Wnt5a is required for proper epithelial-mesenchymal interactions in the uterus

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Accepted 22 January 2004

Development 131, 2061-2072
Published by The Company of Biologists 2004
doi:10.1242/dev.01090

Summary

Epithelial-mesenchymal interactions play a crucial role in the correct patterning of the mammalian female reproductive tract (FRT). Three members of the Wnt family of growth factors are expressed at high levels in the developing FRT in the mouse embryo. The expression of Wnt genes is maintained in the adult FRT, although levels fluctuate during estrous. *Wnt4* is required for Müllerian duct initiation, whereas *Wnt7a* is required for subsequent differentiation. In this study, we show that *Wnt5a* is required for posterior growth of the FRT. We further demonstrate that the mutant FRT has the potential to form the posterior compartments of the FRT using grafting techniques. Postnatally, *Wnt5a* plays a crucial role in the generation of uterine glands and is required for cellular and molecular responses to exogenous estrogens. Finally, we show that *Wnt5a* participates in a regulatory loop with other FRT patterning genes including *Wnt7a*, *Hoxa10* and *Hoxa11*. Data presented provide a mechanistic basis for how uterine stroma mediates both developmental and estrogen-mediated changes in the epithelium and demonstrates that *Wnt5a* is a key component in this process. The similarities of the *Wnt5a* and *Wnt7a* mutant FRT phenotypes to those described for the *Hoxa11* and *Hoxa13* mutant FRT phenotypes reveal a mechanism whereby Wnt and Hox genes cooperate to pattern the FRT along the anteroposterior axis.

Key words: Wnt, Uterus, Mouse, DES, Glands

Introduction

The mammalian female reproductive tract (FRT) is derived from the Müllerian ducts giving rise to the oviducts, uterine horns, cervix and the anterior vagina (Cunha, 1975). The murine FRT is immature at birth and consists of two tubes of simple columnar epithelia surrounded by a mesenchymal sheath fused at the level of the cervix. Distinct cytodifferentiation occurs during postnatal development and differentiation is complete 2 weeks after birth. Uterine horns develop postnatally to form an external myometrium surrounding the mesenchymal (stromal) compartment which contains glands. By contrast, the vagina and cervix do not develop glands and the luminal epithelium undergoes a transition from simple columnar to squamous (stratified) morphology. Experiments in which neonatal epithelium from any part of the FRT is recombined with presumptive uterine or vaginal mesenchyme reveals that the epithelium is developmentally plastic and adopts either a uterine (simple columnar) or vaginal (squamous/stratified) epithelial fate dependent upon the origin of the mesenchyme (Cunha, 1976; Kurita et al., 2001). Recent results using grafts between estrogen receptor α (*Esr1*) mutant and wild-type FRTs demonstrates that *Esr1* function is required in the mesenchyme but not in the epithelium to mediate estrogen-mediated responses in the epithelium (Cooke et al., 1997; Kurita et al., 2000). Taken together, these studies demonstrate that FRT mesenchyme delivers developmental and estrogenic signals to the epithelium.

Wnt genes encode secreted glycoproteins that regulate cell and tissue growth and differentiation (Polakis, 2000) and activate multiple signaling pathways through the frizzled receptors and the cytoplasmic signaling protein, dishevelled (Pandur et al., 2002). We identified three members of the Wnt gene family (*Wnt4*, *Wnt5a*, *Wnt7a*) that are expressed at high levels in the adult FRT throughout development (Miller et al., 1998b). At birth, *Wnt4* expression is restricted to the uterine mesenchyme. By contrast, *Wnt5a* is distributed throughout the mesenchyme of the uterus, cervix and vagina, whereas *Wnt7a* is in the epithelium. During postnatal differentiation, *Wnt5a* and *Wnt7a* become restricted to the uterine horns, whereas *Wnt4* expression is activated in the stratified epithelium of the cervix and the vagina (Miller et al., 1998b). We noted that the levels and the sites of *Wnt* expression fluctuate during estrous, suggesting a continued role in the adult (Miller et al., 1998b).

Mice corresponding to all three Wnt genes expressed in the FRT have been generated. *Wnt4* mutants fail to form Müllerian ducts and die at birth due to numerous defects, thus an analysis of how Wnt4 contributes to later FRT development is unknown (Vainio et al., 1999). *Wnt7a* mutants are viable and exhibit malformations in the FRT including shortened and uncoiled oviducts, hypoplastic uterine horns and a vaginal septum (Miller and Sassoon, 1998; Parr and McMahon, 1998). The

uterus is most affected with a marked reduction in the stromal compartment, accompanied by a lack of uterine glands and a disorganized myometrium. In addition, *Wnt7a* mutant uterine epithelium fails to maintain a normal columnar phenotype and becomes stratified upon puberty (Miller and Sassoon, 1998). It was subsequently demonstrated that fetal diethylstilbestrol (DES) exposure transiently represses *Wnt7a* expression in the Müllerian ducts and is sufficient to recapitulate the *Wnt7a* mutant FRT phenotypes providing a molecular basis for environmental endocrine disruption (Miller et al., 1998a).

In this study, we examined the role of *Wnt5a*, which is expressed in the FRT mesenchyme, and thus is a good candidate as a potential mediator of mesenchymal-epithelial interactions (Miller et al., 1998b; Pavlova et al., 1994). *Wnt5a* mutants die at birth due to a failure to complete anteroposterior body axis development (Yamaguchi et al., 1999). In order to circumvent the neonatal lethality, we grafted neonatal mutant FRT tissue into adult hosts to assess postnatal potential and phenotypes. We find that although the oviduct, uterine and cervical compartments of the FRT develop in the absence of *Wnt5a*, the mutant uterus fails to form glands that are essential for adult function. In addition, we demonstrate that *Wnt5a* is required for the complete repertoire of estrogen-mediated cellular and molecular responses. Furthermore, *Wnt5a* participates in a regulatory loop with *Wnt7a* and is required for correct regulation of *Hoxa10* and *Hoxa11*, which control anteroposterior patterning of the FRT (Benson et al., 1996; Hsieh-Li et al., 1995). These data shed light upon the mechanism by which uterine stroma mediates both developmental and estrogen-mediated changes in uterine epithelium and reveal that *Wnt5a* is required in these processes.

Materials and methods

Mice breeding

Wnt5a mutant mice were obtained from A. McMahon (Yamaguchi et al., 1999) and maintained in a C57BL6/SV129 mixed background. Neonates were obtained after delivery or C-section on day 19 of gestation. *Wnt7a* mutant mice were obtained from B. Parr and A. McMahon (Parr and McMahon, 1998) and maintained in a SV129 background. *Lef1* mutant mice were kindly obtained from R. Grosschedl (van Genderen et al., 1994) and were maintained in a C57BL6/SV129 mixed background. Nude mice in a C57BL6 background were purchased intact or ovariectomized from Taconic, Germantown, NY. All procedures for handling of mice, housing and maintenance were performed according to approved institutional guidelines. All surgical procedures were prior approved by the institution according to NIH guidelines.

Tissue recombination and renal capsule grafting

Wild-type and mutant neonate FRT fragments were grafted under the renal capsule of each kidney of the same adult female nude host to ensure identical hormonal conditions. Grafting procedures were performed as previously described (Cunha, 1976). Adult female hosts were ovariectomized 3-4 weeks prior to grafting where indicated. Separation of the neonatal epithelium from the mesenchyme for recombination between wild-type and *Wnt5a* mutant tissues was performed as previously described (Bigsby et al., 1986). Diethylstilbestrol (DES) administration was delivered i.p. suspended in saline between day 18 to 20 post graft implantation following a previously described protocol (Miller et al., 1998a). Host FRT and neonate grafts were harvested 24 hours after the last injection on day 21, i.e. a developmental stage equivalent to 3-week-old FRT, and were fixed o/n in 4% PAF 4°C and processed for paraffin histology.

Retroviral expression vectors

Wnt cDNAs encoding HA tagged *Wnt4* and *Wnt5a* were inserted in QCX backbone vectors derived from MLV retrovirus and produced as previously described (Julius et al., 2000; Shimizu et al., 1997). Retroviral supernatants were concentrated by ultracentrifugation, 2 hours at 100 ***g*** in a Beckman SW28 rotor. Titer was estimated to 1×10^6 infection unit by *lacZ* staining of NIH3T3 cells infected with a parallel QC-lacZ prep. Western blot and in situ immunofluorescence using anti-HAtag high affinity, rat monoclonal antibody (3F10) from Roche Diagnostics (Mannheim) were performed on infected NIH3T3 to confirm the synthesis of the Wnt factors by the retrovirus. Neonate uterine fragments were infected overnight in retroviral supernatant resuspended in DMEM/20% FCS/4 μg/ml hexadimethrine bromide (H9268, Sigma).

In situ hybridization

In situ hybridizations for the *Wnt*, *Hoxa* and *Msx1* were performed as previously described (Miller and Sassoon, 1998). *Esr1* and *Pgr* probes were kindly provided by G. Cunha. RNA probes were labeled with ^{35}S-UTP. Black and white dark field images were converted to using Adobe Photoshop to allow superimposing upon phase contrast images.

Results

Wnt5a is required for proper anteroposterior development of the FRT

Wnt5a heterozygote mice were crossed to generate a total of 242 neonates. We obtained 40 *Wnt5a*$^{-/-}$ pups, which falls below the number predicted by Mendelian genetics (~60 pups or 16.53% versus 25%), indicating loss of mutant fetuses in utero. Of 40 mutant pups, 17 were males and 19 females revealing no gender bias in survival during gestation. We could not determine the sex of 4 pups because of extreme reduction in posterior development. The anterior Müllerian-derived structures (oviducts and uterine horns) could easily be identified, whereas posterior derived structures (cervix and vagina) were absent (Fig. 1A,B). The uterine horns are either fused at the midline (Fig. 2) or terminate as a blind pouch (Fig. 1B). The *Wnt5a* mutant uterine horns have an undulated lumen and show a 60 to 90% reduction in length when compared with wild types (see also left panels in Fig. 2). The oviducts are less affected and we observe correct narrowing of the anterior uterine horn at the level of the uterotubal junction with the oviduct. The anterior border of expression for *Hoxa10* defines the site of the uterotubal junction (Benson et al., 1996). Using whole-mount in situ hybridization, we confirmed the position of the uterotubal junction in the *Wnt5a* mutant and in the wild type (Fig. 1). Expression of *Wnt7a* and *Msx1* is detected in the epithelium of the Wnt5a mutant FRT (Fig. 1E-H) whereas the expression of Wnt4 is only slightly reduced when compared with wild-type uterus and is not affected in the ureters (Fig. 1I,J). The expression of the *Wnt5a* mutant transcript is not affected in the absence of *Wnt5a* in the FRT (Fig. 1K,L) as previously seen for the *Wnt5a* mutant limb bud (Yamaguchi et al., 1999). We conclude that loss of *Wnt5a* affects posterior growth of the Müllerian duct.

Postnatal development of the Wnt5a mutant FRT

In order to circumvent the perinatal lethality of the *Wnt5a*

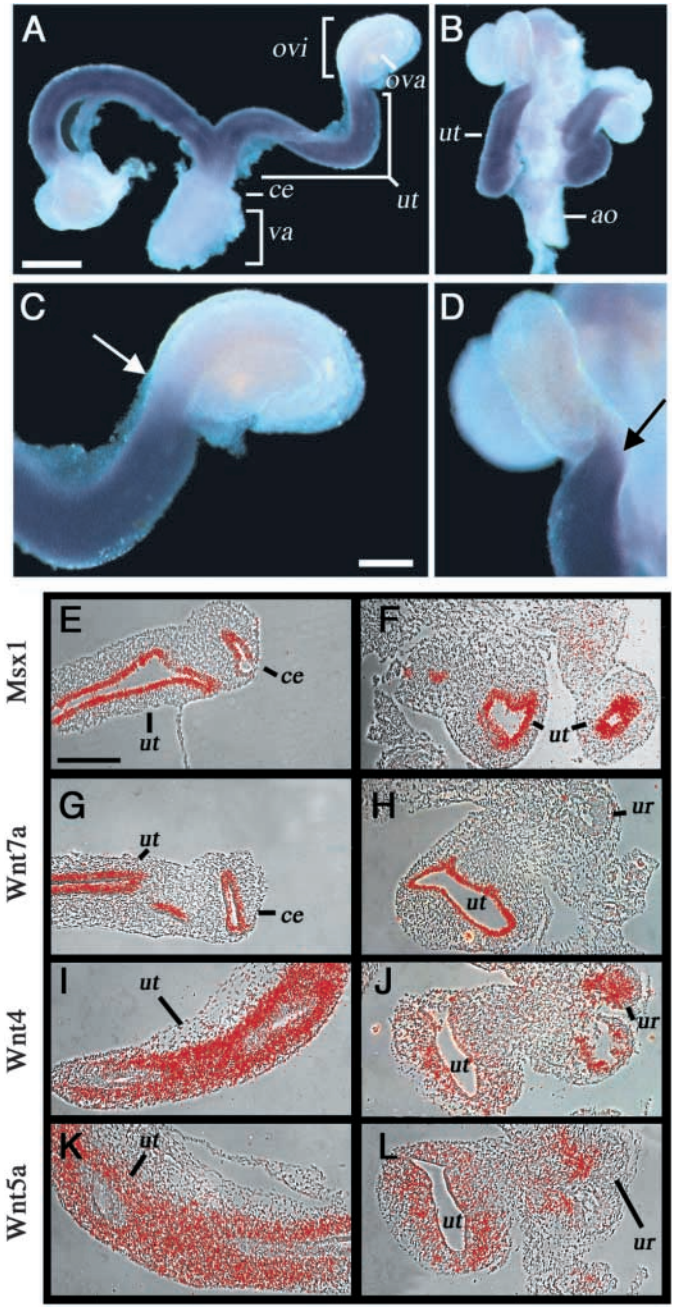

Fig. 1. *Wnt5a* mutant FRTs lack posterior structures. *Hoxa10* whole-mount in situ hybridization of the FRT from wild-type (A,C) and *Wnt5a*$^{-/-}$ (B,D) at P0. The *Wnt5a* mutant FRT lacks cervical and vaginal structures and the uterine horns are short and convoluted. Arrows in C and D indicate the anterior limit of *Hoxa10* expression at the uterotubal junction. Scale bars: 1 mm in A,B; 0.4 mm in C,D. *Msx1* (E,F), *Wnt7a* (G,H), *Wnt4* (I,J) and *Wnt5a* (K,L) ^{35}S in situ hybridization of paraffin wax embedded sections from wild-type (E,G,I,K) and *Wnt5a* mutant (–/–) (F,H,J,L) P0 FRTs. Silver grains are superimposed as red upon a phase contrast image. Ao, aorta; ce, cervix; ova, ovary; ovi, oviduct; ur, ureter; ut, uterus; va, vagina. Scale bar: 200 μm in E-L.

mutant, we grafted newborn (postnatal day 0/embryonic day 19-20) FRT fragments under the renal capsule of cycling *nude* females (8 week old). *Wnt5a*$^{-/-}$, *Wnt5a*$^{+/-}$ and *Wnt5a*$^{+/+}$ FRT tissues were grafted and grown for 3 weeks in the same host to ensure identical host conditions. FRTs were cut into size-matched fragments along the anteroposterior axis prior to grafting (Fig. 2). FRTS harvested from a total of four *Wnt5a*$^{+/+}$, four *Wnt5a*$^{+/-}$ and five *Wnt5a*$^{-/-}$ pups, and were analyzed using grafting procedures with multiple grafts per individual. The ovaries, oviduct and uterine regions, which normally express *Wnt5a,* developed correctly for all genotypes (Fig. 2). The epithelial cells display the normal characteristics of square-shaped ciliated cells for the oviduct and tall columnar cells for the uterus in the *Wnt5a* mutant compared with wild-type grafts. The smooth muscle cell layers formed normally in the absence of *Wnt5a*, although they appear thinner when compared with control grafts. This is in contrast to the *Wnt7a* mutant, which shows a hyperplastic and hypertrophied smooth muscle compartment (Miller and Sassoon, 1998). Smooth muscle myosin heavy chain in situ hybridization revealed correct differentiation of smooth muscle layers in the *Wnt5a* mutant (data not shown). We observed grafts that developed stratified epithelium in two out of five independent *Wnt5a* mutant grafts which were derived from the most posterior portion of the neonate *Wnt5a* mutant Müllerian ducts (Fig. 3A-C). The morphological columnar-to-squamous junction of the epithelium was accompanied at the molecular level by the correct boundary of *Msx1* and *Wnt7a* expression and activation of *Wnt4* in the stratified epithelium (Fig. 3D-F; data not shown). This result reveals the potential to form a cervix in the *Wnt5a* mutant despite the lack of a morphologically defined cervix at birth.

Wnt5a and Wnt7a are required for gland formation

We observed that few glands develop in wild-type uterine grafts grown in adult cycling females. Uterine glands normally appear by 1-2 weeks after birth in situ. In the grafts, a small number of glands appear after 5 weeks of growth under the renal capsule showing an abnormal delay when compared with gland formation in the uterus in situ (data not shown). We reasoned that this delay in glandulargenesis may be caused by precocious exposure of neonatal uterine grafted tissues to adult levels of sex hormones present in the cycling female hosts as perinatal exposure of the FRT to sex hormones is linked to deficient glandulargenesis (Branham et al., 1985a; Branham et al., 1985b; Gray et al., 2001). As normal (postnatal) glandulargenesis occurs in the immature uterus in the absence of high levels of circulating steroid hormones, we placed grafts into female hosts that were ovariectomized 2 weeks prior to the grafting procedures. Under these conditions, numerous and normal appearing glands developed in all the grafts (n=18; Fig. 4A). In addition, the tissue organization of the grafts and the luminal folds were indistinguishable from uteri in situ at an equivalent stage of post-natal development (3 weeks). Thus, this procedure for obtaining normal morphogenesis and maturation of wild-type grafted uterine tissues allows for assessment of the outcome of mutant FRT development, particularly in the case of perinatal lethality. Using ovariectomized hosts, 10 out of 12 grafts derived from eight independent *Wnt5a* mutant mice did not develop glands whereas the remaining two grafts developed very few glands (Fig. 4B; data not shown). The overall morphology of the *Wnt5a* mutant grafts is otherwise normal, although the smooth muscle layers are moderately thinner compared with the wild-type grafts.

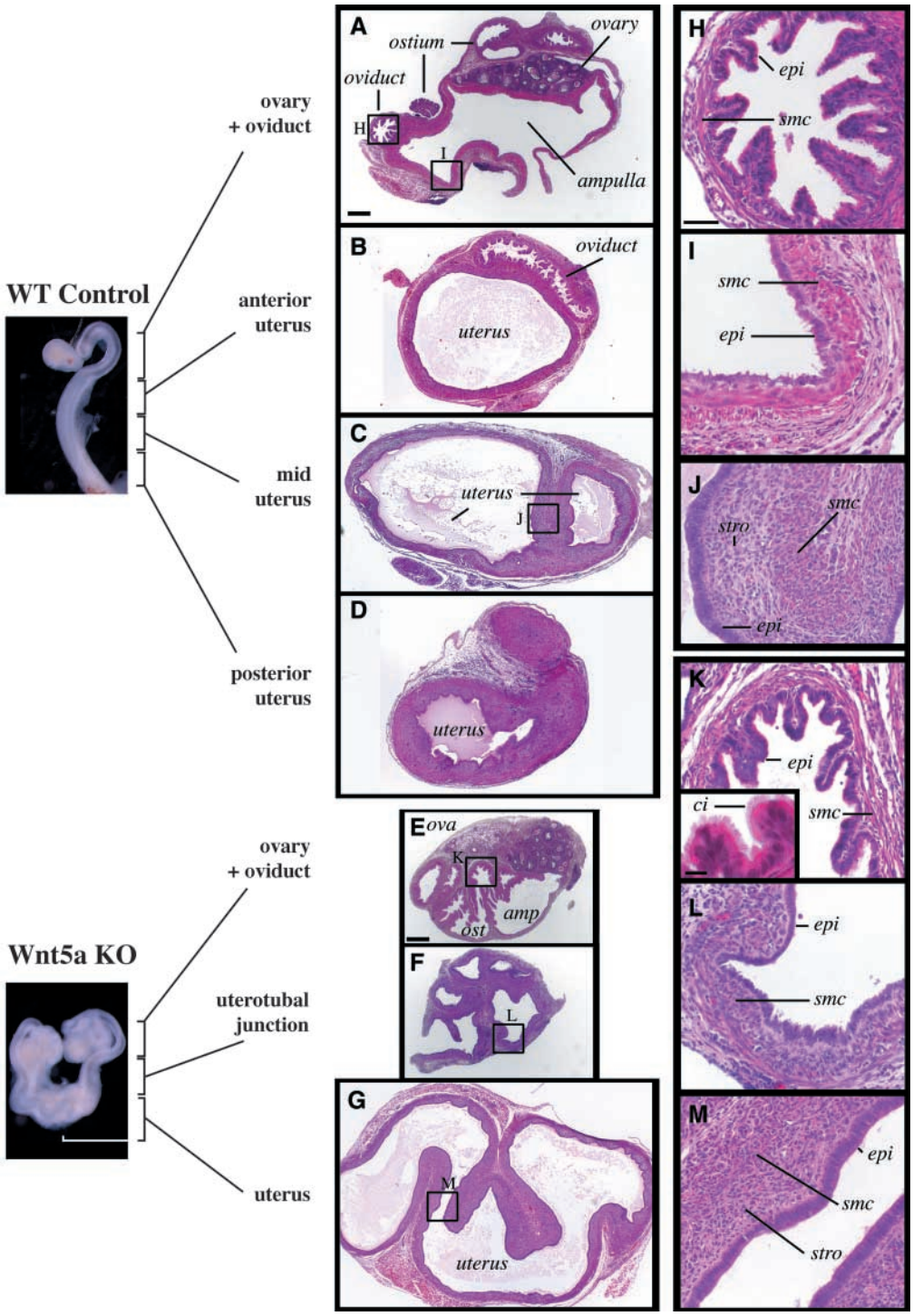

Fig. 2. Postnatal development of the *Wnt5a* mutant FRT grafts. Wild-type and *Wnt5a* mutant P0 FRT were cut into fragments along the anteroposterior axis (as indicated) and grafted under the renal capsule of an intact adult host. Grafts were harvested 3 weeks after grafting into the host. Paraffin sections from wild-type (A-D) and *Wnt5a* mutant (E-G) were stained with Haematoxylin and Eosin. (H-M) High magnification of corresponding boxed area in A-G. All structures formed in the *Wnt5a* mutant displayed normal characteristics of each compartment, including postnatal smooth muscle (smc) differentiation, stromal compartment (stro) and ciliated epithelium (ci) in the oviduct [inset in K (scale bar: 10 μm)]. Scale bar: 250 μm for low magnification; 50 μm for high magnification. ost, ostium; amp, ampulla.

We had previously observed that *Wnt7a* mutant females do not develop uterine glands (Miller and Sassoon, 1998), suggesting a potential genetic interaction between *Wnt7a* and *Wnt5a*. However, we had analyzed the *Wnt7a* uterine phenotype from samples obtained directly from postnatal mutant females. To compare directly our results under the same experimental conditions, we tested *Wnt7a* mutant uterine grafts in ovariectomized hosts. The *Wnt7a* mutant uterine grafts (n=3) fail to develop glands in ovariectomized hosts and recapitulate the myometrial and epithelial phenotypes that have been reported in our previous studies (Miller and Sassoon, 1998) (Fig. 4C).

Lef1, a mediator of canonical Wnt signaling and gland formation, is not required for uterine glandulargenesis

Lef1 is a transcription factor that interacts with β-catenin and mediates the canonical Wnt pathway (McKendry et al., 1997). *Lef1* is expressed in the Müllerian duct mesenchyme (Oosterwegel et al., 1993); however, *Lef1* mutant mice die

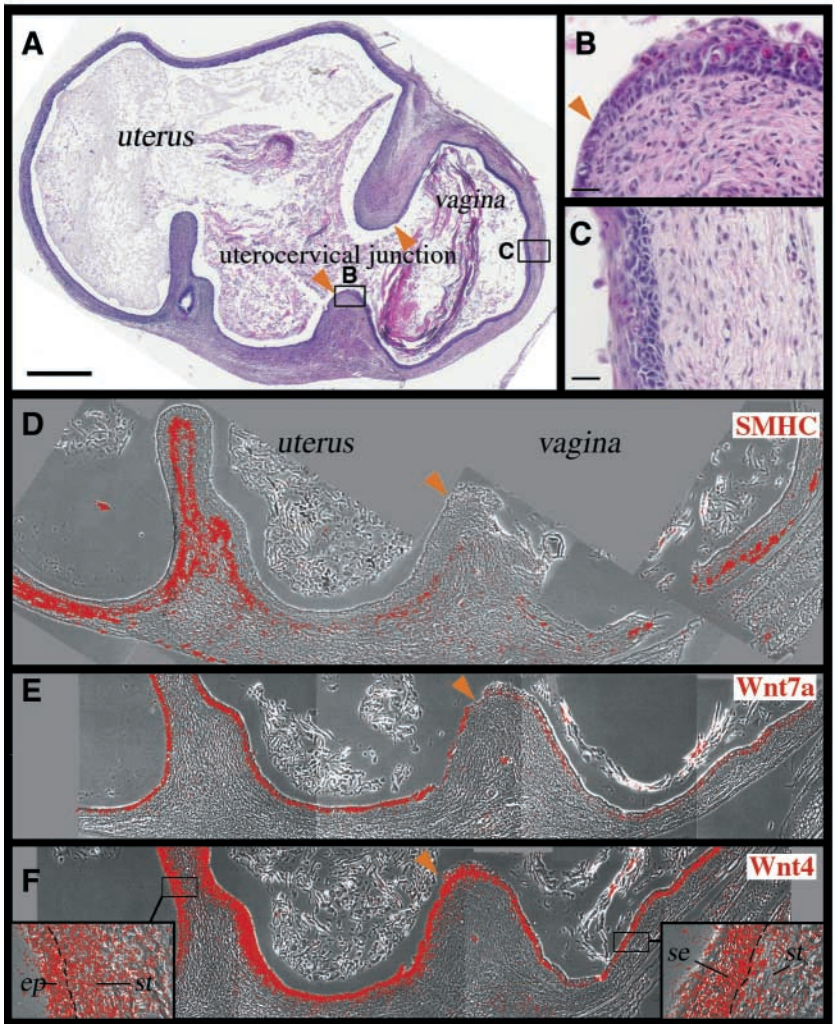

Fig. 3. Uterocervical junction forms in the *Wnt5a* mutant despite the lack of an identifiable cervix at birth. (A) Haematoxylin-Eosin staining of a *Wnt5a* mutant posterior graft at low magnification. Scale bar: 400 μm. (B,C) High magnification of boxed areas in A showing the transition from simple columnar epithelium to stratified epithelium (arrowhead; scale bar: 40 μm). The transition is accompanied by a correct formation of thick smooth muscle layers in the uterine area and sparse smooth muscle bundles in the vaginal region as shown by smooth muscle myosin heavy chain in situ hybridization (D, *SMHC*). (E) *Wnt7a* also shows a normal and sharp boundary of expression at the level of the uterocervical transition and *Wnt4* (F) expression shows the correct pattern of epithelial (ep) and stromal (st) expression in the uterus (proestrus stage) and stratified epithelium (se) in the vagina (see insets).

several days after birth prior to cytodifferentiation of the FRT and uterine gland formation. As *Lef1* is required for glandulargenesis in the mammary gland and mediates numerous epithelial-mesenchymal interactions during development (van Genderen et al., 1994), we evaluated the potential role of *Lef1* in uterine postnatal development using grafting procedures. We find that *Lef1* is not required for uterine development. Moreover, glandulargenesis proceeds normally in *Lef1* mutant grafts (n=2). In addition we generated grafts (n=2) from double *Wnt7a/Lef1* mutants that are indistinguishable from *Wnt7a* mutant grafts (Fig. 4D,E). Taken together, these results show that *Wnt5a* and *Wnt7a* are required for gland formation in the uterus and participate in a signaling pathway that does not require *Lef1*.

Wnt5a is required in the stroma to induce gland formation

Uterine gland formation initiates on postnatal days 7-9 (P7-9). and they continue to grow and increase in number until puberty. By P15, *Wnt7a* is expressed exclusively in luminal epithelium but not in glandular epithelium (Fig. 4G). *Wnt7a* is also expressed in the deep folds of the luminal epithelium that start to form between P5 and P7 (Brody and Cunha, 1989). In wild-type grafts, we observe a sharp boundary of *Wnt7a* expression at the transition between luminal and glandular epithelium (Fig. 4J). *Wnt4* expression is restricted to the stroma between the luminal epithelium and adjacent uterine glands (Fig. 4I). *Wnt5a* expression is abundant throughout the stroma that extends from the subepithelial region up to the inner smooth muscle layer. This domain of expression includes stroma surrounding the folding luminal epithelium and more distal glands (Fig. 4H). In addition, low but detectable levels of *Wnt5a* expression are observed in luminal and glandular epithelium. These patterns of expression suggest that *Wnt7a* and *Wnt5a* act in juxtaposed compartments to control gland formation. The sharp boundary of *Wnt7a* expression at the site of glandular invagination suggests a mechanism whereby *Wnt7a* is repressed locally to allow luminal epithelium to participate in gland formation. If this model is correct, then *Wnt5a* may provide a permissive environment for proper regulation of *Wnt7a*. To determine if *Wnt5a* expression is required in the stroma to mediate gland formation, we performed recombinant graft experiments using wild-type and *Wnt5a* mutant (–/–) uterine fragments (Fig. 5A). When wild-type stroma is recombined with wild-type epithelium or *Wnt5a* mutant epithelium, recombinant grafts form glands (Fig. 5B,D). When *Wnt5a* mutant stroma is recombined with *Wnt5a* mutant or wild-type epithelium, no glands developed, except for one mutant mesenchyme/wild-type epithelium graft that developed a single gland (Fig. 5C,E; data not shown). These results demonstrate that *Wnt5a* is required in the stroma for glandulargenesis.

The inability of the *Wnt5a* mutant FRT to form glands may reflect a requirement for *Wnt5a* directly at sites of gland formation or an earlier requirement during fetal development to promote the survival of a unique population of cells in the FRT that direct this process. To test these possibilities, uterine fragments were infected with retroviral vectors that are expressed only in the stroma, based on histological markers such as human placental alkaline phosphatase or β-

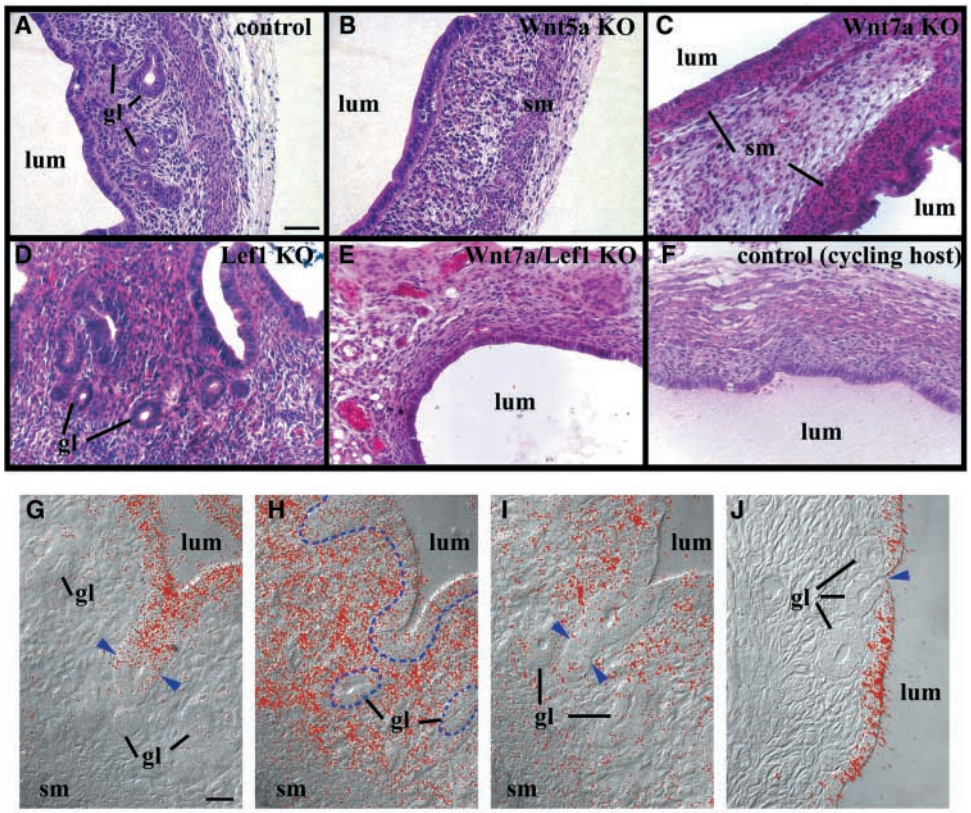

Fig. 4. *Wnt5a* and *Wnt7a* are required for gland formation in grafts grown into ovariectomized hosts.
(A-F) Haematoxylin-Eosin staining of grafts grown for 3 weeks in ovariectomized hosts (A-E) and cycling host (F). Note the presence of glands (gl) in the control *Wnt5a*$^{+/-}$ graft (A) and *Lef1*$^{-/-}$ (D) but not in the *Wnt5a*$^{-/-}$ (B), *Wnt7a*$^{-/-}$ (C) and *Wnt7a/Lef1*$^{-/-}$ (E), nor the wild-type graft grown in a cycling host (F). Scale bar: 50 μm. (G-J) Pattern of Wnt gene expression during gland formation in the wild-type uterus at P15 (G-I, adjacent sections) and in a 3 week-old wild-type graft (J). Lumen (lum) and smooth muscle (sm) are indicated.
(G) *Wnt7a* is expressed in the 'invaginated' luminal epithelium but not in the glandular epithelium (gl). In a section that passes through the site of invagination (J), we observe a boundary of *Wnt7a* expression between the luminal epithelium and the glandular epithelium. *Wnt5a* is expressed in the stroma surrounding the glands (H). Low levels of *Wnt5a* expression are also detected in the epithelium. *Wnt4* expression is most abundant in the subepithelial stroma (I). Scale bar: 20 μm.

galactosidase (data not shown). Ectopic *Wnt5a* expression in *Wnt5a* mutant neonatal uterine grafts rescues gland formation in discrete regions of the uterine grafts in three out of three independent grafts whereas no glands are formed in uterine grafts derived from the same tissues infected with an empty vector (n=2) (Fig. 5F,G). Interestingly, *Wnt4* overexpression is unable to rescue gland formation in the *Wnt5a* mutants (n=2) (Fig. 5H) demonstrating that Wnt4, which is also expressed in the uterine mesenchyme is unable to substitute for Wnt5a and that specific roles likely exist for each ligand in this system.

Wnt5a is required for Wnt7a and Hoxa repression by DES

We evaluated the morphological and cellular responses of the *Wnt5a* mutant uterine graft to the potent estrogen, DES (diethylstilbestrol), which normally elicits pronounced cellular and molecular changes in uterine tissue. Grafts were allowed to grow in ovariectomized hosts for 18-20 days followed by three daily injections of DES or saline followed 24 hours by harvesting the grafts. Wild-type (n=3) and *Wnt5a*$^{+/-}$ grafts (n=6) responded to DES exposure with the typical changes associated with the estrogenic response, i.e. hypertrophy and hyperplasia of the luminal and glandular epithelial cells and the distension of the stromal compartment which is associated with the changes in vascular permeability that occur upon estrogenic compounds exposure (Korach and McLachlan, 1995) (Fig. 6, compare B with A). All *Wnt5a* mutant grafts (n=4) exposed to DES responded by a normal increase in cellularity and thickness of the epithelial compartment (although no glandular response is measured) (Fig. 6D,H). The average luminal epithelium height was assayed in three independent grafts of each genotype. The epithelium height changed from 19.4±3.8 μm in the absence of DES to 38.4±0.2 μm after DES exposure in the *Wnt5a* mutant graft (compare Fig. 6G,H), showing no significant difference with the wild-type grafts, 20.1±1.5 μm in saline conditions to 38.3±5.1 μm after DES exposure (Fig. 6E,F). By contrast, the global response of the *Wnt5a* mutant grafts was abnormal in appearance. The uterine walls of the mutant grafts did not enlarge in response to DES, but instead underwent an unusual dilation. In addition, we note that two out of the four *Wnt5a* mutant DES exposed grafts did not display stromal edema (Fig. 6D; data not shown).

We analyzed the expression of *Wnt7a* in saline and DES exposed *Wnt5a* mutant grafts and compared the pattern with wild-type grafts grown in the same hosts (Fig. 6I-L). We observe that *Wnt7a* is expressed throughout the luminal epithelium of 3-week-old *Wnt5a*$^{+/-}$ control and *Wnt5a*$^{-/-}$ mutant grafts grown in saline injected ovariectomized host (Fig. 6I,K). *Wnt5a*$^{+/-}$ and wild-type grafts show the expected downregulation of *Wnt7a* following exposure to DES (Fig. 6J; data not shown). By contrast, exposure to DES is unable to repress *Wnt7a* in the *Wnt5a* mutant graft (Fig. 6L). In addition *Hoxa10* and *Hoxa11*, which are also repressed by DES exposure in utero (Block et al., 2000; Miller et al., 1998a) are not repressed by DES exposure in the *Wnt5a* mutant grafts (Fig. 6M-T, compare P with N and T with R).

To determine if prolonged exposure to high levels of a synthetic estrogen adequately reproduces the endogenous regulation of *Wnt7a* and the Hoxa genes by *Wnt5a*, we analyzed uterine grafts grown in intact cycling hosts that were

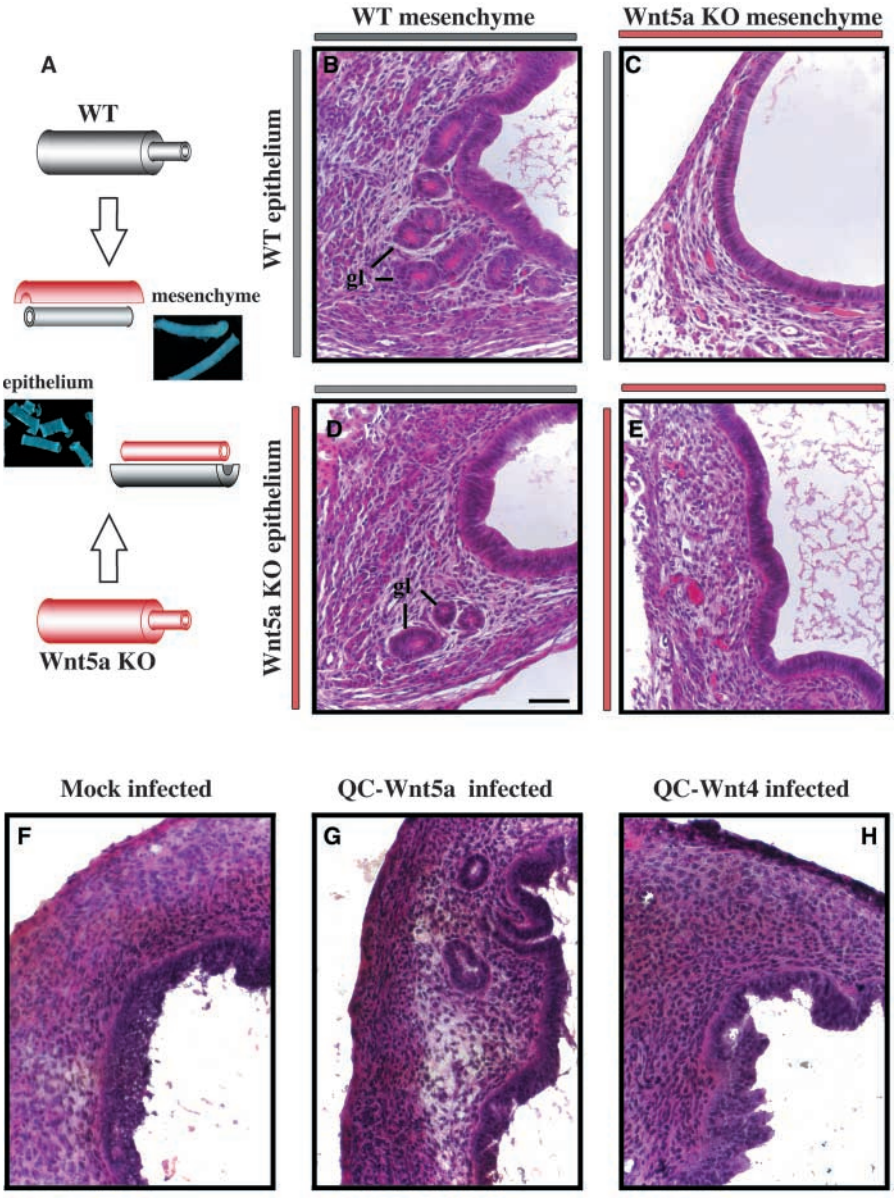

Fig. 5. Stromal *Wnt5a* expression is required for gland formation in the uterus. (A) Schema of the recombinant graft procedure. The mesenchymal sheath is separated from the epithelial tube by mild trypsin digestion and gentle mechanical manipulation. The mesenchyme (mes) from either wild-type or *Wnt5a*$^{-/-}$ is recombined with wild-type or *Wnt5a*$^{-/-}$ epithelium (epi) and grafted under the renal capsule of an adult host. (B-E) Haematoxylin-eosin staining of the recombinants. Glands (gl) form in the wild-type mes/wild-type epi (B) and wild-type mes/*Wnt5a*$^{-/-}$ epi (D) but not in the *Wnt5a*$^{-/-}$ mes/wild-type epi (C), or in the *Wnt5a*$^{-/-}$ mes/*Wnt5a*$^{-/-}$ epi (E). (F-H) Haematoxylin-Eosin staining of grafts (frozen sections) derived from the same *Wnt5a*$^{-/-}$ individual infected at birth by the retroviral backbone (control, F), *Wnt5a* expressing retrovirus (G) and a *Wnt4* expressing retrovirus (H). We observe that *Wnt5a* rescues the formation of glands whereas *Wnt4* does not. Scale bar in D: 50 μm.

sacrificed at different period of the estrous cycle. Expression patterns of *Wnt7a*, *Hoxa10* and *Hoxa11* are normal in *Wnt5* mutant grafts harvested at diestrus from the host, when levels of estrogen are low and levels of progesterone are high (Fig. 7A). During proestrus, when the levels of estrogen are high and the levels of progesterone are low, we find that *Wnt7a* levels remain high in *Wnt5a* mutants (Fig. 7B). Similarly, *Hoxa10* and *Hoxa11* remain high during proestrus in the *Wnt5a* mutant grafts. These results show that estrogen mediated repression of *Wnt7a* in the epithelium and repression of *Hoxa10 and Hoxa11* in the stroma is dependent upon *Wnt5a* expression.

One simple mechanism underlying abnormal estrogen responses in *Wnt5a* mutant FRTs is that estrogen receptor expression is altered. However, we find that *Esr1* is correctly expressed in the *Wnt5a* mutant uterine grafts grown in ovariectomized hosts and expression is activated in epithelium and the smooth muscle in both controls and *Wnt5a* mutant grafts upon DES exposure (Fig. 8A-F). Expression of *Esr1* is also normal in mutant grafts grown in an intact cycling and harvested at different stages of the estrous (data not shown).

The progesterone receptor (*Pgr*) gene is a major target of estrogen signaling. In mice, *Pgr* is repressed by estrogen in the uterine epithelium in adult females (Kurita et al., 2000). We found that *Pgr* is repressed by DES in the ovariectomized adult host consistent with previously reported observations (Fig. 8K,L). In contrast to adult host uteri, *Pgr* is not repressed by DES in the epithelium of control grafts, rather it is repressed in the stroma and activated in the epithelium and smooth muscle layers (Fig. 8N). The differences in regulation of *Pgr* between the 3-week-old wild-type grafts and the adult host may reflect the previously noted differences in the estrogenic response between prepubertal females and sexually mature females (Korach and McLachlan, 1995); however, we note that *Pgr* gene expression and regulation in the *Wnt5a* mutant grafts is identical to control grafts (Fig. 8O,P). These results show that key aspects of the genomic response to estrogenic signals is preserved in the *Wnt5a* mutant. We then analyzed *Wnt5a* expression following DES exposure. *Wnt5a* transcripts are restricted primarily to the stroma in control grafts grown in mock-injected ovariectomized hosts (Fig. 8G), whereas *Wnt5a* mutant transcripts are present in both the stroma and epithelium in mutant grafts (Fig. 8I). DES exposure increases *Wnt5a* levels in the epithelium and the smooth muscle in both control and *Wnt5a* mutant grafts (Fig. 8H,J) revealing that *Wnt5a* signaling is not required for the regulation of its own gene product by estrogen. Therefore, in contrast to *Wnt7a*, which is primarily repressed by DES exposure, *Wnt5a* undergoes a

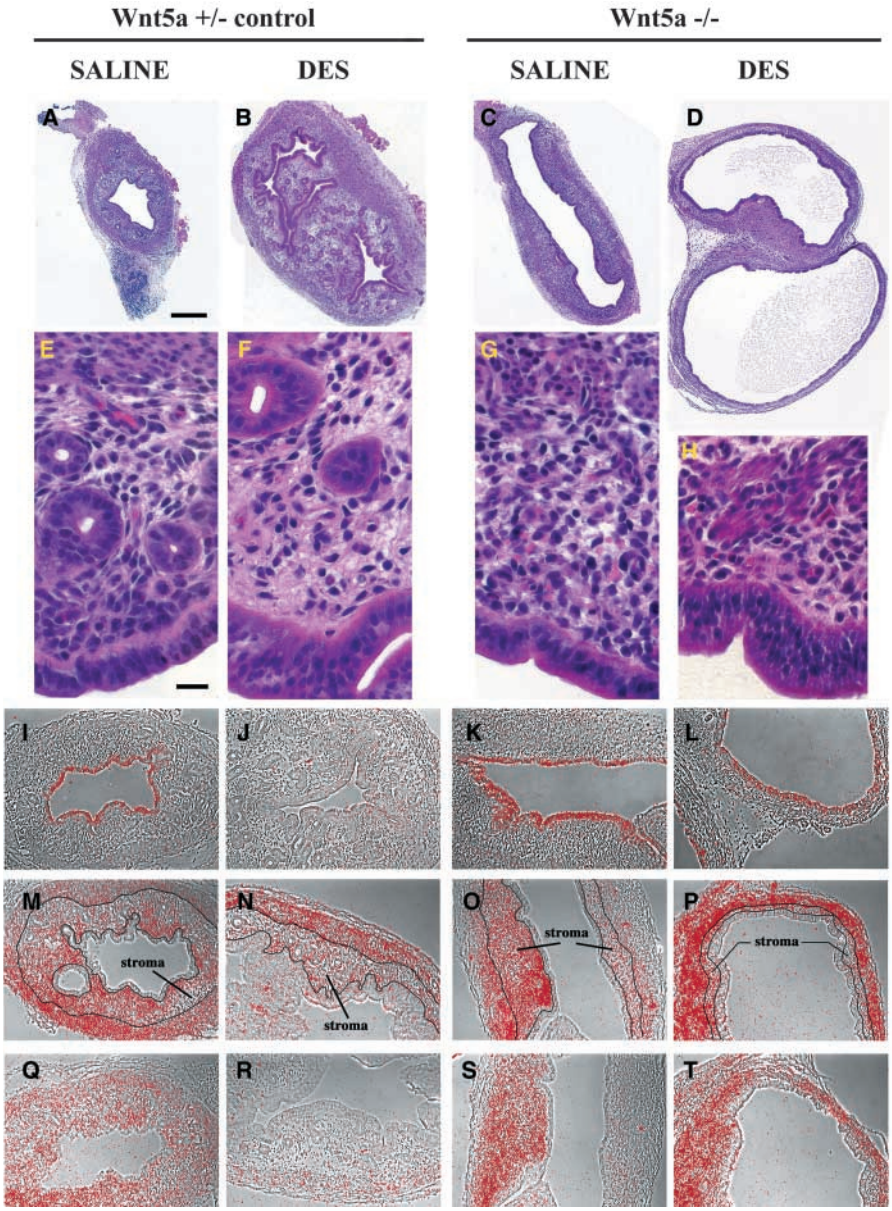

Fig. 6. *Wnt5a* is required for the uterotrophic response and for DES-mediated repression of *Wnt7a*. Neonate (P0) uterine horns from control and *Wnt5a*$^{-/-}$ individuals were separated into two pools of grafts that were grown in two ovariectomized hosts for 3 weeks. Each host received control (two left-hand columns) and mutant (two right-hand columns) grafts. For each experiment, one host was injected intraperitoneally daily from day 18 to day 20 with DES resuspended in saline and one host was injected with saline alone, as indicated. Hosts were sacrificed on day 21 and the grafts harvested for analyses. Results are shown for a *Wnt5a*$^{+/-}$ individual and a *Wnt5a*$^{-/-}$ individual. (A-H) Haematoxylin-Eosin staining at low magnification (A-D; scale bar: 250 μm) and high magnification (E-H; scale bar: 20 μm). Note the aberrant uterotrophic response in the *Wnt5a*$^{-/-}$ graft (D) showing enlarged lumen and thin uterine walls when compared with the *Wnt5a*$^{+/-}$ graft (B). The *Wnt5a*$^{-/-}$ epithelium does show an increase in height and thickness in response to DES (H). (I-T) In situ hybridization for *Wnt7a* (I-L), *Hoxa10* (M-P) and *Hoxa11* (Q-T). *Wnt7a* is repressed by DES in the *Wnt5a*$^{+/-}$ control graft (J) but not in the mutant (L). *Hoxa10* and *Hoxa11* are strongly repressed by DES in the subepithelial stroma of the *Wnt5a*$^{+/-}$ control graft (N,R) but not in the *Wnt5a*$^{-/-}$ graft (P,T). Lines delineate the limits between the luminal epithelium, the stroma and the myometrium.

spatial change in expression similar to the situation observed with *Esr1*. This result and the fact that *Esr1* itself is correctly regulated in the *Wnt5a* mutant (Fig. 8A-F) suggest that *Wnt5a* regulation by estrogenic stimuli is genetically downstream of *Esr1*.

To determine if genes expressed in the epithelium other than *Wnt7a* are misregulated in the *Wnt5a* mutant, we analyzed the regulation of *Msx1*, a homeobox gene whose expression is maintained specifically in the luminal and glandular uterine epithelium of the adult (Pavlova et al., 1994). We found that DES represses *Msx1* in the *Wnt5a* mutant grafts as in control grafts (Fig. 8Q-T) indicating that *Msx1* regulation by estrogen is *Wnt5a* independent in marked contrast to what is seen with other patterning genes examined in this study. Thus, *Msx1* represents a potential developmental and hormone-sensitive pathway that is not subject to control by Wnt genes.

Discussion

The role of the Wnt genes in the developing FRT

The adult FRT expresses multiple members of the Hox and homeogene families, Bmp4, and several members of the Wnt gene family (Ma et al., 1998; Miller et al., 1998b; Pavlova et al., 1994; Taylor et al., 1997; Ying and Zhao, 2000). Mouse mutants generated to the *Hoxa10*, Hox*a11* and *Wnt7a* genes reveal a requirement for these genes during postnatal development (Benson et al., 1996; Hsieh-Li et al., 1995; Miller and Sassoon, 1998; Parr and McMahon, 1998). In cases where gene mutation leads to perinatal lethality, postnatal FRT development has not been examined. Using grafting techniques as a means to circumvent the neonatal lethality of the *Wnt5a* mutant, we find that *Wnt5a* is required to appropriately establish the development of the posterior region of the FRT. *Wnt5a* mutant FRTs have short and coiled uterine horns of normal diameter and lack defined cervical/vaginal structures. These findings are in contrast to our previous observations for the *Wnt7a* mutant FRT, which shows complete posterior development whereas the uterine horns are atrophic (Miller and Sassoon, 1998) (this study). Although the *Wnt5a* and *Wnt7a* phenotypes differ, they share specific characteristics described for the FRT of different *Hox* gene mutants (Fig. 9A). In the *Hoxa13* mutant, the caudal region of the Müllerian ducts does not develop (Warot et al.,

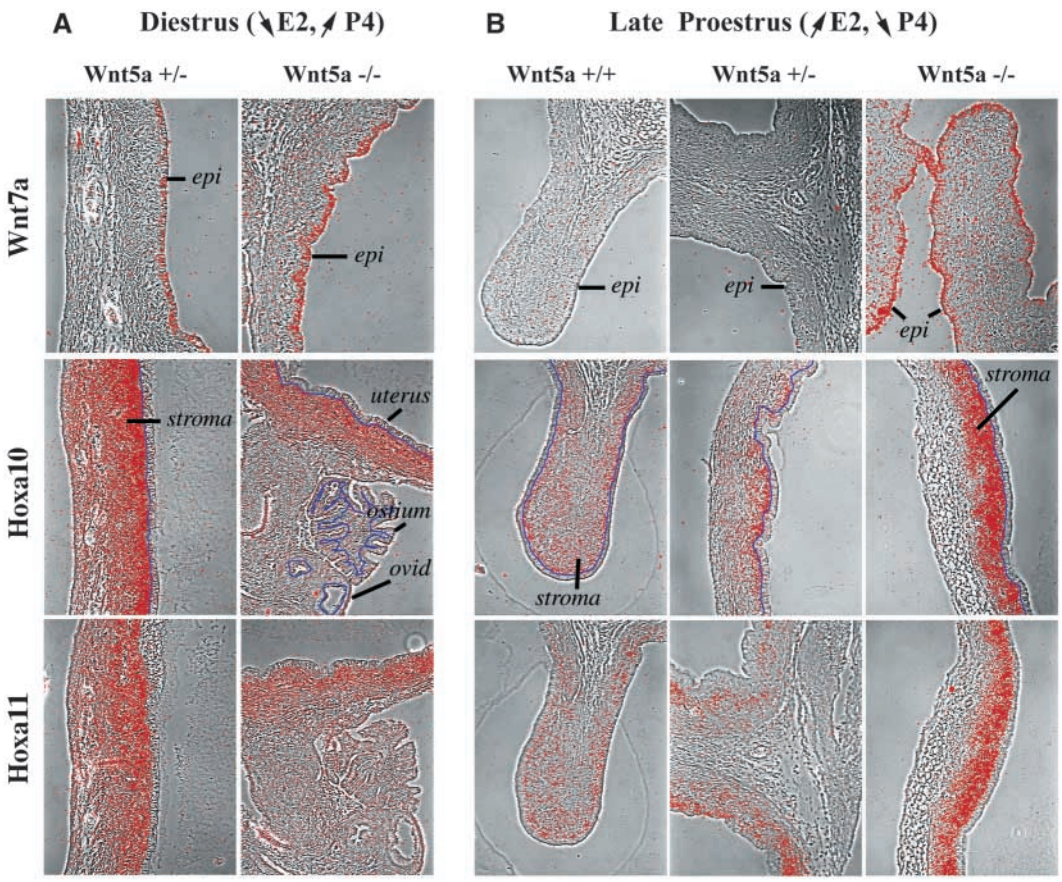

Fig. 7. Regulation of *Wnt7a*, *Hoxa10* and *Hoxa11* genes is deficient in the *Wnt5a* mutant during estrous. Genotypes are indicated at the top and in situ probes on the left. (A) Control and *Wnt5a*$^{-/-}$ grafts were grown for 3 weeks in a cycling host that was sacrificed at diestrus when the level of circulating estrogen is low. *Wnt7a* and *Hoxa* genes expression is normal in the *Wnt5a*$^{-/-}$ graft. Note the expected lack of expression of *Hoxa10* and *Hoxa11* genes in the anterior mutant FRT, i.e. tubular and ostium (infundibulum) region of the oviduct. Lines in the *Hoxa10* photographs delineate the limit between the epithelium and the stroma. (B) As in A but the host was sacrificed at late proestrus when the level of circulating estrogen is high. *Wnt7a* and *Hoxa10* and *Hoxa11* are downregulated in the control wild-type and *Wnt5a*$^{+/-}$ grafts but not in the *Wnt5a*$^{-/-}$ grafts.

1997). In fact other aspects of the phenotypes of posterior *Hoxa* gene mutants and *Wnt5a* mutant are similar as the growth of the genital tubercle and the limb buds is also severely affected in the double *Hoxa13/d13* mutant as in the *Wnt5a* mutant (Warot et al., 1997). Taken together, the phenotypic similarities of *Wnt5a* and *Hoxa13/d13* mutant FRTs suggest that they may act in a common pathway during development to regulate posterior growth of the Müllerian ducts (Fig. 9A). Whereas a phenotypic similarity between *Wnt5a* and posterior *Hoxa* mutant FRTs is found, we note that the atrophic uterine horns of the *Wnt7a* mutants and the reduction of the glandular and stromal compartments in the adult resemble the phenotypes described for the *Hoxa11* and *Hoxa10/Hoxa11* transheterozygotes mutant FRTs (Branford et al., 2000) (Fig. 9A). We reported previously that *Hoxa10* and *Hoxa11* expression is normal at birth in the *Wnt7a* mutant FRT; however, *Hoxa10/11* expression is not maintained in the mature FRT (Miller and Sassoon, 1998). Similarly, the expression of *Wnt7a* is normal in the *Hoxa11* mutant neonates and subsequently declines (data not shown). Therefore *Wnt7a* and *Hoxa11* are independently activated but then maintain expression of each other. These genetic analyses suggest that in addition to their homeotic functions along the anteroposterior axis (Benson et al., 1996; Branford et al., 2000; Hsieh-Li et al., 1995), *Hoxa10* and *Hoxa11* participate in a common morphogenetic pathway with *Wnt7a* that directs growth along the radial axis of uterine horn and subsequent stromal/epithelial differentiations required to generate the glandular compartment (Fig. 9A).

Role of Wnt signaling in uterine glandulargenesis

We found that *Wnt5a* provides a specific signal derived from stromal cells that permits the luminal epithelium to form uterine glands. Little is known regarding the developmental mechanisms that direct gland formation in the FRT (Gray et al., 2001). Analyses of the *Wnt7a* and *Wnt5a* mutants demonstrate the requirement of both genes in glandulargenesis of the uterus (Miller and Sassoon, 1998) (this study). The fact that *Wnt7a* is expressed in uterine epithelium and that *Wnt5a* is expressed in uterine stroma is consistent with longstanding observations that cytodifferentiation of the uterus requires epithelial-mesenchymal paracrine interactions. Although *Wnt5a* is expressed throughout the uterine mesenchyme, we observed that *Wnt7a* is downregulated specifically in the invaginating epithelium that gives rise to the glands during postnatal development. Based upon these observations, we propose that highly regionalized repression of *Wnt7a* is required to allow luminal epithelium to change fate, invaginate and form glands and that *Wnt5a* is required for this downregulation (see model in Fig. 9B). Although global hormonal repression of *Wnt7a* and local repression of *Wnt7a* during pre-pubertal development may well reflect completely different pathways, we find that *Wnt5a* is required for downregulation of *Wnt7a* in response to DES.

The complete loss or transient repression of *Wnt7a* expression during perinatal FRT development leads to global disorganization of the uterine epithelium and a disruption of gland formation later in adult life (Miller et al., 1998a; Miller and Sassoon, 1998). This is in contrast to *Wnt5a* mutant FRT,

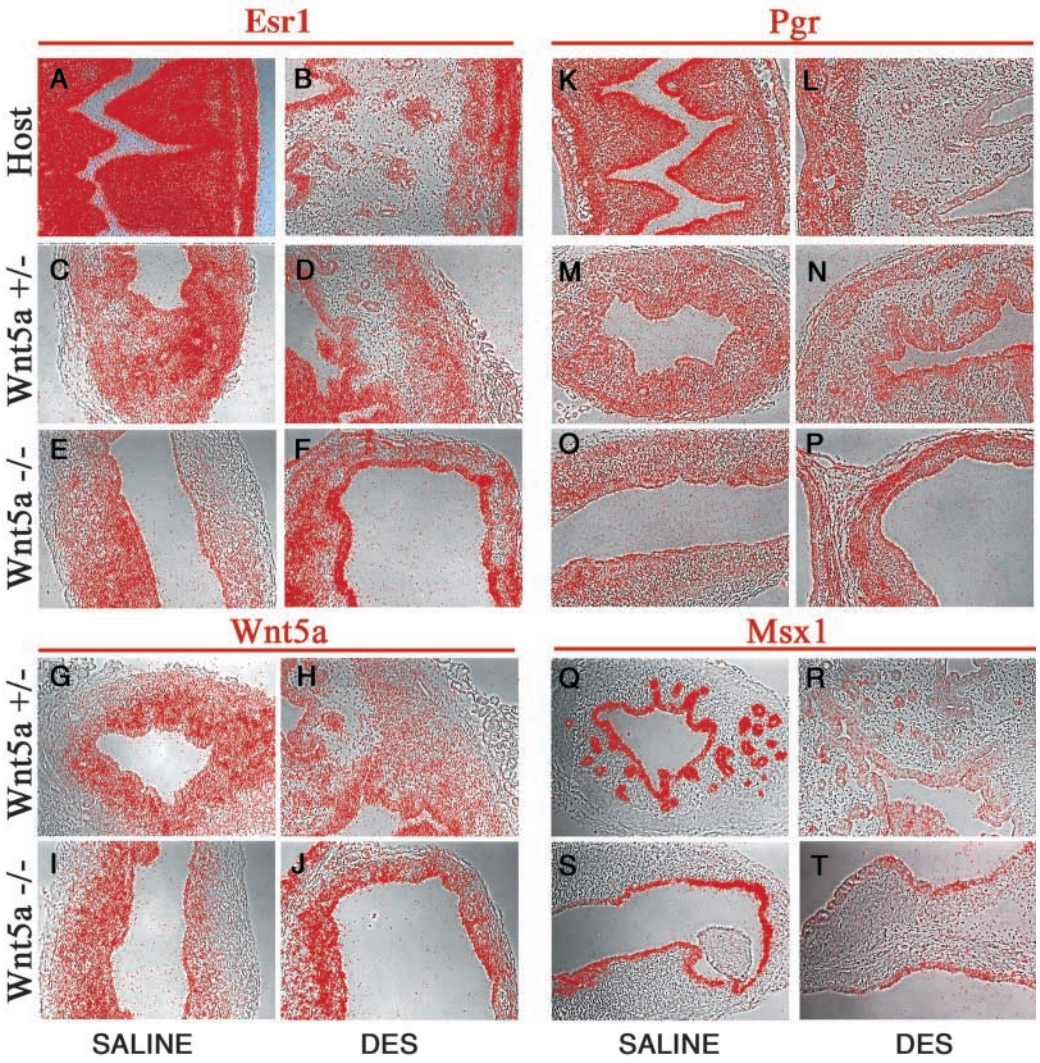

Fig. 8. Estrogen signaling is intact in *Wnt5a* mutant grafts. In situ hybridization for *Esr1* (A-F), *Wnt5a* (G-J), *Pgr* (K-P) and *Msx1* (Q-T) from host uteri and grafts grown in saline or DES conditions, as indicated at the bottom. Genotypes are indicated on the left. *Esr1* expression increases to very high levels in adult mice after ovariectomy (A) but is repressed after prolonged exposure to DES, except in the epithelium and the smooth muscle layer (B). Although lower in 3-week-old grafts, *Esr1* expression is similarly regulated in both control *Wnt5a*$^{+/-}$ (C,D) and *Wnt5a*$^{-/-}$ grafts (E,F). *Wnt5a* is downregulated in the stroma and activated in the epithelium and smooth muscle, and *Wnt5a* mutant transcript is correctly regulated even in absence of *Wnt5a* product (H,J). *Pgr* gene regulation is also identical in control and mutant grafts although *Pgr* is not downregulated in the epithelium from grafts (N,P) as in the host (L), probably because of stage difference between immature 3-week-old grafts and sexually mature host uterus. *Msx1* is repressed by DES in both control (R) and *Wnt5a* mutant grafts (T).

which maintains a normal columnar epithelial phenotype but still fails to form glands. These observations suggest that *Wnt7a* is required to maintain a columnar epithelial phenotype and if downregulation of *Wnt7a* is blocked, gland formation will not occur as seen in the *Wnt5a* mutant. Alternatively, if *Wnt7a* expression is disrupted, epithelial cells may attempt to participate in gland formation giving rise to an abnormal multilayered epithelium that is not permissive for gland formation. Chimeric analyses in mice has shown that uterine glands are monoclonal in origin (Lipschutz et al., 1999), raising the intriguing possibility that the repression of *Wnt7a* may occur in a single cell that then gives rise to a gland (see model in Fig. 9B). We note that *Wnt5a* is expressed throughout the mesenchyme, suggesting that an additional factor may cooperate with *Wnt5a* to restrict glandulargenesis at specific sites of the luminal epithelium. Alternatively, it is possible that *Wnt7a* repression is a stochastic event that occurs in a unique cell and that *Wnt5a* is simply required for subsequent growth. Experiments to address these models are in progress.

Neither *Wnt7a* nor *Wnt5a* has been clearly linked to the canonical Wnt signaling pathway that requires members of the *lef1/tcf1* family. In the chick limb bud, β-catenin and *Lef1* retroviral infections induce morphogenetic outcomes similar to *Wnt3a* infection and distinct from *Wnt7a* overexpression (Kengaku et al., 1998). *Wnt5a* has been implicated in Ca^{2+} signaling and has been demonstrated to antagonize canonical Wnt signaling (Miller et al., 1999; Topol et al., 2003). Our results demonstrate that *Lef1* is dispensable for uterine morphogenesis and gland formation, suggesting that canonical Wnt signaling is not required for Wnt signaling in the uterus. We note that *Tcf1* is also expressed in the uterus and may rescue the lack of *Lef1* in the uterus, although it does not do so in many other structures dependent upon epithelial-mesenchymal interactions previously examined. The early embryonic lethality of the double *Tcf1/Lef1* mutants precludes analyses of FRT development in the double mutant (Galceran et al., 1999).

Wnt5a is required for downregulation of Wnt7a and Hoxa genes by estrogenic stimuli

It has been demonstrated that estrogen induction of uterine epithelial proliferation is dependent upon *Esr1* expression in the stroma which then signals via an unknown ligand to the epithelium (Cooke et al., 1997; Kurita et al., 2000). We have demonstrated previously that DES represses *Wnt7a* in the neonate FRT (Miller et al., 1998a) and it was later

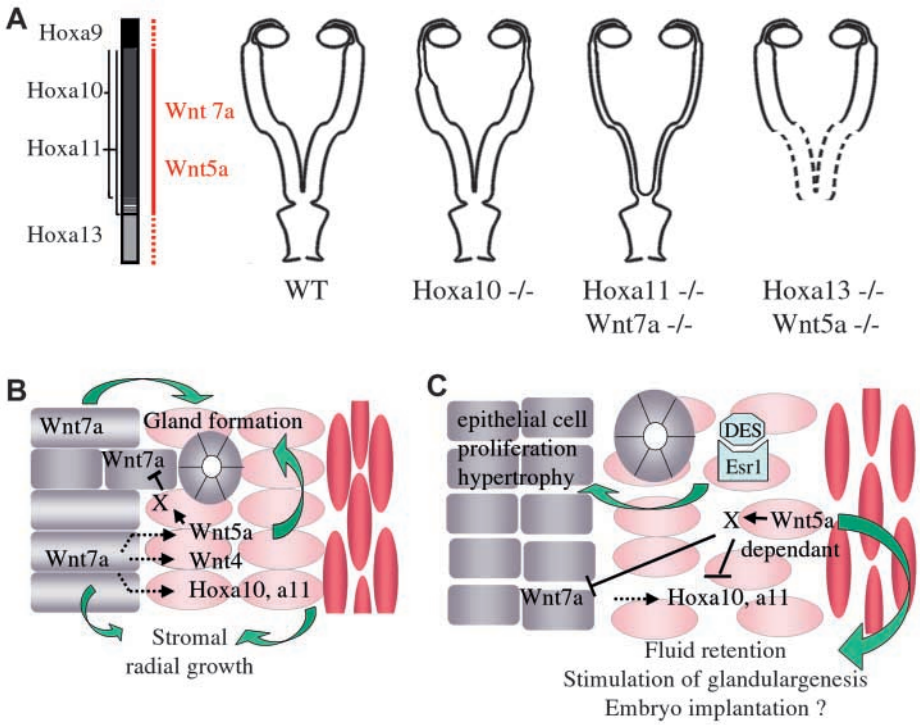

Fig. 9. Role and regulation of Wnt genes during FRT morphogenesis and estrogenic response. (A) Schematic comparison of Hoxa code and Hoxa mutant phenotypes with *Wnt5a* and *Wnt7a* mutant phenotypes in the FRT. During fetal development, all the Hoxa genes, *Wnt7a* and *Wnt5a* are expressed all along the anteroposterior axis of the FRT (not shown). At birth, domains of Hoxa genes expression start to regionalize along the anteroposterior axis of the FRT (see left diagram). The regionalization of *Hoxa10* to the uterine horn slightly precedes regionalization of *Wn7a* and *Wnt5a* also to the uterine horns that occur a few days after birth. The *Hoxa10* mutant phenotype presents a bona fide homeotic transformation of the anterior 25% of the uterine horn into an oviduct-like structure. Loss of *Hoxa11*, or one allele of each *Hoxa10* and *Hoxa11* genes, or loss of *Wnt7a* affects primarily the uterine horns; however, *Wnt7a* phenotype can also affect the oviduct and the vagina. Loss of *Hoxa13* or *Wnt5a* affects the caudal growth of the Müllerian ducts and the growth of the genital tubercle (not shown). (B) Postnatal uterine morphogenesis. *Wnt7a* is required for correct epithelial organization, the radial growth and patterning of the adjacent mesenchymal cells, and the organization of the smooth muscle layers. *Wnt7a* is required for maintenance (dotted arrows) of high levels of *Wnt5a*, *Wnt4*, *Hoxa10* and *Hoxa11* genes. *Wnt5a* signals cooperate with an unknown factor X to allow *Wnt7a* downregulation during gland formation (this study). (C) *Wnt5a*-dependant and *Wnt5a*-independent uterotrophic response to DES. DES binding to stromal *Esr1*, downregulates *Wnt7a* in the epithelium through a factor X that is functional or present only when *Wnt5a* is expressed. The factor X could be the same or different to the factor X required for *Wnt7a* repression during glandulargenesis. DES, through factor X, represses the levels of *Hoxa10* and *Hoxa11* in the stroma either directly or through repression of *Wnt7a*. Correct *Wnt5a* dependant downregulation of *Wnt7a* and Hoxa genes by prolonged estrogenic signal may be involved in the stimulation of glandulargenesis, fluid retention by the stroma and possibly preparation of the uterine wall for embryo implantation.

demonstrated that *Wnt7a* repression requires the expression of *Esr1* in the FRT (Couse et al., 2001). We observe here that downregulation of *Wnt7a* and *Hoxa10* and *Hoxa11* genes by estrogens is abolished in absence of *Wnt5a* (Figs 6, 7). However, *Wnt5a* mutant uterine grafts undergo an abnormal dilation and show an increase in epithelial thickness following DES exposure. Based on these results, we propose that there are *Wnt5a* dependant and independent responses to estrogenic stimulation (Fig. 9C). Factors such as *Msx1* may be part of a *Wnt*-independent regulatory response to estrogen as shown in this study. Candidate factors that link *Wnt5a* to estrogenic signaling may include *Wnt7a* and Hoxa genes that are misregulated in the *Wnt5a* mutant FRT. *Wnt7a* and Hoxa genes are developmental factors required for normal morphogenesis of the FRT (Branford et al., 2000; Gendron et al., 1997; Hsieh-Li et al., 1995; Miller and Sassoon, 1998; Parr and McMahon, 1998; Warot et al., 1997), and are expressed throughout adult life (Benson et al., 1996; Lim et al., 1999; Ma et al., 1998; Miller et al., 1998b; Pavlova et al., 1994). Expression of *Hoxa10* in the uterus is required for successful embryo implantation through the regulation of PGE2 receptors subtypes EP3 and EP4 (Benson et al., 1996; Lim et al., 1999). The expression of genes involved in Wnt signaling is modified during the implantation period (Kao et al., 2002; Paria et al., 2001; Pavlova et al., 1994). We note that *Wnt7a* mutant females are sterile although their ovaries are functional following transplantation into wild-type recipients (Parr and McMahon, 1998). Taken together, these data implicate uterine Wnt gene expression as crucial regulators of uterine adult function.

A system for the analysis of lethal mutant FRTs

We found that wild-type neonate uterine grafts grown in cycling hosts show highly impaired and delayed gland formation. By contrast, neonate uterine fragments grown in ovariectomized hosts develop normally and form uterine glands. We conclude that precocious exposure to endogenous adult levels of ovarian hormones is sufficient to disrupt crucial perinatal patterning events in the FRT. Indeed, precocious exposure to DES, 17 β-estradiol, progestin or tamoxifen alter FRT morphogenesis and glandulargenesis (Branham et al., 1985a; Branham et al., 1985b; Cunha et al., 1991; Gray et al., 2001). The mechanisms underlying how hormonal teratogens permanently alter FRT development have not been completely elucidated; however, these studies support a model whereby precocious exposure to estrogens exerts a teratogenic effect upon the FRT through a perturbation of patterning gene expression in the FRT and a permanent change in gene regulation in response to hormone challenge.

We thank T. Kurita and G. Cunha (UCSF) for teaching us the renal capsule grafting technique and fruitful discussions. We thank Drs B. Kaiser (Bronx Hospital) and L. Carta (Mount Sinai Hospital) for

discussions and technical advice. This work was supported by NIH-NIA R01 AG13784 to D.S. and J.K., and a grant awarded by NIEHS through the Superfund Basic Research Program (P42 ES07384) to D.S.

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