only one of the three Wnt genes that is not expressed in the vagina prior to birth (Chapter 3 and [139]). This suggests that Wnt-4 regulates early compartmentalization events, or that the expression of Wnt-4 is governed by unidentified genes which set up the initial boundaries during compartmentalization. It is difficult to understand how Hoxa gene expression could be regulating Wnt-4 expression. For instance, Hoxa-13 is expressed in the vagina both prenatally and postnatally (data not shown and [129, 132]). If Hoxa-13 represses Wnt-4 expression in the vagina prenatally, why is Wnt-4 expressed in the vagina post-natally when Hoxa-13 is still maintained in the vagina? It seems likely that factors in addition to the Hox genes dictate the expression patterns of the Wnt genes and thus play roles in compartmentalization.

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The developmental origin of the human vagina has long been a source of contention. In mice, it is generally accepted that the anterior vagina develops from the Müllerian ducts and the posterior vagina develops from the urogenital sinus. The human situation remains unresolved. Recently, Boutin and Cunha [18] have suggested that the epithelium of the Müllerian -derived vagina replaces the epithelium of the sinus vagina. At 15.5 d.p.c., Wnt-4 is expressed in the epithelium of the sinus vagina, but not within the epithelium of the Müllerian -derived vagina (data not shown and [139]). By 17.5 d.p.c., Wnt-4 is not expressed within the epithelium of either the Müllerian-derived or the sinus-derived vagina [139]. This suggests that the Müllerian epithelium replaces the sinus epithelium at a very early age in the mouse. Since Wnt-4 expression delineates sinus vagina and Müllerian-derived epithelium at early stages, Wnt-4 may serve as a marker in the human to determine the origin of the vaginal tissues.

Conserved Mechanisms Underlying Patterning of the Limb and the Female Reproductive Tract

Specific signaling molecules that guide the development and function of the female reproductive tract are also known to be involved in the development of other structures such as the limb [31, 127, 132, 142]. Moreover, the observation that expression of the same signaling molecules in limb and in female reproductive tract development allows for speculation regarding conservation of molecular pathways during development in different structures [170, 171]. Wnt-7a is expressed in the dorsal ectoderm of the limb [77, 82]). Although loss of Wnt-7a affects dorsal-ventral limb patterning, it also affects anterio-posterior limb patterning [82]. This result and work by others [172-175] suggest that the development of the three axes of the limb are interconnected, and that Wnt-7a plays roles in governing two of the axes. Similarly, Wnt-7a functions in multiple axes in the female reproductive tract. Loss of Wnt-7a affects anterio-posterior patterning and radial patterning in the female reproductive tract [142]. Again, this suggests that the two axes are inter-

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connected. Another similarity between the limb and the female reproductive tract is that Wnt-7a maintains expression of a homeobox gene in each case [84, 85, 142]. This signaling mechanism has not only been conserved between structures within the same organism, but has been maintained throughout evolution.

Although some of the same molecules appear to function in both systems, such as the Hox and Wnt gene families, there are many other molecules which seem to participate only in the formation of the limb. Sonic hedgehog, FGF's, Engrailed-1, and the BMP's all play roles in limb formation. Their roles in the formation and function of the urogenital system are still unknown. Sonic hedgehog interacts with FGF's, Wnt-7a, and Engrailed-1 in the patterning of the limb [82, 85, 172, 176]. Although sonic hedgehog clearly is important in the limb, expression is not detected in the female reproductive tract from 15.5 d.p.c. to adulthood (Miller and Sassoon, data not shown). Similarly, only low levels of BMP-2 and FGF-2 and -4 can be detected in the female reproductive tract. Engrailed-1 represses Wnt-7a expression in the ventral limb ectoderm [85, 176]. Expression of Engrailed-1 is critical to maintain dorsal-ventral patterning in the limb, however, Engrailed-1 is not expressed in the female reproductive tract (Miller and Sassoon, data not shown). It is not surprising that different molecular pathways are involved in the patterning of the limb and the reproductive tract since the two structures are also quite different.

Potential Relationships between Wnt-7a and Various Human Syndromes

At least 9 human syndromes exist that involve malformations of both the limbs and the Müllerian ducts [177, 178]. The phenotypes described for the various syndromes do not correspond precisely to the limb and reproductive malformations seen in the Wnt-7a mutant mice [82, 142, 169]. One caveat in comparing human and murine genetics is that mutations/deletions in the same gene often give rise to related but nonetheless different phenotypes [179]. This may reflect differences in specific developmental programs. For example, mice normally have bicornuate uteri whereas humans normally do not. Therefore

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a mutation in the human gene which results in a bicornuate uteri may show no phenotype in a mouse carrying the same mutation. Thus the involvement of WNT-7a in a human genital/limb syndrome seems probable. For instance, Halal has reported a case of an autosomal dominant trait which results in duplication of the uterus and vagina along with upper limb hypoplasia, and either polydactyly or syndactyly [177]. While the Wnt-7a murine defects are autosomal recessive, most of the limb-genital syndromes are autosomal dominant disorders. The genetics of hand-foot-genital syndrome in mice and in humans show the same difference [75, 122]: inheritance is autosomal dominant in humans and autosomal recessive in mice. This suggests that the difference in inheritance patterns between mice and humans does not necessarily rule out mutations of the same gene. The difference in inheritance between species may reflect different mutations. For instance, the human mutations may result in dominant-negative proteins, and therefore we observe an dominant effect, while the truncated proteins observed in mice with targeted deletions would only show a phenotype in the homozygous form. Therefore, the same gene could be mutated in different species, resulting in similar syndromes with different patterns of inheritance.

We have determined that DES treatment down-regulates Wnt-7a, and that Wnt-7a expression is lowest during the stages of the estrous cycle with the highest levels of estrogen. However, we do not fully understand the connection between Wnt-7a and the steroid hormones. Estrogen may act on Wnt-7a through estrogen responsive elements in the Wnt-7a gene, or its actions may be mediated through other signaling molecules. It is clear that low levels of Wnt-7a mimic the effects of unopposed estrogen treatment since glandular hyperplasia develops in both situations [142, 150, 168]. One fifth of post-menopausal women develop cystic glandular hyperplasia [180]. This condition can lead to endometrial carcinoma [150, 181]. We do not understand the molecular mechanisms by which glandular hyperplasia develops and becomes cancerous. It is possible that Wnt-7a is involved in this process. Wnt-7a may acquire mutations with time, so that lower levels of

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Wnt-7a are expressed in many older women. Although this may be sufficient to induce glandular hyperplasia, other molecules could also be involved. Preliminary data suggests that glandular hyperplasia of the uterus occurs much earlier in Wnt-7a $^{+/-}$/Wnt-5a $^{+/-}$ compound heterozygote mice (Miller and Sassoon, data not shown). Many of the cases of glandular hyperplasia may reflect this type of compound mutation. Additionally, one could speculate that the cases of glandular hyperplasia which become cancerous may reflect loss of heterozygosity in one or more of the Wnt genes.

Since Wnt genes control growth, patterning, and maintain tissue boundaries, the loss of specific Wnt gene expression later in life could result in tumor formation in humans as well. We have preliminary observations that older Wnt-7a mutant mice develop smooth muscle derived uterine tumors (Miller and Sassoon, data not shown). In this regard, Wnt-7a represses tumor formation by controlling the growth of specific cell types (see Figure 7.1). In the absence of functional Wnt-7a, smooth muscle cells display abnormal and ultimately hyperplastic growth. Leiomyomas, smooth muscle derived endometrial tumors, express high levels of the estrogen related genes [182]. The authors theorized that the tumors resulted due to this maintained responsiveness to estrogen. We have noted a similar situation in the Wnt-7a mutant mice (Miller and Sassoon, data not shown). Adult mice normally have low levels of the estrogen receptor in the uterine myometrium, while the Wnt-7a mutant myometrium has high level of the estrogen receptor expression. A further understanding of the molecular basis of smooth muscle derived endometrial tumors in the Wnt-7a mutant mice may aid in our understanding of leiomyomas, which develop in 20-30% of women over the age of 30 [182]. In this regard, the Wnt-7a mutant mice will be informative for understanding the molecular control of smooth muscle development. One exciting direction of the studies in the laboratory is to determine the specific connection between Wnt gene signaling and these common human pathologies.

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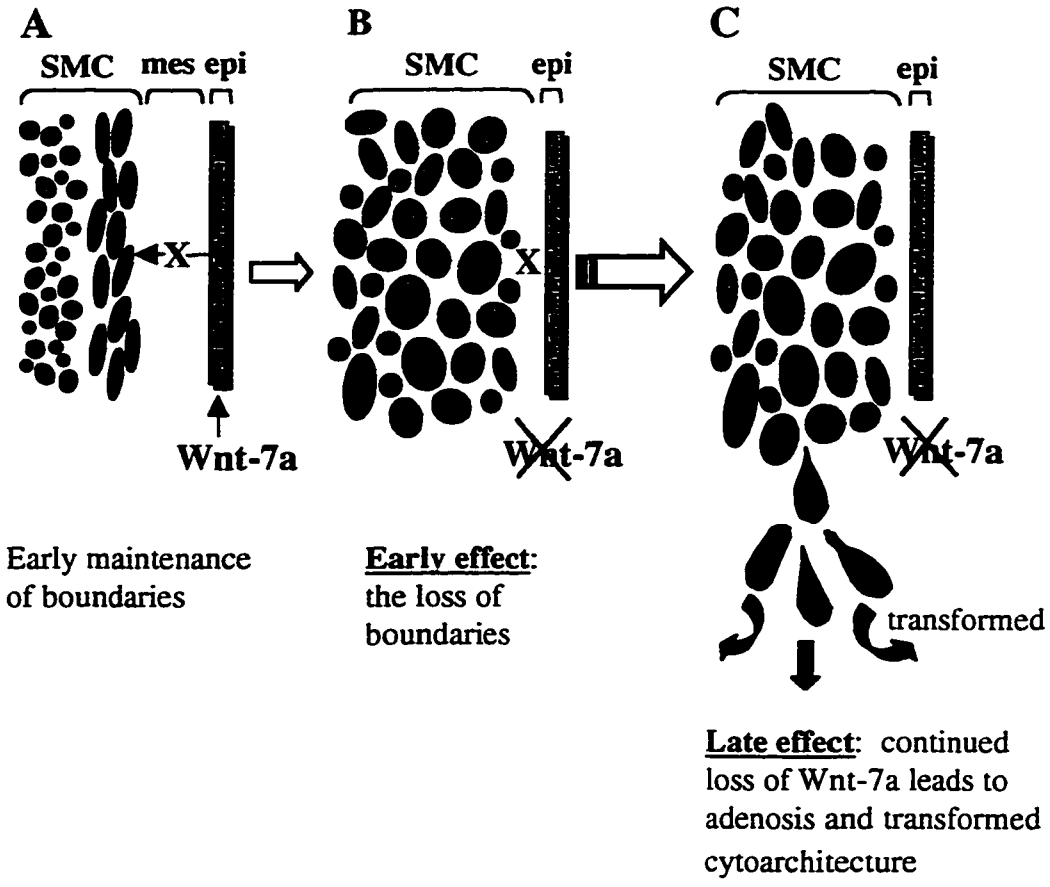

Figure 7.1 Loss of Wnt-7a expression has different effects in the uterus depending on the timing. **(A)** Wnt-7a serves to maintain tissue boundaries. **(B)** Loss of Wnt-7a at an early timepoint results in loss of tissue boundaries in the uterus. We observe the loss of the stroma and proliferation and disorganization of the smooth muscle cell layers. Therefore the phenotype of the Wnt-7a null mouse at early stages resembles the phenotype of the DES exposed mice. **(C)** Continued loss of Wnt-7a in the null mice results in vaginal adenosis and the transformation of smooth muscle cells.

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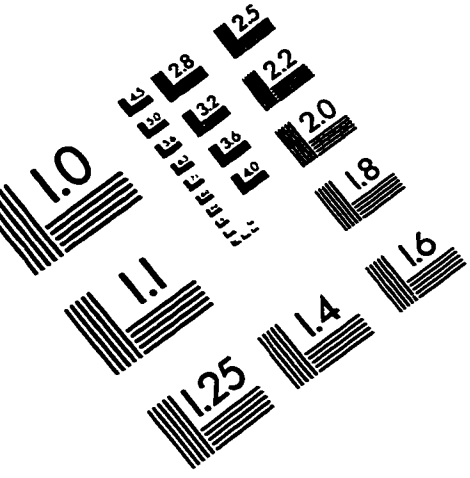

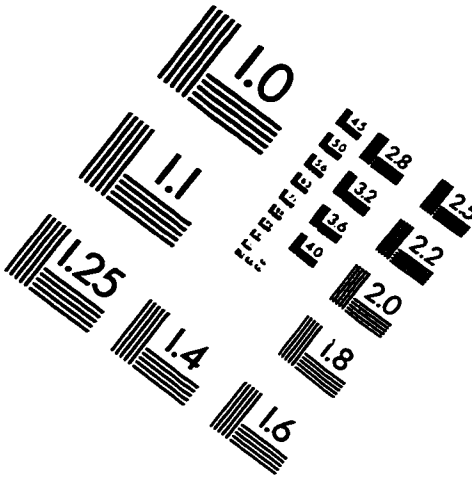

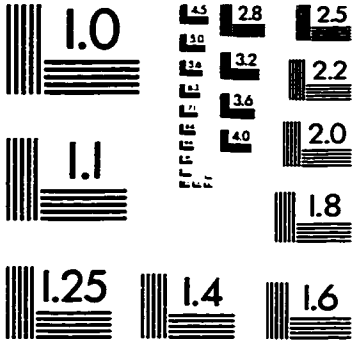

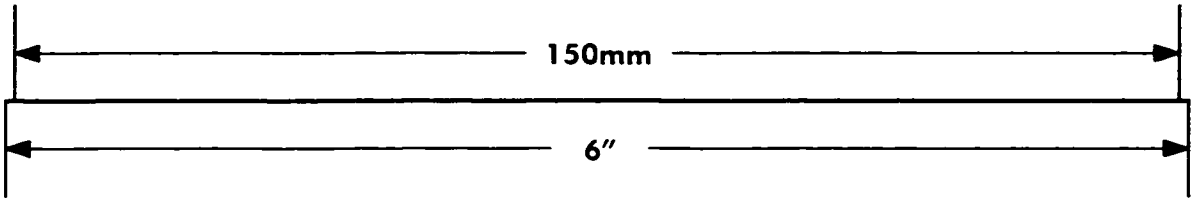

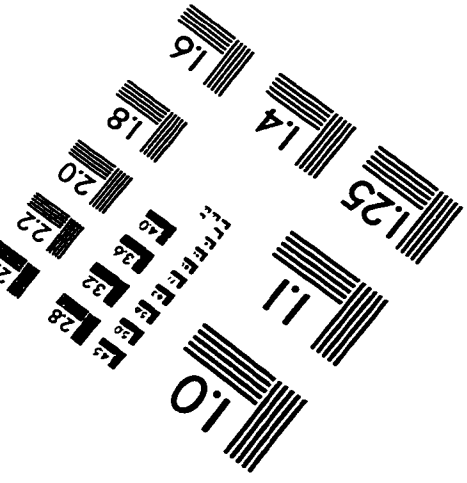

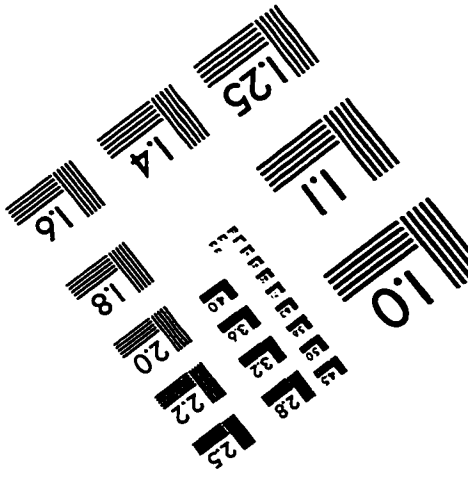

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