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Developmental Contribution of Wnt-signal-responsive Cells to Mouse Reproductive Tract Formation

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In mammals, the müllerian duct (MD) is an embryonic tubular structure that gives rise to the female reproductive tract (FRT). The MD originates from the coelomic epithelium (CoE) and takes on a rostral to caudal shape to establish the primary structure of the FRT under the regulation of morphogenetic signals. During these developmental processes, the MD and its derivatives require proper regulation of the Wnt-signaling-pathway. Here, to investigate the developmental contribution of FRT primordia under the influence of the Wnt-signaling, genetic lineage tracing was carried out using TopCreER/Rosa-LacZ mice to follow the fate of Wnt-signal-responsive cells during reproductive tract formation. TopCreER-marked-LacZ+ cells, arising from the Wnt-signal-responsive progenitors in CoE, give rise to spatially restricted MD and the uterine luminal epithelium. Similarly, the progeny from LacZ+ mesenchymal cells surrounding the MD contribute to both the uterine smooth muscle and stroma. Furthermore, in males, the Wnt-signal-responsive MD mesenchyme develops into the epididymis. These results show, for the first time, evidence of the sequential involvement of reproductive tract progenitors under the influence of Wnt-signal throughout the developmental term. This study provides a precise outline for assessing the lineage relation between the reproductive tract and the cell fate of its primordia in a temporally regulated manner.

Key words: Wnt signal, reproductive tract, uterus, genetic lineage tracing, müllerian duct

I. Introduction

During embryogenesis, the müllerian duct (MD) and the wolffian duct (WD) form the female and male reproductive tracts, respectively [2, 7, 16, 21, 26, 27, 29, 30]. In the mouse, the WD is first formed from the intermediate mesoderm by embryonic day 9 (E9) [17, 28]. Subsequently, the MD starts to form by the invagination of the surface epithelium of the mesonephros, also called the coelomic epithelium (CoE). It then extends caudally along the WD towards the cloaca around E11.5 in both the male and female embryo. The MD degenerates later by the anti-müllerian hormone signaling pathway in the male [3, 15, 16, 26, 30]. In the female, MD formation continues to form the primordium of the female reproductive tract (FRT). The MD, then, finally matures into the distinct female reproductive organs, including the oviduct, uterus, cervix, and upper part of the vagina, through epithelia-mesenchymal tissue interactions under the influence of various growth factors and hormonal regulations during postnatal, juvenile, and

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adult stages [7, 20, 21].

Mouse genetic studies have indicated that several signaling factors regulate MD development [16, 26, 30]. Of these, Wnt signaling has been considered to be one of the key signals, since the MD and its derivatives fail to develop normally in the event of its dysregulation [1, 5, 8, 9, 22, 24, 31, 33, 38, 43]. Analysis of Wnt4-null mutant mice suggests that Wnt4 in the mesonephric region is essential for MD invagination and elongation [40]. Mutations of the Wnt4 gene are associated with human congenital disorders that involve several defects in FRT [4]. Differentiation of the MD into the mature FRT is strongly influenced by the epithelial-mesenchymal interactions between the MD epithelium and mesenchyme [20, 21]. Several Wnt ligands, including Wnt4, Wnt5a, and Wnt7a, participate in these tissue interactions during the late embryonic and early postnatal stages [5, 22, 24, 31, 33]. β-catenin, a critical cytoplasmic downstream effector of the Wnt signaling pathway, is involved in tissue differentiation during the formation of female reproductive organs [1, 38, 43]. Upon Wnt-ligand mediated stimulation, stabilized β-catenin translocates into the nuclei, where it interacts with the Tcf/Lef molecular complex to activate the transcription of its target genes [23, 25, 35, 41, 42]. Dysregulation of β-catenin in the developing FRT results in severe differentiation defects in the endometrium and myometrium [1, 38, 43]. Anomalous Wnt signaling causes severe developmental abnormalities and is often associated with human congenital disorders in the urogenital field [6]. However, little is known as to how the developmental process of embryonic FRT primordia, CoE and MD, which have received Wnt signals, is spatiotemporally controlled during the prenatal and postnatal stages.

Genetic lineage tracing based on mouse Cre-loxP recombination system has been used to reliably investigate distinct cell/tissue fate by following the long term differentiation process for progeny of genetic marked cells [11, 19]. In this study, to address the contribution of Wnt-signal-activated cells during the development of the FRT, Wnt-signal-responsive cells were identified and followed by using the previously generated TopCreER mouse strain that expressed tamoxifen-inducible Cre recombinase under the transcriptional control of the Wnt-signal-responsive promoter.

II. Materials and Methods

Animals

The Rosa-LacZ (Stock No. 012429) and TopCreER (Stock No. 016236) were obtained from the Jackson Laboratory [34, 35, 44]. The mice were maintained in a mixed genetic background. All animal experimental procedures and protocols were approved by the Committee on Animal Research of Ehime University (Permit No. 05-KU-25-16). Mice were sacrificed by cervical dislocation.

Genetic lineage tracing experiments

Tissue lineage analyses were conducted by using compound TopCreER/Rosa-LacZ mice. The TopCreER male mice were crossed with Rosa-LacZ homozygous Cre indicator female mice. Stock solutions of tamoxifen (TM) (Sigma) were prepared at a concentration of 20 mg/mL in corn oil. TM inducible CreER mediated gene recombination in the embryos (E10.5–E15.5) was induced by one oral gavage to pregnant mice at 100 mg/kg body weight. Control experiments were carried out with corn oil alone, with no evidence of Cre mediated gene recombination. No overt teratogenic effects were observed after TM administration under these conditions (data not shown). We monitored the post-pubescent reproductive activity of some animals exposed to TM as embryos and found that they had normal reproductive activity (data not shown).

Histological analysis

Mouse tissues were dissected and fixed in 4% PFA/PBS for 2 days at 4°C, dehydrated through ethanol, and embedded in paraffin. Eight-μm serial sections were then prepared for histological analysis. Immunohistochemical analyses were carried out by standard procedures using the following antibodies (Ab) [14]: anti-beta-catenin (1:1000, cat. no. C19220, BD Biosciences) [43], anti-Lef1 (1:200, cat. no. 2230, CST) [43], anti-SMA (1:100, cat. no. M0851, Dako) [32], and anti-ERα (1:100, cat. no. 1115-1, Epitomics) [36].

X-gal staining

LacZ reporter gene expression was detected as previously described [12–14]. Dissected tissues were fixed in 0.8% paraformaldehyde (PFA) and 0.02% glutaraldehyde in PBS for 2 days at 4°C and washed five times in PBS before X-gal staining by standard procedures [12, 13]. Individual tissue samples were then rinsed in 70–100% ethanol, embedded in paraffin wax, sectioned at 6 μm, counterstained with eosin, and immunohistochemically stained.

III. Results

Contribution of Wnt-signal-responsive cells in the mesonephric region and MD mesenchyme to the newborn uterus

To genetically mark cells responding to Wnt signaling at particular developmental stages, TopCreER mice, a Wnt responsive transgenic reporter line, were used [34, 35] (Fig. 1a). Twenty-four hr after the induction of Cre activity by injection of TM at E10.5, E12.5 and E15.5, lacZ expression was detected in a small number of cells of the CoE and in mesenchymal cells surrounding the MD (Fig. 1b–g). These expression patterns of the LacZ were consistent with the expression pattern of Lef1, which reflects endogenous Wnt signal activity, in the mesonephric region and the embryonic uterus (Supplementary Fig. 1) [23]. At E11.0, the mesonephric region was composed of the CoE and

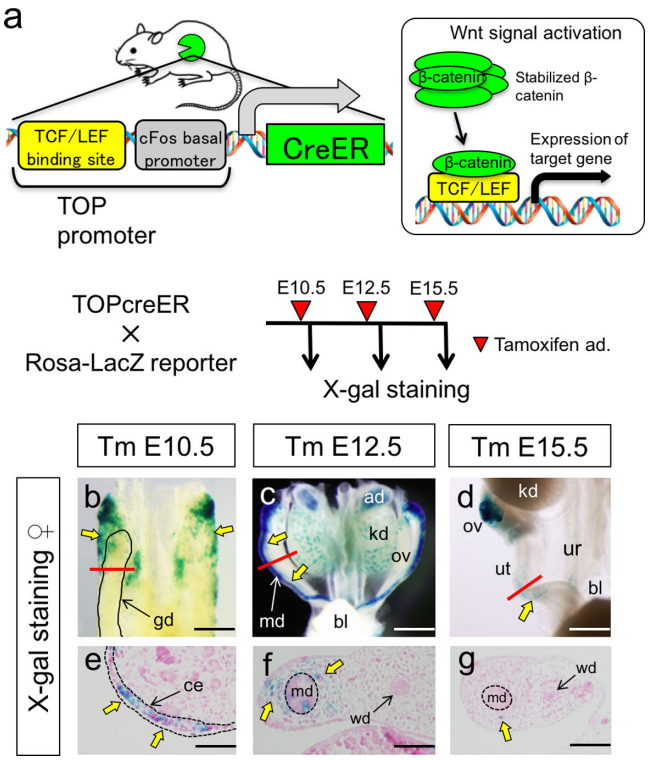

Fig. 1. TopCreER allele marks Wnt-signal-responsive cells in the mesonephric region and embryonic uterus. (**a**) Schema of the promoter construct of TopCreER transgenic mice and transcriptional activation by Wnt signaling components, β-catenin and Tcf/Lef1. (**b–g**) Whole-mount and section images of X-gal-stained TopCreER/Rosa-LacZ specimens. Red lines in (**b**), (**c**), and (**d**) indicate sites of transverse sections in (**e**), (**f**), and (**g**), respectively. β-galactosidase activity (yellow arrows) showing Wnt-signal-responsiveness is detectable in the coelomic epithelium (**b, e**) and mesenchymal cells surrounding the müllerian duct (**c, d, f, g**). The upper panel shows the experimental schedule for tamoxifen administration used in (**b**)–(**g**) to analyze the contribution of Wnt-signal-responsive cells to the embryonic uterus. gd, gonad; ce, coelomic epithelium; md, müllerian duct; bl, bladder; kd, kidney; ad, adrenal; ov, ovary; ut, uterus; ur, ureter; wd, wolffian duct. Bars = 500 μm (**b–d**), 100 μm (**g**) and 50 μm (**e, f**).

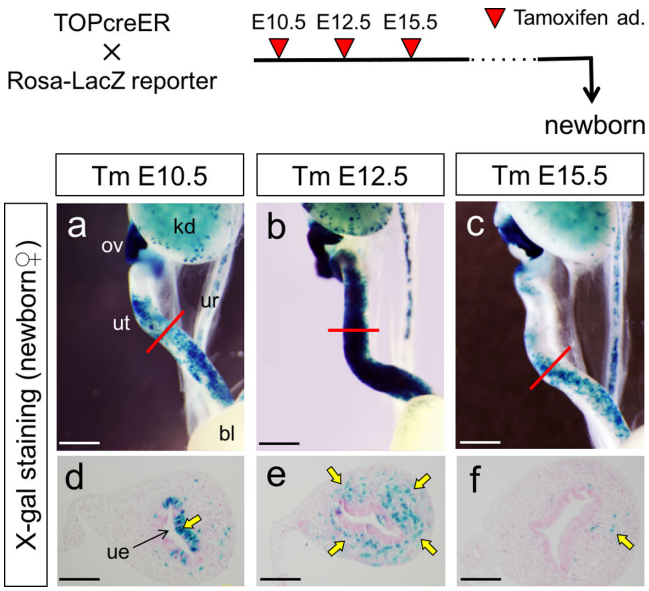

Fig. 2. Contribution of embryonic Wnt-signal-responsive cells in the mesonephric region to neonatal uterus (**a–c**) Whole-mount images of X-gal-stained TopCreER/Rosa-LacZ uterus. (**d–f**) X-gal stained sections of embryonic uterus. Red lines in (**a–c**) indicate sites of transverse sections in (**d–f**). β-galactosidase activity (yellow arrows) showing Wnt-responsiveness is detectable in the uterine epithelium (**d**) and its surrounding mesenchyme (**e, f**) at the newborn stage. Upper panel shows experimental schedule for TM administration used in (**a**)–(**f**) to analyze the contribution of Wnt-signal-responsive cells to the embryonic uterus. bl, bladder; kd, kidney; ov, ovary; ut, uterus; ur, ureter; ue, uterine epithelia. Bars = 500 μm (**a–c**) and 100 μm (**d–f**).

undifferentiated mesenchymal cells, where LEF1 and β-catenin, Wnt signal downstream effectors, were detected (Supplementary Fig. 1a and b). After E12.5, mesonephric mesenchymal cells had committed to the uterine stroma elements via mesenchymal proliferation and differentiation. At this stage, the LEF1 and β-catenin were mainly localized in the mesonephric mesenchyme and MD surrounding mesenchymal cells (Supplementary Fig. 1b, c, e, f).

After TM treatment at E10.5, E12.5, and E15.5, TopCreER/Rosa-LacZ mice were harvested and X-gal stained at the newborn stage (Fig. 2). In TopCreER/Rosa-LacZ mice at E10.5, TM treatment labeled the newborn uterine epithelium (Fig. 2a, d), and in those at E12.5, it labeled a much broader uterine mesenchyme (Fig. 2b, e).

Contribution of embryonic Wnt-signal-responsive cells to adult reproductive tract tissues

By following up the embryonic marked cells responding to Wnt signaling at adult stages, we examined the uterus six wk after TM induction at E10.5, E12.5, and E15.5, and detected various remarkably labeled uterine tissues (Fig. 3). In TopCreER/Rosa-LacZ mice treated with TM at E10.5, the endometrial epithelium was labeled and positive for E-cadherin (Fig. 3a, d, g). In those treated at E12.5, immunohistochemical analysis revealed significantly stained SMA-positive uterine smooth muscle cells (Fig. 3b, e, h). And in those treated at E15.5, the uterine stromal cells were marked (Fig. 3c, f, i). Rosa-LacZ mice treated with TM showed few or no lacZ-positive cells in the adult uterus (Supplementary Fig. 2). In males, embryonic Wnt-signal-responsive cells in the MD region contributed remarkably to the male reproductive tract tissues (Fig. 4). Forty-eight hr after the induction of Cre activity by TM injection at E12.5, lacZ expression was detected in a large number of mesenchymal cells surrounding the WD (Fig. 4a, c), and 24 hr after TM induction, lacZ expression was detected in the MD mesenchyme as in females (data not shown). TM treatment at E12.5 revealed a marked epididymal mesenchyme in the newborn (Fig. 4b, d) and mesenchymal cells in the adult epididymal smooth muscle were, by histochemical analysis, SMA-positive (Fig. 4e, f).

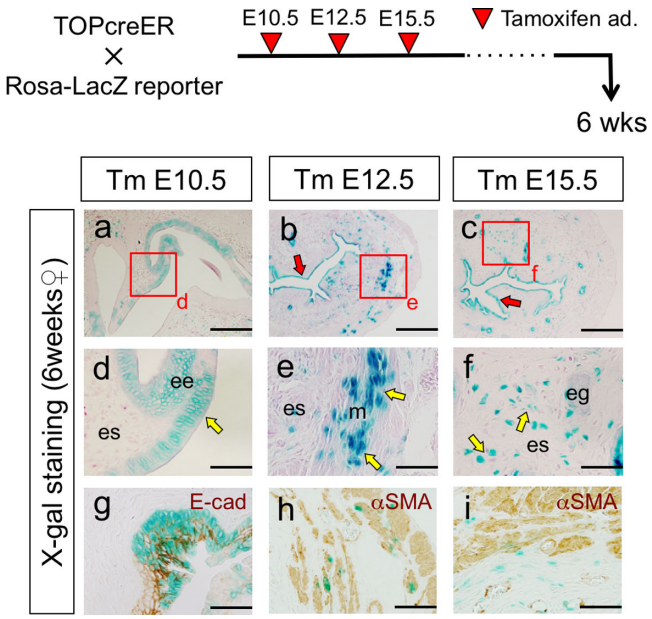

Fig. 3. Contribution of embryonic Wnt-signal-responsive cells in the mesonephric region to adult uterus β-galactosidase activity showing embryonic Wnt-signal-responsiveness is detectable in the mouse uterus at 6 wk of age. Experimental schedule for tamoxifen administration used in (**a**)–(**i**) to analyze the developmental contribution of embryonic Wnt-signal-responsive cells to the adult uterus. (**a–f**) β-galactosidase activity (yellow arrows) showing embryonic Wnt-signal-responsiveness is detectable in the mouse uterus at 6 wk of age. Red arrows indicate non-specific background staining of LacZ (**b, c**). Supplementary Figure 2 shows results of non-specific background staining of LacZ in Rosa-LacZ reporter mouse (Cre driver line negative). (**g–i**) Immunohistostaining of X-gal sections of the endometrium and myometrium region with anti-E-cadherin Ab and anti-α SMA Ab confirms that β-galactosidase positive cells are localized in the endometrial epithelial cells (**g**) and uterine smooth muscle cells (**h**). (**i**) Most X-gal stained signals are detected at the fibroblastic cells in the uterine stromal layer. (**d–f**) shows higher magnification view of red boxes in (**a–c**). ee, endometrial epithelium; es, endometrial stroma; m, myometrium; eg, endometrial gland. Bars = 100 μm (**a–c**) and 50 μm (**d–f**).

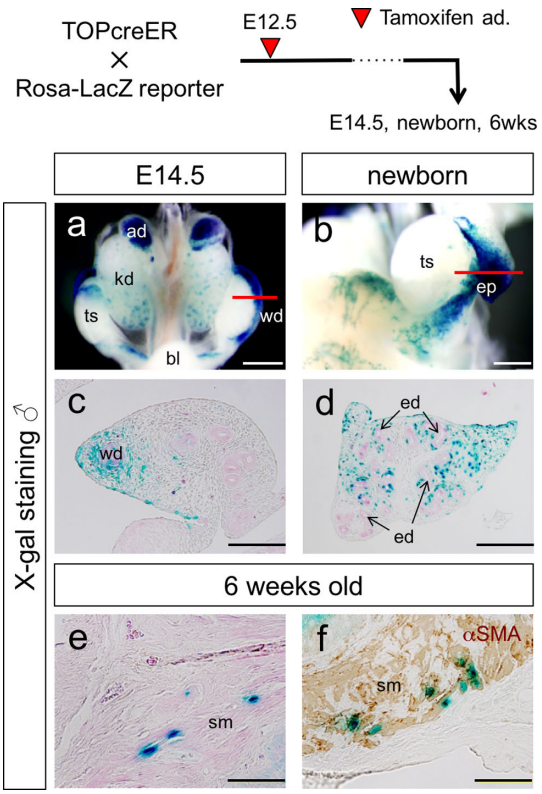

Fig. 4. Contribution of embryonic Wnt-signal-responsive cells in müllerian duct mesenchyme to adult epididymis. (**a–f**) Whole-mount and sections images of X-gal-stained TopCreER; Rosa-LacZ epididymis. (**a–d**) β-galactosidase activity showing Wnt-signal-responsiveness is detectable in the wolffian and epididymal mesenchyme at E14.5 and at the newborn stage. (**e, f**) At 6 weeks of age, the adult epididymis shows β-galactosidase activity of embryonic Wnt-signal-responsiveness in smooth muscle cells. Immunohistostaining of X-gal stained sections of the adult epididymis with anti-α SMA Ab confirms that β-galactosidase positive cells are localized in epididymal smooth muscle cells (**f**). Experimental schedule for TM administration used in (**a–f**) to analyze the contribution of embryonic Wnt-signal-responsive cells to the male reproductive organs is shown in upper panel. Red lines in (**a**) and (**b**) indicate sites of transverse sections in (**c**) and (**d**). bl, bladder; kd, kidney; ad, adrenal; ts, testis; wd, wolffian duct; ep, epididymis; ed, epididymal duct; sm, smooth muscle. Bars = 500 μm (**a, b**), 100 μm (**c, d**) and 50 μm (**e, f**).

IV. Discussion

The results of the present study have demonstrated that Wnt-signal-responsive cells in MD derivatives, epithelial structures of the FRT including oviduct and uterus, contribute to various tissues of the adult reproductive tract in a time-dependent manner (Fig. 5). Using an irreversible genetic marking system, a certain cell lineage was traceable for a considerable period of time. This study provides, for the first time, experimental evidence supporting previous reports on developmental lineage of reproductive organ formation, and corroborated that the MD originates from the earliest Wnt-signal-responsive progenitors located in the embryonic CoE. The results also demonstrated that, after MD formation, Wnt-signal-responsive cells surrounding the MD give rise to the uterine smooth muscle and stromal domains (epididymal smooth muscle in males) probably through proliferation and migration processes.

Previous mouse gene targeting analyses of the Wnt signaling pathway have shown that Wnt-signal-responsiveness is a crucial prerequisite during the prenatal and postnatal stages of reproductive organ development [1, 5, 9, 22, 24, 31, 33, 38, 40, 43]. As suggested by the aforementioned studies, temporally regulated Wnt-signal-responsiveness in the developing uterus may play crucial roles in the formation of MD derivatives during prenatal and postnatal stages. Our data also revealed that the embryonic CoE and the MD mesenchyme are direct targets of Wnt signaling. Cells in the CoE and the mesenchyme surrounding the MD are positive for TopCreER/Rosa-LacZ reporter mice, and the Wnt-signal-responsive cells contribute to the construction of the MD and its derivatives until

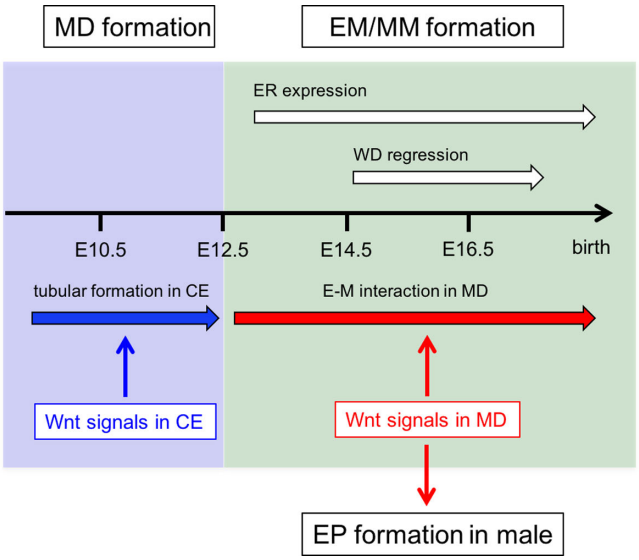

Fig. 5. Activation of Wnt signaling and lineage contribution of the Wnt-responsive cell populations to reproductive tract formation. We proposed that the coelomic epithelium derivatives show spatiotemporal selectivity of tissue lineage contribution of Wnt-signal-responsive cells during sequential development and maturation of the female reproductive tract (FRT). The embryonic FRT developmental processes consist of two major steps. Step 1: establishment of the müllerian duct (MD) by tubular formation of the coelomic epithelium. Step 2: epithelial-mesenchymal interaction between MD and its mesenchyme for uterus differentiation. At early stages, Wnt/b-catenin signaling is activated in embryonic coelomic epithelium committed to the MD lineage with tubular-genesis. At late stages, the MD mesenchyme receives a Wnt-signal derived from the MD or its mesenchyme, and then differentiates into the uterine stroma and muscle in an estrogen-signal-dependent manner. In the male, Wnt-signal-responsive cells in the mesenchyme surrounding the MD contribute to epididymal (EP) smooth muscle formation. The horizontal scale represents mouse developmental stages. The red and blue arrows represent the cellular events in the coelomic epithelium (CE) and MD, respectively.

the adult stage. A further point to consider is whether the main Wnt ligand sources in CoE and MD, as well as for Wnt4 and Wnt7a, actually confer Wnt-signal-responsiveness to TopCreER/Rosa-LacZ reporter mice. However, as we have shown in the present study, fate mapping analyses with the use of the TopCreER mice line provides a powerful tool for assessing the developmental potential of Wnt-responsive cell lineages during the development of reproductive organs at the embryonic, postnatal, and adult stages. Future studies are needed to define the precise developmental role of other signaling cascades, which is important for understanding reproductive organ formation (non-canonical Wnt-signaling through the Ca^{2+}-PLC-JNK pathway, Hedgehog, Bone morphogenetic proteins, etc.) within Wnt-signal-responsive cell lineages (e.g., mouse genetically conditional gene mutation with the use of the TopCreER line) [10, 18, 37].

The surprising finding in this study is that some of the smooth muscle cells in adult epididymis can be shown to have originated from an embryonically labeled TopCreER precursor in the MD mesenchyme. Like that of the female, the male MD mesenchyme is also marked by Wnt-signal-responsiveness at E12.5 and gives rise to smooth muscle populations of the epididymis. Previous studies on persistent Müllerian duct syndrome showing the lack of MD regression in the male, with description of Wnt signaling, have investigated the potential role of Wnt-signal-responsiveness of MD derivatives Wnt-responsiveness in the development of the adult epididymis [39]. Interestingly, constitutively activated β-catenin in the embryonic MD mesenchyme with the use of Amhr2cre mice, the MD mesenchyme-specific cre driver line, could result in focal MD retention and adjacent WD obstruction caused by the differentiation anomaly of the epididymal smooth muscles. The domains are derived from the MD mesenchyme, a region where TopCreER-mediated recombination is found during embryogenesis. Our data will contribute to resolving the important pending question, raised in previous reports, as to why Wnt-signal-responsiveness to dysregulation of the MD mesenchyme causes epididymal malformation in the persistent MD syndrome.

To summarize, this is the first study conducted with the use of genetic fate mapping to identify the lineage contribution of Wnt-signal-responsive cells to the development of reproductive organs. The major finding in this analysis was that Wnt-signal-responsive cells in the CoE and the MD mesenchyme exhibited specific lineage contribution to the various tissues of the mouse uterus in a time-dependent manner. Moreover, Wnt-signal-responsive cells in the MD mesenchyme also contributed to the epididymal smooth muscle cells of the male reproductive tract. The aberrant Wnt signaling in MD derivatives can be observed in a wide range of gynecological diseases. Our study may help to shed light on this pathogenesis. The disturbance of the Wnt or other signaling pathways resulting in a permanent anomaly of the affected MD derivatives during embryogenesis may be one of the causative mechanisms of gynecological diseases in humans [6].

V. Conflict of Interest

All authors declare that they have no competing interests.

VI. Author Contributions

RH, GY and SK conceived and designed the experiments; RH performed the experiments; RH, RK, AM, GY and SK analyzed the data; RH, RK and SK contributed reagents/materials/analysis tools; RH, RK, AM, GY and SK wrote the paper.

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