Non-canonical Wnt signaling regulates cell polarity in female reproductive tract development via van gogh-like 2

Alysia L. vandenBerg and David A. Sassoon*

Wnt signaling effectors direct the development and adult remodeling of the female reproductive tract (FRT); however, the role of non-canonical Wnt signaling has not been explored in this tissue. The non-canonical Wnt signaling protein van gogh-like 2 is mutated in loop-tail (Lp) mutant mice (Vangl2Lp), which display defects in multiple tissues. We find that Vangl2Lp mutant uterine epithelium displays altered cell polarity, concomitant with changes in cytoskeletal actin and scribble (scribbled, Scr1) localization. The postnatal mutant phenotype is an exacerbation of that seen at birth, exhibiting more smooth muscle and reduced stromal mesenchyme. These data suggest that early changes in cell polarity have lasting consequences for FRT development. Furthermore, Vangl2 is required to restrict Scr1 protein to the basolateral epithelial membrane in the neonatal uterus, and an accumulation of fibrillar-like structures observed by electron microscopy in Vangl2Lp mutant epithelium suggests that mislocalization of Scr1 in mutants alters the composition of the apical face of the epithelium. Heterozygous and homozygous Vangl2Lp mutant postnatal tissues exhibit similar phenotypes and polarity defects and display a 50% reduction in Wnt7a levels, suggesting that the Vangl2Lp mutation acts dominantly in the FRT. These studies demonstrate that the establishment and maintenance of cell polarity through non-canonical Wnt signaling are required for FRT development.

KEY WORDS: Vangl2, Wnt, Female reproductive tract, Uterus

INTRODUCTION

Mammals possess male and female reproductive tract (FRT) anlage during early development (E13.5) (Yin and Ma, 2005). The Müllerian duct epithelium in mice remains undifferentiated until ~5 days after birth and consists of columnar epithelium surrounded by mesenchyme (Boutin et al., 1992; Cunha, 1976c; Glasser et al., 2002). Subsequently, the posterior epithelium stratifies in the presumptive cervix and vagina, while anterior epithelium forms the uterine horns and oviducts (Kitajewski and Sassoon, 2000; Yin and Ma, 2005). By 1 week of development, uterine epithelial glands and smooth muscle form, and by 2 weeks the FRT is differentiated. Epithelial-mesenchymal interactions underlie FRT development (Cunha et al., 2004; Cunha et al., 1992b; Kitajewski and Sassoon, 2000). In tissue recombination experiments, grafting perinatal Müllerian mesenchyme from presumptive vagina to epithelium from any part of the FRT induces stratified epithelium (Cunha, 1976b; Cunha et al., 1992b; Pavlova et al., 1994), demonstrating that the mesenchymal signals dictate epithelial fate. Mesenchyme grown alone develops entirely into smooth muscle (Boutin et al., 1992; Cunha et al., 1992a).

Wnt signaling molecules, including Wnt4 (Bernard and Harley, 2007; Miller et al., 1998b), Wnt5a (Mericskay et al., 2004; Miller et al., 1998b), Wnt7a (Carta and Sassoon, 2004; Miller et al., 1998a) and Wnt9b (Carroll et al., 2005), regulate FRT development. Wnt9b signals upstream of Wnt4 and both direct Müllerian tube formation (Carroll et al., 2005). At birth, Wnt5a mutants lack a cervix and vagina, and uterine horns are shortened and fuse at the midline or terminate in a blind pouch (Mericskay et al., 2004). Neonatal Wnt7a mutants have a septate vagina, with small diameter uterine horns and a lack of oviduct coiling (Miller and Sassoon, 1998). Both Wnt5a (Mericskay et al., 2004) and Wnt7a (Miller et al., 1998a) mutant postnatal uterine tissues fail to form glands, and Wnt7a mutants display hyperplastic and disorganized smooth muscle with stratified uterine epithelium.

Wnt5a expression in the mesenchyme (Mericskay et al., 2004) and Wnt7a expression in the epithelium of the oviduct and uterus (Miller and Sassoon, 1998) are required for proper FRT development (Mericskay et al., 2004; Miller and Sassoon, 1998). In Wnt7a mutants, Wnt5a is expressed ectopically in epithelium and its expression declines by 12-16 weeks (Mericskay et al., 2004) and Wnt4 expression is misregulated (Miller et al., 1998a). By contrast, Wnt5a mutants display a reduction in the expression of Wnt4a, whereas Wnt7a expression is unaffected unless challenged with estrogen (Mericskay et al., 2004).

Wnt molecules signal through three known pathways: the canonical β-catenin-dependent pathway (Logan and Nusse, 2004), non-canonical CnKa-mediated signaling (Wang and Malbon, 2003), and non-canonical signaling orchestrating cell migration and movement (Mlodzik, 2002). Transgenic mice lacking β-catenin in the mesenchyme of the FRT do not recapitulate the Wnt5a or Wnt7a mutant phenotypes (Arango et al., 2005; Deutscher and Hung-Chang Yao, 2007), suggesting that canonical Wnt signaling involves both pathways. We previously noted that Wnt7a knockout mice display changes in cell orientation of the FRT at birth (Miller et al., 1998a; Miller et al., 1998b). FRT developmental defects have been described for loop-tail mice that contain a point mutation in van gogh-like 2 (Vangl2Lp) (Kibar et al., 2001), including a septate vagina (Murdoch et al., 2001; Strong and Hollander, 1949), cell polarity defects in the cochlea (Montcouquiol et al., 2003) and disruptions in cardiac outflow tract (Phillips et al., 2005) owing to defects in cell movement (Phillips et al., 2008).

Drosophila Strabismus (Van Gogh – FlyBase), the homolog of van gogh-like 2, is required for the planar cell polarity (PCP) establishment of eyes and wing bristles (Kibar et al., 2001; Wolff and Rubin, 1998). Core non-canonical Wnt signaling components, which are conserved in Xenopus, Drosophila and mammals,
Developmentally coordinate to regulate developmental processes requiring cell movement, including convergent extension, PCP in *Drosophila* eyes and wing bristles and in mouse cerebellum, and murine epidermal hair patterning (Klein and Mlodzik, 2005). These signaling components have been investigated in *Xenopus* and *Drosophila*, and recent studies suggest a related function in mice. The proteins involved in neural tube closure include *Wnt5a* and *Wnt11b* (Hardy et al., 2008), frizzled 6 (*Fzd6*) (Guo et al., 2004; Wang et al., 2006b) and downstream non-canonical Wnt signaling components such as mouse *Vangl2* (Montcouquiol et al., 2003; Phillips et al., 2005) and dishevelled 1 (*Dvl1*) and *Dvl2* (Hamblet et al., 2002). PCP in mouse cerebellum uses the same signaling module, as shown by defects observed in *Vangl2* mutants and in *Fzd3*; *Fzd6* and *Dvl1*; *Dvl2* double mutants (Montcouquiol et al., 2003; Wang et al., 2005; Wang et al., 2006b). PCP also functions in the murine epidermis: *Fzd6* mutants have whorled hair patterns (Guo et al., 2004) and *Vangl2* mutants display a loss of hair follicle polarization (Devéneport and Fuchs, 2008).

Murine *Vangl2* contains multiple PDZ domains, which mediate scribble (scribbled, Scrb1) binding (Montcouquiol et al., 2006b). Scrb1 also contains multiple PDZ domains and functions in cell polarity and migration in different capacities through interactions with multiple binding partners. In polarized epithelium, Scrb1 binds lethal giant larva 2 (*Lgl2*) (Kallay et al., 2006) and functions with discs large (Dlg) to restrict the Par3-Par6 (Par) complex to the apical membrane (Macara, 2004). The Par complex, in turn, restricts the Scrb1 complex to the basolateral membrane (Bilder et al., 2003; Tanentzapf and Tepass, 2003). In *Drosophila*, loss of either Lgl, Dlg or Scribble induces expansion of the Par-expressing apical domain (Bilder et al., 2000), suggesting that these two groups of proteins reciprocally maintain the apical and basolateral domains. In mammalian MDCK cells, E-cadherin-mediated cell adhesion is disrupted by the loss of Scrb1 and restored by expression of an E-cadherin-catenin fusion protein (Qin et al., 2005), and Scrb1 interacts with the tight junction protein ZO-2 (Tip2) as determined by immunoprecipitation (Metais et al., 2005), suggesting that it plays a role in cell-cell adhesion. In a process that is not well understood, Scrb1 is required to establish cell polarity for the migration of epithelial cells (Dow et al., 2007) and T-cells (Ludford-Menting et al., 2005), which might involve the ability of Scrb1 to bind p21-activated kinase (Pak) interacting exchange factor (*B-PiX; Arhgef7*) (Audebert et al., 2004), which activates GTPases such as CDC42/Rac (Sinha and Yang, 2008). Other Vangl2 binding interactions in mice include the epithelial tight junction protein Magi3 (Laura et al., 2002; Yao et al., 2004) and Dvl1, Dvl2 and Dvl3 (Torban et al., 2004). The *Vangl2* mutation genetically interacts with genes involved in neural tube closure in mice including *Vangl2* (Montcouquiol et al., 2003; Phillips et al., 2005) and dishevelled 1 (*Dvl1*) and *Dvl2* double mutants (Montcouquiol et al., 2003; Wang et al., 2005; Wang et al., 2006b). PCP also functions in the murine epidermis: *Fzd6* mutants have whorled hair patterns (Guo et al., 2004) and *Vangl2* mutants display a loss of hair follicle polarization (Devéneport and Fuchs, 2008).

**Materials and Methods**

**Mouse breeding.** *Wnt7a* and loop-tail mice were obtained from Jackson Labs (Bar Harbor, Maine, USA); loop-tail mice were maintained on a CBA background, and *Wnt7a* mice were bred to the loop-tail line for double-heterozygote analysis. Mice were housed under standard conditions with 12-hour light-dark cycles.

**RESULTS**

**At birth, *Vangl2* mutant female reproductive tracts display gross morphological and histological defects similar to those of *Wnt7a* mutants.** E18.5 *Vangl2* mutant FRTs displayed several overt defects in their gross morphology as compared with wild-type FRTs (Fig. 1). There was a striking defect in the fusion of uterine horns at the level of the cervix (Fig. 1C, B), as previously described (Murdoch et al., 2001; Strong and Holland, 1949). *Vangl2* mutant mice also lacked...
Vangl2 regulates uterine development

Fig. 1. Vangl2Lp mutants have gross morphological defects at E18.5. ~50% of Vangl2Lp heterozygote adult females are infertile and have a septate vagina. Wild-type (A) and Vangl2Lp/Lp mutant (B,C) E18.5 mouse female reproductive tract (FRT). The mutant demonstrates a lack of oviduct coiling and a septate vagina (arrows in C). (D) Wild-type adult FRT. (E) About 50% of Vangl2Lp heterozygote FRTs are greatly enlarged and fluid-filled, indicating a vaginal blockage. ovi, oviduct; ov, ovary; ut, uterine horn; cx, cervix; vg, vagina; bl, bladder. Scale bars: 1 mm in A,B; 0.5 mm in C; 5 mm in D,E.

Oviduct coiling (Fig. 1B) and uterine horns appeared shortened (Fig. 1B). We found that about half of the adult female heterozygous Vangl2Lp mice were infertile; many exhibited an increased vaginal and anal opening space. As infertile females reached 4 to 6 months of age, approximately half displayed an enlarged abdomen owing to the presence of fluid-filled and grossly extended uterine horns (Fig. 1D). We noted, however, that the heterozygous Vangl2Lp cervix and vagina appeared normal. In one case (n=3), the outer vaginal opening formed a blind pouch that did not connect to the internal reproductive organ (data not shown).

Plastic semi-thin sections taken from Vangl2Lp mutant and wild-type littermate controls at E18.5 demonstrated several striking differences (Fig. 2A,B). First, Vangl2Lp mutant uterine tissue displayed a disorganized cellular structure in comparison with wild-type littersmates. Specifically, we observed differences in the orientation and organization of mesenchymal cells. Mesenchymal cells underlying the epithelium aligned their cell axes with the underlying epithelium (Fig. 2A,C), whereas mesenchymal cells in Vangl2Lp mutant uteri were misaligned (Fig. 2B,D). We observed differences in the organization of the Vangl2Lp mutant epithelium (Fig. 2B) and an overall rounder lumen (n=5), although the wild type does have a round lumen early in development. The edges of the epithelial cells facing the lumen appeared smoother and more closely packed together in the Vangl2Lp mutant compared with the wild type.

Higher resolution examination of the Vangl2Lp mutant epithelium using transmission electron microscopy (EM) revealed that the epithelium of mutant uteri (Fig. 2D) does not appear columnar as compared with the wild type (Fig. 2C). Instead, the mutant epithelium consisted of multiple cell layers and rounder nuclei. Although higher magnification did not reveal defects in either desmosomes or tight junctions per se, we observed electron-dense fibrillar-like structures in the area of the tight junctions of Vangl2Lp mutant epithelium (Fig. 2F), in contrast to the situation in the wild type (Fig. 2E). These results prompted us to observe epithelial cell tight junction markers more closely.

E-cadherin staining reveals a defect in uterine epithelial morphology in Vangl2Lp mutants

E-cadherin is expressed ubiquitously in epithelial cell types (Butz and Larue, 1995). E-cadherin localization appeared normal in Vangl2Lp mutant epithelium as compared with wild type (Fig. 2G,H). However, as shown in Fig. 2I,J, we noted ectopic epithelial cell layers in the Vangl2Lp image. In rare cases (n=3 images from two different mutants), these abnormal epithelial cells appeared to lose contact with neighboring cells and reside in the lumen (Fig. 2H, arrow). Generally, there were 2 to 4 layers of rounded epithelial cells in Vangl2Lp mutant uteri, rather than the 1 to 2 layers of elongated columnar epithelial cells in the wild type. Given these changes in Vangl2Lp mutant epithelium, it seemed possible that proliferation might be altered; however, immunofluorescence (IF) staining for the Ki67 (Mki67) proliferation marker revealed no significant differences between mutant and wild-type sections at E18.5 (data not shown).

Vangl2 protein localizes to the apical edges of epithelial cell membranes and to glands

We performed IF analysis on uterine sections using an antibody to the Vangl2 protein (Fig. 3) (Montcouquiol et al., 2006b). At E18.5, Vangl2 protein was found in uterine epithelial cells and appeared to be membrane localized and enriched at the lateral edges near the luminal apical edge of wild-type epithelial cells (Fig. 3A,C). In 1-month-old uterine samples, Vangl2 protein was localized throughout the entire periphery of the epithelial membrane, was concentrated at lateral cell edges and enriched at the apical portion of these cell-cell contacts (Fig. 3D,E). Also, Vangl2 protein localized to the cell membranes within glands (Fig. 3E), but with uniform distribution throughout the membrane. The same Vangl2 localization pattern was observed in reproductively mature 2-month-old adult uterine tissue, and in E18.5 vaginal epithelium (data not shown). As shown in Fig. 3B, almost undetectable levels of Vangl2 staining were observed in Vangl2Lp mutants, consistent with previous observations (Montcouquiol et al., 2006b).

Vangl2Lp mutant uteri display defects in cytoskeletal actin polarization

Given the gross morphological changes in Vangl2Lp mutant uteri, and the demonstrated involvement of the Vangl2 protein in PCP, we undertook a molecular analysis of Vangl2Lp mutants to characterize protein localization for known polarity markers and Vangl2-interacting proteins in the FRT. Phalloidin staining of filamentous cytoskeletal actin demonstrated that actin polarizes to the apical edges of epithelial cells in wild-type uteri at E18.5 (Fig. 4A,C), whereas in Vangl2Lp mutants the polarization of cytoskeletal actin was markedly reduced (Fig. 4B,D). Serial examination of z-plane optical sections revealed gross cytoskeletal actin polarity defects throughout the section (compare Movie 1 with Movie 2 in the supplementary material). However, IF localization of Cdc42 and
RhoA proteins did not reveal any differences between the wild type and Vangl2<sup>−/−</sup> mutant (data not shown), suggesting that the defect in cytoskeletal actin polarization is not due to any detectable defect in the localization of either of these polarity proteins.

**Vangl2<sup>−/−</sup> mutant epithelium has defects in scribble localization**

Alterations in the morphology and cellular organization of mutant epithelium prompted us to further explore whether the integrity of cell-cell interactions was impaired in Vangl2<sup>−/−</sup> mutants. IF localization was performed against the tight junction protein ZO-1. No differences were detected between mutant and wild type (data not shown). Scrib1 protein interacts with Vangl2 and this interaction can be detected by protein co-immunoprecipitation (Kallay et al., 2006; Montcouquiol et al., 2006a). IF localization on E18.5 uterine sections (Fig. 4E-H) revealed an accumulation of Scrib1 at the apical edges of Vangl2<sup>−/−</sup> mutant epithelial cells (Fig. 4H) as compared with wild type (Fig. 4G), with an apparent increase in the intensity of Scrib1 staining in mutants. Scrib1 functioned together with Lgl2 and Dlg to establish apical/basolateral polarity (Bilder et al., 2003; Humbert et al., 2003; Tanentzapf and Tepass, 2003) and is localized to the basolateral membrane of MDCK epithelial cells and human uterine cervical epithelial tissues (Nakagawa et al., 2004). This result suggests that Vangl2 protein is required to restrict Scrib1 to the basolateral domain of the uterine epithelial membrane.

**Grafting to enable observation of postnatal development of Vangl2<sup>−/−</sup> FRT**

As Vangl2<sup>−/−</sup> mutants die at birth, a grafting technique using ovariectomized nude mouse hosts was used to observe postnatal FRT development (Cunha, 1976a). Briefly, the grafting technique consists of placing fetal reproductive tissues under the renal capsule of the host nude mouse and then harvesting the grafted tissue at various time points. Our previous studies show that 2 weeks is sufficient time to obtain completely normal tissue architecture when grown in ovariectomized hosts, while avoiding precocious estrogen exposure during reconstitution of the tissue and cellular morphology (Mericskay et al., 2004). As expected, wild-type E18.5 tissue developed all of the normal histological features of wild-type FRT in situ, including glands and smooth muscle. By contrast, grafts of mutant tissue generated all the proper cell types but showed an overall exacerbation of the disorganized phenotype seen at birth (Fig. 5). The histological defects seen in Vangl2<sup>−/−</sup> heterozygote and mutant grafted uterine tissue were generally an exacerbation of the phenotype observed at E18.5 (Fig. 2). Specifically, the epithelium became highly pseudostratified, and other unusual epithelial morphologies were present in both Vangl2<sup>−/−</sup> heterozygotes (Fig. 5C-H) and homozygotes (Fig. 5I-N) that were not present in wild-type grafts (Fig. 5A,B). Accellular material accumulated in the lumen of a Vangl2<sup>−/−</sup> heterozygote (Fig. 5C,D). We also observed pseudostratified epithelium (Fig. 5F,H) with a large band of Eosin-stained material towards the basolateral cell edge (compare Fig. 5F with 5B). What appeared to be cell protrusions into the lumen were occasionally seen at high magnification in Vangl2<sup>−/−</sup> heterozygous (Fig. 5H) and homozygous (data not shown) mutants. An increase in the number and size of lipid-like vesicles or vacuoles was noticeable in homozygous (Fig. 5J) and heterozygous (Fig. 5F,H) mutants. Only some of these vesicles stained positively with Oil Red O (data not shown), suggesting that they are not composed entirely of lipid vesicles. Finally, regions of epithelium that appeared hyperpolarized were observed in homozygous mutants (Fig. 5K,L). Delaminated epithelial cells were observed in two of four

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**Fig. 2. Vangl2<sup>−/−</sup> mutants display alterations in cytoarchitecture at E18.5, including the loss of typical columnar epithelial cell morphology.**

(A, B) Wild-type (A) and Vangl2<sup>−/−</sup> mutant (B) mouse uterine semi-thin cross-sections reveal a change in uterine lumen shape in the mutant (it is more rounded) as well as disorganized mesenchyme and non-columnar epithelium. (C-F) Transmission EM of wild type (C,E) and Vangl2<sup>−/−</sup> mutant (D,F) reveals ultrastructural changes associated with the loss of columnar uterine epithelial cell morphology in mutants. Near the tight junctions, electron-dense fibrillar structures are seen in the mutant (F, arrows), but are rarely visible in wild type (E).

(G-J) Immunofluorescent confocal staining of E18.5 wild-type (G,I) and mutant (H,J) uterine sections with E-cadherin antibody. Note the nearly detached epithelial cell (H, arrow). E-cadherin is enriched at the apical edge (J, arrows); note increased number of cell layers of E-cadherin-staining epithelium in the Vangl2<sup>−/−</sup> mutant (J, arrows). Scale bars: 10 μm in C,D,G,H; 5 μm in A,B,I,J; 1 μm in E,F.
independent grafts (Fig. 5M,N). To assess the overall phenotypic changes observed in H&E-stained grafts, a qualitative analysis was performed on blinded tissue sections (Table 1). We concluded that \textit{Vangl2}\textsubscript{Lp} heterozygotes demonstrate minor changes in pseudostratification and in epithelial vesicles compared with wild-type grafts. \textit{Vangl2}\textsubscript{Lp} mutants, however, had a marked reduction in total mesenchyme and increase in smooth muscle (Table 1), as observed by H&E staining.

**Postnatal grafts of \textit{Vangl2}\textsubscript{Lp} FRT have altered F-actin, E-cadherin, scribble and smooth muscle actin staining**

IF staining of uterine tissue grafted for 2 weeks demonstrated abnormal localization of polarity markers. Filamentous actin is normally localized as a faint band of membrane staining at the apical edge of wild-type cells (Fig. 6B). In \textit{Vangl2}\textsubscript{Lp} homozygous mutant grafts, stronger actin staining was apparent, which extended to adjacent cells (Fig. 6E) rather than being localized to apical membranes directly adjacent to the lumen. Filamentous actin staining was evident around the entire cell periphery in some epithelial cells of homoygous mutants (see Fig. 6E, arrows), whereas
Vangl2Lp mutants had uneven E-cadherin staining and had lost the E-cadherin enrichment at regions of cell-cell contact (Fig. 6F, arrows). Colocalization analysis of these markers demonstrated that filamentous actin has a more limited apical expression domain than E-cadherin in wild type (Fig. 6A), whereas in Vangl2Lp mutants the actin and E-cadherin staining overlapped (Fig. 6D).

Scrb1 distribution was perturbed in postnatal grafted heterozygous and homozygous Vangl2Lp mutant tissue. In the wild type, Scrb1 was localized predominantly to regions where epithelial cells invaginate (Fig. 6G) and to puncta at cell-cell contacts where tight junctions are located (Fig. 6J), adjacent to regions that stained strongly for Vangl2 localization (Fig. 3D,E), consistent with previous observations that Scrb1 binds Vangl2 (Kallay et al., 2006; Montcouquiol et al., 2003). By contrast, the entire multi-layered population of pseudostratified epithelium (Fig. 6H,K) and homozygous (Fig. 6I,L) Vangl2Lp mutant epithelium stained the entire membrane more strongly than in wild type (Fig. 6G,J). Apically located heterozygous and homozygous mutant epithelial cells had increased Scrb1 at cell-cell contacts (Fig. 6H,I,L) and the expression domain was larger than in the wild type (Fig. 6J, arrow); in the underlying layers of mutant epithelial cells, Scrb1 was uniformly localized throughout the entire cell membrane (Fig. 6H,I,L).

H&E staining of grafts showed an increase in smooth muscle in postnatal grafted Vangl2Lp tissue (Fig. 5; Table 1). IF confirmed that smooth muscle actin-stained tissue was increased in postnatal grafted heterozygous (Fig. 6N) and homozygous (Fig. 6O) Vangl2Lp mutant tissue, as compared with wild type (Fig. 6M), with a corresponding decrease in total mesenchyme (double-headed arrows in Fig. 6M,N; arrowheads in Fig. 6O).

Vangl2Lp mutant uteri have reduced Wnt7a expression
Several Wnt signaling members function in FRT development and, given the similarities in gross morphology between Vangl2Lp mutants and Wnt7a mutants, we measured the expression of several Wnt genes using quantitative RT-PCR at E18.5 (Fig. 7). Both heterozygous and homozygous Vangl2Lp mutants displayed a significant reduction in Wnt7a (Fig. 7A). Diethylstilbestrol (DES) downregulates Wnt7a expression as previously described (Mericssay et al., 2004; Miller and Sassoon, 1998). We observed that DES reduced Wnt7a transcription to very low levels: 9% of wild type, compared with a 52-60% reduction in Vangl2Lp mutants. This suggests that the Vangl2Lp mutation partially perturbed Wnt7a expression. Neither Wnt5a nor Wnt4 expression was altered in either heterozygous or homozygous Vangl2Lp mutants (Fig. 7B,C). However, perinatal DES exposure notably altered Wnt4 and Wnt5a expression as expected. This result suggests that the moderate disruption of Wnt7a expression in Vangl2Lp heterozygous and homozygous mutants was not sufficient to alter expression of the other Wnt genes in neonatal tissues.
### Table 1. Phenotypic characterization of postnatal grafted tissue

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Vangl2Lp mutants grafts have an increased index of abnormal phenotypes as observed by H&E staining. Column headings refer to histological phenotypes observed by microscopic observation of the entire section of H&E-stained grafted tissue and designate the following: Glands, the total number of glands; Pseudostratified, the degree of pseudostratified epithelium; Vesicles, lipid vesicles or vacuoles of increased size and/or number; Mesenchyme to muscle, the proportion of total mesenchyme versus total muscle (> indicates more mesenchyme than muscle); Cell protrusions, epithelial cell protrusions or extensions projecting into the lumen. The phenotypic index was determined by calculating the average value for each genotype as shown in the “average” row.

Lp, Vangl2Lp mutation; WT, wild type; n.a., not applicable; n.d., not done.

### Table 2. Ratio of pups obtained from Wnt7a and Vangl2Lp crosses

<table>
<thead>
<tr>
<th>Number of mice</th>
<th>Progeny</th>
<th>WT; WT</th>
<th>Vangl2Lp/+; Wnt7a+/-</th>
<th>Wnt7a-/-</th>
<th>Vangl2Lp/+; Wnt7a-/-</th>
<th>Vangl2Lp/+; Wnt7+/+</th>
<th>Vangl2Lp/-/-; Wnt7+/+</th>
<th>Vangl2Lp/-/-; Wnt7a-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>53</td>
<td>Female</td>
<td>16</td>
<td>0</td>
<td>22</td>
<td>6</td>
<td>3</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>Male</td>
<td>7</td>
<td>0</td>
<td>12</td>
<td>3</td>
<td>0</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>91</td>
<td>Total</td>
<td>23</td>
<td>0</td>
<td>34</td>
<td>9</td>
<td>3</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>% Female</td>
<td>30.2</td>
<td>0.0</td>
<td>41.5</td>
<td>11.3</td>
<td>5.7</td>
<td>11.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Male</td>
<td>18.4</td>
<td>0.0</td>
<td>31.6</td>
<td>7.9</td>
<td>0.0</td>
<td>42.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Total</td>
<td>25.3</td>
<td>0.0</td>
<td>37.4</td>
<td>9.9</td>
<td>3.3</td>
<td>24.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Expected</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
<td>25.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Difference</td>
<td>-12.8</td>
<td>12.5</td>
<td>-12.4</td>
<td>2.6</td>
<td>9.2</td>
<td>0.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Wnt7a+/+ females were crossed to Wnt7a-/-; Vangl2Lp/+; Wnt7a+/- double heterozygotes. Expected Mendelian ratios are given in the penultimate row. \( \chi^2 < 0.01 \).

Lp, the Vangl2Lp mutation; WT, wild type; Wnt7a is Wnt7a-/-.

Genetic interactions between Vangl2Lp and Wnt7a

When Wnt7a heterozygote females were crossed to Wnt7a+/-; Vangl2Lp/+ double heterozygotes we observed a genetic interaction between the Vangl2Lp and Wnt7a alleles (Table 2). The expected ratio of Vangl2Lp/+; Wnt7a+/- offspring is 12.5%, whereas we obtained 3.3% of mice with this genotype, suggesting either a semi-lethal interaction when one allele of Vangl2Lp is in the context of a homozygous Wnt7a mutation, or an enhancement of the Vangl2Lp phenotype in the Wnt7a mutant background. There was a gender bias with double-heterozygous offspring: 11.3% females and 42.1% males were obtained, versus an expected ratio of 25%. This suggests that male pups might have reduced lethality.

### DISCUSSION

#### Morphological defects of Vangl2Lp mutants partially overlap with those of Wnt7a mutants

We observe phenotypic overlap among Wnt7a and Vangl2Lp mutants, consistent with observations that Wnt7a levels are lower in Vangl2Lp mutants. Nonetheless, a partial decrease in Wnt7a expression in Vangl2Lp mutants cannot explain all of the gross morphological defects observed. The uterine horns of Vangl2Lp mutants fail to fuse at the cervix, as previously described (Strong and Sassoon, 1998), and oviduct coiling is lacking, similar to what we and others observe in Wnt7a mutants (Miller et al., 1998a; Miller and Sassoon, 1998; Parr and McMahon, 1998). However, other phenotypes observed suggest that additional pathways are affected in Vangl2Lp mutants. Uterine horns appear shortened in Vangl2Lp mutants, consistent with a role in convergent extension and rostral-caudal lengthening, as observed in shortened Xenopus embryos with a loss-of-function in Trilobite (homolog of Van Gogh) (Darken et al., 2002; Goto and Keller, 2002; Jessen et al., 2002; Park and Moon, 2002), as well as the shorter temporal bones and cochlea in Vangl2Lp mice (Montcouquiol et al., 2003). Although the uterine horns of Wnt7a mutants are smaller in diameter (Miller and Sassoon, 1998), this defect is not found in Vangl2Lp mutants. Wnt7a heterozygous uterine tissues that most closely resemble the partial decrease in Wnt7a expression observed in Vangl2Lp mutants display increased gland formation in the postnatal uterus (Miller and Sassoon, 1998), but they display neither the epithelial pseudostratification nor the increase in smooth muscle observed in Vangl2Lp mutants.

We observe semi-penetrant infertility of Vangl2Lp heterozygotes, whereas Wnt7a heterozygotes are fertile. The uterine block observed in Vangl2Lp adult heterozygotes is not due to the septation observed in Wnt7a mutants, consistent with distinct Vangl2Lp and Wnt7a mechanisms. The uterine blockage accompanied by the lack of a proper vagina closely resembles vaginal agenesis, which is present in ~1/5000 human births. This can be accompanied by painful symptoms if endometrial uterine tissue remains, as it does in ~7-10% of cases (Rackow and Arici, 2007). Therefore, Vangl2 is a candidate gene for vaginal agenesis in humans.

#### Changes in the cell polarity of neonatal reproductive tissues have lasting consequences for female reproductive tract development

Histological, EM and IF examination of Vangl2Lp mutants reveal numerous defects in the cellular organization of neonatal Vangl2Lp mutants. Mesenchymal cells adjacent to the epithelium align their cell axes with the underlying epithelium, whereas mesenchymal
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Fig. 6. Mutant Vangl2Lp grafted postnatal uterine tissue displays hyperpolarized actin and abnormal E-cadherin distribution. (A-F) The wild type (A-C) has a thin and even domain of actin polarization towards the lumen, as compared with the Vangl2Lp mutant (D-F) in which the actin staining (red) is uneven (compare B with E, arrows). E-cadherin (green) is enriched at the apical domain of lateral cell junctions in wild-type epithelium (C, arrows), but this localization is lost in the mutant (F, arrows). (G-L) Scrb1 localization is perturbed in grafted postnatal Vangl2Lp mutant uterine tissue. The wild type (G,I) demonstrates discrete points of Scrb1 localization (green) to apical regions (lumen to the left in G,I) of epithelial cell-cell contact (I, arrows). By contrast, in Vangl2Lp heterozygotes (H,K,L), the expanded pseudostratified layer of epithelial cells localize Scrb1 in a non-polarized fashion, including some Scrb1+ epithelial cells in the middle of the lumen (H, arrows) and uniform membrane localization (H,I,K,L). Scrb1 localization is abnormal in the highly pseudostratified Vangl2Lp mutant epithelium (L). (M-O) Increase in smooth muscle layer with corresponding decrease in number of mesenchymal cells in Vangl2Lp mutants. Wild type (M), Vangl2Lp/+ (N) and Vangl2Lp/Lp (O) grafted mouse uterine postnatal tissue sections were stained for laminin (green) and smooth muscle actin (red). Laminin delineates the basal lamina at the border between epithelium and mesenchyme. Double-headed arrows indicate the width of the mesenchyme (M,N). The homozygous mutant (O) displays smooth muscle directly adjacent to the basal lamina (no mesenchyme). Scale bars: 10 μm in G-I,M-O; 5 μm in A-F,J-L.

cells in Vangl2Lp uteri are misaligned, similar to Wnt7a mutant uteri (Miller et al., 1998a; Miller and Sassoon, 1998). The loss of normal columnar epithelium in Vangl2Lp uteri at birth and the rounded lumen are features unique to these mutants. Changes in uterine elasticity due to altered cell-cell contacts or loss of cell rigidity could explain the changes in the lumen shape of Vangl2Lp mutants.

A reduction in polarized filamentous cytoskeletal actin was seen in neonatal Vangl2Lp mutant epithelium as compared with wild type. Defects in polarized cell movement are observed in Vangl2Lp mutant embryonic heart (Phillips et al., 2005), as well as actin cytoskeletal defects in mutant myocardial cells (Phillips et al., 2008). Hair follicles within Vangl2Lp mutant epithelium fail to polarize and mislocalize a number of cellular markers, including E-cadherin and Celsr1 [homolog of Drosophila Flamingo (Starry night)] (Devenport and Fuchs, 2008). In postnatal Vangl2Lp mutant uterine grafts, cytoskeletal actin staining becomes uneven and the protein is more strongly expressed in a wider domain than in wild-type epithelium. These data suggest that cytoskeletal remodeling events are important for early uterine patterning.

There is ample evidence that Scrb1 and Vangl2 interact in other murine tissues. Scrb1 interacts with Vangl2 as determined by protein co-immunoprecipitation (Kallay et al., 2006; Montcouquiol et al., 2006a), and mice mutant for Scrb1 mislocalize Vangl2 in the hair cells of the cochlea (Montcouquiol et al., 2006b). Also, mutants of Vangl2Lp or Scrb1 demonstrate the same cardiac development defects as double heterozygotes, suggesting that Vangl2 and Scrb1 act in the same developmental pathway in the heart (Phillips et al., 2007). We observe Scrb1 localized to the apical edges of Vangl2Lp...
mutant neonatal epithelium, suggesting that the domain of Scrb1 expression is expanded when the Vangl2Lp mutation is present. Murine Scrb1 is basolaterally localized in MDCK cells (Nagasaki et al., 2006) and human SCRB1 is similarly localized in human uterine cervical epithelial tissues (Nakagawa et al., 2004). Scrb1 also functions in apical/basolateral cell polarity with Lgl2 and Dlg by antagonizing the activity of the apically localized Par complex (Bilder et al., 2003; Macara, 2004; Tanentzapf and Tepass, 2003). We propose that the Vangl2Lp mutation provokes disruption of Scrb1 localization and that Scrb1 is no longer restricted to the basolateral domain of epithelial cells. Scrb1 has been shown to interact with Lgl2 as well as with Vangl2, and the analysis of macromolecular complexes containing Scrb1 have suggested that it organizes the intracellular face of the lateral plasma membrane by acting as a ‘retaining wall’ (Kallay et al., 2006). The fibrillar-like structures seen at the cellular junctions of Vangl2Lp mutants in EM might result from an abnormal accumulation of proteins due to the presence of Scrb1 at the apical cell edge. Scrb1 expression in the FRT is dynamic. In postnatal grafted tissue, Scrb1 localization becomes concentrated at cellular junctions. This dynamic localization might reflect multiple roles for Scrb1, as it is essential for directed epithelial cell migration (Dow et al., 2007; Ludford-Menting et al., 2005; Qin et al., 2005). These data suggest a model in which Vangl2 and Scrb1 function together in a complex, and Vangl2 is required to restrict Scrb1 to the basolateral domain in neonatal uterine epithelial cells and to concentrate Scrb1 expression at areas of cell-cell contact in postnatal epithelial cells (Fig. 8).

E-cadherin is an epithelial marker (Butz and Larue, 1995) and interacts with β-catenin to promote cell junction adhesion (Gumbiner, 1997). E-cadherin interacts with scribble (Navarro et al., 2005; Qin et al., 2005). Whereas neonatal Vangl2Lp mutant epithelium loses normal columnar morphology, it retains normal E-cadherin localization; however, polarity defects of mutant tissues become marked in postnatal tissues. In addition to defects in polarized filamentous actin and Scrb1 localization, we observe an increased domain of E-cadherin expression in Vangl2Lp mutant epithelium. This increase in E-cadherin expression, coupled with an increase in the domain of Scrb1 expression, might alter the integrity of cellular junctions in postnatal tissues. When spindle pole orientation is perpendicular to the basement membrane in mouse skin, the epithelium becomes stratified rather than columnar (Lechler and Fuchs, 2005), supporting a model whereby cell division perpendicular to the basement membrane directs stratification. In this context, we observe changes in the alignment of cell axes in both Wnt7a and Vangl2Lp mutant epithelium and in the underlying mesenchymal cells. Wnt7a mutants also display altered epithelial cell morphology, becoming stratified in reproducibly mature adult female uterine tissues. However, Wnt7a mutants lack glands, whereas Vangl2Lp mutant grafts form glands, highlighting another unique feature of Vangl2Lp mutants. Taken together, these data suggest that Wnt7a and Vangl2Lp mutant phenotypes are partially overlapping and that early changes in cell polarity of neonatal FRT can be linked to the ultimate pseudostratiﬁcation of the Vangl2Lp postnatal mutant epithelium.

In postnatal Vangl2Lp mutant tissues, defects in the localization of Scrb1, E-cadherin and polarized filamentous cytoskeletal actin become more pronounced, suggesting that defects observed in neonatal undifferentiated FRT tissues have permanent effects upon postnatal development. Both the homoygous and heterozygous Vangl2Lp mutant postnatal grafts exhibit a range of histological defects. Characterization with molecular markers demonstrates uneven, and occasionally massively increased, cytoskeletal actin staining, an increased and abnormal domain of E-cadherin expression in epithelial cells, the loss of specific Scrb1 localization to regions of cell-cell contact, and an increased domain of Scrb1 expression. These changes appear sufficient to alter the developmental program of the FRT.

**The Vangl2Lp mutation provokes a reduction of Wnt7a expression in female reproductive tissues**

There is precedent for a hierarchical regulation of Wnt proteins in the FRT (Mericskay et al., 2004; Miller et al., 1998b; Miller and Sassoon, 1998), as well as in other tissues such as muscle (Tabakhsh et al., 1998). Loss of Wnt7a provokes the misexpression of Wnt5a in mesenchyme and epithelium and leads to its eventual complete loss in adult tissues, and provokes the loss of Wnt4 expression in the mesenchyme (Miller and Sassoon, 1998). Also, Wnt5a mutants have reduced Wnt4 expression (Mericskay et al., 2004). This suggests that in general, the loss of Wnt signaling affects the expression of other Wnts in the developing FRT. Since the Vangl2Lp mutation affects the expression of Wnt7a, which is also expressed in epithelium, this
suggests that the mutation causes a disruption of Wnt signaling in the epithelium, which is nonetheless mild compared with signaling disruption by DES. DES, an estrogenic compound known to disrupt Wnt7a expression (Ma and Sassoon, 2006; Miller et al., 1998a), perturbs the expression of three Wnts—Wnt7a, Wnt5a and Wnt4—in neonatal FRT.

Three pieces of evidence support a model in which the Vangl2Lp mutation acts in a dominant manner in the developing FRT. First, both heterozygous and homozygous Vangl2Lp mutants show an almost identical decrease in Wnt7a expression. Second, the presence of either one or two copies of mutant Vangl2Lp provokes similar changes in postnatal grafted FRT tissues, as observed by H&E, actin, E-cadherin and Scrib1 staining. Finally, we obtained fewer Vangl2Lp/#; Wnt7a–/– compound mutant mice than expected. However, we cannot rule out the possibility that the Vangl2Lp allele might cause increased lethality in the Wnt7a mutant background, as we obtain more Wnt7a heterozygous mice than the expected ratio (37.4% versus 25%) and fewer Vangl2Lp/# heterozygous mice than expected (0% versus 12.5%). Taken together, these three lines of evidence suggest that the Vangl2Lp mutation acts in a dominant manner in the developing FRT (Fig. 8). Finally, our results reveal that both the canonical and non-canonical pathways participate in FRT development and play a key role in maintaining and governing adult FRT function. Perturbations in the levels of Wnt ligands and/or their pathway effectors have profound effects upon reproductive function and tissue integrity.

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Vangl2 regulates uterine development


