Neonatal Wnt-dependent Lgr5 positive stem cells are essential for uterine gland development

Wnt signaling is critical for directing epithelial gland development within the uterine lining to ensure successful gestation in adults. Wnt-dependent, Lgr5-expressing stem/progenitor cells are essential for the development of glandular epithelia in the intestine and stomach, but their existence in the developing reproductive tract has not been investigated. Here, we employ Lgr5-2A-EGFP/CreERT2/DTR mouse models to identify Lgr5-expressing cells in the developing uterus and to evaluate their stem cell identity and function. Lgr5 is broadly expressed in the uterine epithelium during embryogenesis, but becomes largely restricted to the tips of developing glands after birth. In-vivo lineage tracing/ablation/organoid culture assays identify these gland-resident Lgr5high cells as Wnt-dependent stem cells responsible for uterine gland development. Adjacent Lgr5neg epithelial cells within the neonatal glands function as essential niche components to support the function of Lgr5high stem cells ex-vivo. These findings constitute a major advance in our understanding of uterine development and lay the foundations for investigating potential contributions of Lgr5+ stem/progenitor cells to uterine disorders.
The majority of the female reproductive tract, comprising the oviduct, uterus, cervix, and upper vagina, develops from the Mullerian duct (Md) during embryogenesis. These organs remain relatively immature at birth and undergo further development during prepuberty to ensure fertility in adulthood. Particularly in uterus, gland development is essential for successful pregnancy as their secretions and products impact implantation, stromal cell decidualization, and placentation development.

Uterine glands are essential for proper uterine function as they secrete various factors, such as leukemia inhibitory factor (LIF) that are important for endometrial receptivity and embryo implantation. Their development postnatally, involving budding, tubalogenesis, coiling and branching of luminal epithelium, orchestrated by interactions with the underlying stroma. Genetic knockout studies have identified multiple, predominantly Wnt-related, genes required for development of the uterine epithelium, but the cellular origins of the glandular epithelium (GE) remain poorly understood. Although single cell sequencing studies have recently documented extensive cellular heterogeneity within the developing mouse uterus epithelium, it is currently unknown whether dedicated endometrial stem/progenitor cells are present.

Leucine-rich repeat containing G-protein-coupled receptor-5 (Lgr5) is a facultative component of the Wnt receptor complex that marks Wnt-regulated stem cells responsible for the development, maintenance, and regeneration of multiple epithelia. In healthy adult mouse uterus, endogenous Lgr5 expression is low, but is markedly upregulated in response to ovarioectomy and downregulated by sex hormone stimulation. In contrast, Lgr5 is robustly expressed in prepubertal mouse endometrium. The identity and function of these endometrial Lgr5+ populations are currently unknown.

Here, we employ non-variegated reporter, in vivo lineage tracing and in vivo ablation mouse models, together with ex vivo organoid culture technologies, to document Lgr5-expressing cells in the developing uterus from embryo to postnatal puberty and to evaluate their physiological roles in driving uterine development. We show that Lgr5 is broadly expressed in the Md during embryogenesis, but becomes largely restricted to the tips of developing glands after birth. These region-restricted Lgr5-high endometrial cells are Wnt-responsive stem/progenitor cells that are indispensable for uterine gland development. We further identify a distinct hierarchy among developing endometrial cells, with Lgr5-high stem/progenitor cells being supported by an epithelial niche that comprises differentiated cells expressing essential Wnt ligands.

Results

Lgr5 expression persists through endometrial development. To evaluate endogenous Lgr5 expression in female reproductive tracts during development, we examined tissues at various time points during mouse embryogenesis. At embryonic day 12.0 (E12.0), when Md formation is initiated by invagination of the coelomic epithelium, nascent Lgr5 expression is observed within the coelomic epithelium, as documented by highly sensitive RNA in situ hybridization (ISH) (Fig. 1a; dashed red line). Of note, Lgr5 was also expressed in the Wolffian duct (Wd) at this time point (Fig. 1a; dashed black line). As a complementary approach, we employed independent Lgr5-2A-enhanced green fluorescent protein (EGFP) reporter mice (Fig. 1b). Unlike our previous, variegated Lgr5-driven reporter mouse model (Lgr5-EGFP-ires-CreERT2), this new model maintains physiological Lgr5 expression levels and consequently faithfully reports all endogenous Lgr5+ populations (Supplementary Fig. 1a). Lgr5-EGFP expression was detected in coelomic epithelium at E12.0 and colocalized with expression of Lim1, a marker of both Md and Wd at this age (Fig. 1c). At E12.5, during elongation of the Md, uniform Lgr5-EGFP expression was maintained throughout the duct, as well as in Wd (Fig. 1d, e). At postnatal day 0 (P0), robust Lgr5-EGFP expression in the uterus was restricted to the epithelium, where it was broadly distributed (Fig. 1f). In contrast, Lgr5-EGFP was weakly expressed in oviduct and upper vagina, both of which originate from Md (Supplementary Fig. 1b, c). These data define the origin of Lgr5 expression in the developing female reproductive tract as cells in the nascent Md. At the time of birth, expression is maintained within the epithelial lining of the developing uterus, as well as in oviduct and upper vagina.

Lgr5+ Mullerian duct cells generate multiple tissues. We have previously employed in vivo lineage tracing to document the endogenous stem/progenitor cell identity of Lgr5+ populations in a variety of tissues. Here, we adopted the same strategy to evaluate the stem/progenitor cell potential of the embryonic Lgr5+ cells identified within the developing reproductive tract. Lineage tracing was initiated in E11.5 Lgr5-2A-CreERT2; R26tdTomato mice (Fig. 2a) by IP injection of a single 0.2 mg/g body weight dose of Tamoxifen (TAM) to pregnant females. After 24 h, tdTomato reporter gene expression was activated in single cells within the Lim1+ Md, consistent with the localization of endogenous Lgr5+ cells at this stage (Fig. 2b). Note that tdTomato (tdTom+) expressing cells were also evident within the Wd, coinciding with endogenous Lgr5 expression (Fig. 2b; dashed white line). After 48 h, there was a marked expansion of the Lgr5+ cell-derived tdTom+ tracing units in Lim1+ populations as Md elongation progressed. In contrast, tdTom+ cells were lost from the Wd, likely reflecting the degeneration of this tissue from E13.5 onwards in females (Fig. 2c; dashed white line). At P90, when the uterus had fully matured, contiguous tdTom+ patches of Lgr5+ cell-derived progeny were evident throughout the epithelia of oviduct, uterus, and upper vagina (Fig. 2d). These results identify the Md-resident Lgr5+ cells as being embryonic stem/progenitor cells contributing to the development and maintenance of the epithelia of the female reproductive tract.

Lgr5 expression is enriched in postnatal endometrial glands. We next evaluated Lgr5 expression in female reproductive tracts after birth. EGFP expression in Lgr5-2A-EGFP reporter mice showed that Lgr5 expression becomes restricted to the uterus and silenced in oviduct and upper vagina by postnatal day 7 (P7) (Fig. 3e, g; Supplementary Fig. 2e, f, i, j). Thus, we performed detailed Lgr5 expression analyses on uterus to formally document the localization and identity of the Lgr5+ populations during its development. Q-PCR analyses of wild-type uterus tissues harvested between P3 to early adulthood (P28) revealed that Lgr5 expression dramatically increased in the first 2 weeks after birth, before gradually declining to low levels at P28 (Fig. 3a). Of note, dioestrous stage in adulthood showed the highest expression during each estrous cycle, implicating a likely hormonal influence on Lgr5 expression in vivo (Fig. 3b). Detailed Lgr5 ISH analyses identified endogenous Lgr5 transcripts distributed at random throughout the developing endometrium at P3, prior to the onset of adenogenesis (Fig. 3d). At P7, which coincides with the onset of gland development, Lgr5 expression presented as a gradient, with highest levels present within the nascent gland buds (Fig. 3f). From P14 to P28, this expression gradient became more pronounced, with Lgr5-high cells predominantly restricted to the tips of developing gland epithelia (GE) (Fig. 3h, j, l). In contrast, Lgr5 expression was greatly reduced post-puberty, with only the
luminal epithelium (LE) of the dioestrus uterus presenting readily detectable levels of \textit{Lgr5} transcripts (Supplementary Fig. 2a–d). An identical \textit{Lgr5-EGFP} expression profile was documented using \textit{Lgr5-2A-EGFP} reporter mice, further validating this model as a faithful reporter of endogenous \textit{Lgr5} expression in the reproductive tract (Fig. 3h–k). Of note, \textit{Lgr5}\textsuperscript{high} cells were localized to the anti-mesometrial side of the tissue, consistent with a previous report that Wnt signaling is active in this region (Supplementary Fig. 3a, b)\textsuperscript{18}. We further employed RNA ISH to document the expression of the highly related genes \textit{Lgr4} and \textit{Lgr6}, which are known to modulate Wnt signaling on stem cells in other tissues\textsuperscript{19}. In P14 uterus, \textit{Lgr4} expression was readily detectable in the epithelium, but \textit{Lgr6} expression was absent. \textit{Lgr4} expression was slightly higher in LE than in GE, in contrast to the expression pattern of \textit{Lgr5} (Supplementary Fig. 3c). In the mature uterus, \textit{Lgr4} expression was detected both in LE and GE, whereas \textit{Lgr6} expression was only detected in stroma during dioestrus and pro-oestrus (Supplementary Fig. 3d).

\textbf{Stage-dependent role of \textit{Lgr5}$^+$ stem cells in endometrium.} To evaluate the stem cell potential of endometrial \textit{Lgr5}$^+$ cells in prepubertal uterus, we initiated in vivo lineage tracing using 4-hydroxytamoxifen (4OHT) or TAM at a single, low dose that does not impact glandular development or uterine function in \textit{Lgr5-2A-CreERT2}; \textit{R26-tdTomato} mice at P7 or P14

\textbf{Fig. 1} \textit{Lgr5} is expressed in the early female reproductive tract during embryogenesis. \textbf{a} RNA ISH for \textit{Lgr5} in coelomic epithelium at E12.0. \textbf{b} The \textit{Lgr5-2A-EGFP} mouse model employed to evaluate endogenous \textit{Lgr5} expression. \textbf{c} Co-IF for \textit{Lgr5-EGFP} and Lim1 in coelomic epithelium at E12.0. \textbf{d} Confocal z-stack image of a whole-mount E12.5 Müllerian duct (highlighted by the red dashed line). Yellow box indicates the region magnified in \textbf{e}. \textbf{e} Endogenous EGFP fluorescence in E12.5 \textit{Lgr5-2A-EGFP} mouse at Md. \textbf{f} Immunostaining for \textit{Lgr5-EGFP} and E-cadherin in P0 uterus. Dashed red lines indicate Md, and dashed black or white lines indicate Wd, respectively. Md, Müllerian duct; Wd, Wolffian duct; Scale bars, 50 \textmu m. All images are representative of three independent mice.
(Supplementary Fig. 4a–e), and traced Lgr5+ cell-derived progeny to various ages. When tracing was initiated at P7, the tdTomato reporter gene was activated in both LE and developing GE, consistent with the location of endogenous Lgr5+ cells at this stage (Fig. 4a; 1 day post injection). One week later, multiple contiguous tdTom+ tracing units had expanded deep into the outer stromal area as endometrial glands developed (Fig. 4b; 7 days post injection). At P56, extensive dTom+ tracing units were readily evident throughout both the LE and GE endometrial compartments (Fig. 4c). Approximately 10% of the P7-P56 dTom+ cells co-expressed Foxa2, a specific marker of GE20, indicating a long-term contribution of P7 Lgr5+ cells to the development and maintenance of both the endometrial LE and GE (Fig. 4f, h). In contrast, when tracing was initiated from P14, the tdTomato reporter was predominantly activated at the tips of developing GE (Fig. 4d; 2 days post injection), reflecting the endogenous distribution of Lgr5high cells at this stage. At later time points, expanding tracing units were largely Foxa2+, consistent with a restricted contribution of P14 Lgr5high cells to GE development and maintenance (Fig. 4e, g, h and Supplementary Fig. 4f). These Lgr5 lineage-tracing data collectively indicate that endometrial Lgr5+ cells in prepubertal uterus are initially functioning as stem/progenitor cells responsible for the development of both the LE and GE, but gradually convert to being a...
dedicated stem cell pool for the developing GE as the uterus matures. Together with the long-term tracing results, this suggests that the stem/progenitor cells in LE and GE exist independently around P14. In addition, co-IF for Lgr5-EGFP and Foxa2 in P14 uterus showed that endogenous Lgr5 and Foxa2 expression is largely mutually exclusive, indicating that Lgr5 expression in the endometrial glands is downregulated once the cells adopt a Foxa2+ glandular cell fate (Fig. 4i). Of note, Lgr5-driven lineage tracing was strictly confined to the K8+ epithelial compartment of the endometrium (Fig. 4j, k) and
**Fig. 3** Lgr5 is dynamically expressed in the developing uterus after birth. **a** QPCR analysis of Lgr5 expression in uterus at various neonatal stages. Data from three independent mice are presented as mean ± s.e.m. **b** QPCR analysis for Lgr5 on adult uterus (P90) from different estrous stages. Data from three independent mice are presented as mean ± s.e.m. **c** Cartoon depicting the tissue sectioning method. **d, f, h, j** RNA ISH for Lgr5 on uterus from various ages during prepuberty period. **e, g, i, k** Immunostaining for EGFP in Lgr5-2A-EGFP mouse uterus at various ages. Red asterisks indicate Lgr5$^{\text{high}}$ cells at the tips of developing glandular epithelium (GE). Boxed enlargements highlight Lgr5 expression within the developing glands at the different developmental stages. LE, luminal epithelium; St, stroma. Scale bars, 100 μm. All images are representative of three independent mice. **l** Cartoon depicting the endogenous Lgr5 expression profiles at P7 and P14.

**Fig. 4** Pre-pubertal Lgr5$^+$ populations are stem/progenitor cells for the developing uterus. **a-e** Endogenous tdTom fluorescence in Lgr5-2A-CreERT2; R26-tdTomato mouse uterus traced from P7 to P8 a, P7–P14 b, P7–P56 c, P14–P16 d, and P14–P56 e, respectively. Insets with solid and dashed lines indicate developing glandular epithelium (GE) and luminal epithelium (LE), respectively. White arrows indicate differentiated GE. St Stroma. **f, g** Co-IF for tdTom and Foxa2 in Lgr5-2A-CreERT2; R26-tdTomato mouse traced from P7 to P56 f and P14–P56 g. **h** Comparison of the percentage of Foxa2$^+$ cells within tdTom$^+$ tracing units in P7–P56 and P14–P56 traced mice. Three independent fields from each mouse were analyzed. Data are represented as mean ± s.e.m. of the average of five independent mice. Data were tested for significance using unpaired two-tailed t-test ($P = 9E^{-05}$, ***$P < 0.001$). **i** 3D image of P14 Lgr5-2A-EGFP mouse uterus, co-stained for Epcam and Foxa2. **j, k** Co-IF for tdTom and K8 in Lgr5-2A-CreERT2; R26-tdTomato mouse traced from P7 to P56 j and P14–P56 k. Insets with solid and dashed lines indicate mature GE and LE, respectively. Scale bars, 100 μm.
was absent from the oviduct and upper vagina (Supplementary Fig. 2m–p).

To investigate any contribution of adult Lgr5+ cells to maintenance of the adult endometrium, we administered TAM to adult Lgr5-2A-CreERT2; R26-tdTomato mice at dioestrus stage. TdTom+ reporter gene activity was activated in scattered cells throughout the LE after 3 days (Supplementary Fig. 4g), as expected from the endogenous Lgr5 expression pattern in dioestrous females (Supplementary Fig. 2b). To evaluate any contribution of adult Lgr5+ cells to estrus-driven endometrial epithelial renewal, we traced for a further 14 days, encompassing three complete estrus cycles. In contrast to the neonatal tracing results, there was a marked reduction in tdtom+ cells within the adult LE (Supplementary Fig. 4h). After 1 year of tracing, tdtom expression in the uterus was negligible, identifying adult Lgr5+ cells as likely being short-lived differentiated cells rather than self-renewing stem/progenitor cells contributing to adult LE homeostasis (Supplementary Fig. 4i).

**Lgr5+ cells are indispensable for uterine gland development.** To further explore the contribution of Lgr5+ cells to endometrial development, we employed the Lgr5-DTR-EGFP mouse model [23], which expresses an Lgr5+ cell-driven Diphtheria toxin (DT) receptor-EGFP fusion gene (Fig. 5a). The DTR-EGFP transgene reports endogenous Lgr5 expression and facilitates selective ablation of Lgr5-expressing cells by administration of DT. Lgr5-DTR-EGFP mice were treated with DT at P7 and harvested at various ages (Fig. 5b). At 24 h post-injection, cleaved Caspase3+ cells were evident throughout the endometrium, including nascent glands, and Lgr5-DTR-EGFP expression was absent (Fig. 5c, Supplementary Fig. 5a, b), indicating efficient ablation of the endometrial Lgr5+ population. One week post-DT administration, there was a marked reduction in Foxa2+ glands compared with the wild-type control (Fig. 5d–f; P14), and the number of Foxa2+ glands was greatly reduced in mature uterus (Fig. 5h, i). In contrast, formation of Foxa2+ glands was not impaired by DT treatment of wild-type mice. Of note, the number of Foxa2+ glands is sustained in adulthood irrespective of estrous cycle, highlighting the validity of comparing gland numbers in mature uterus (Supplementary Fig. 5c, d). Treating Lgr5-DTR-EGFP mice with DT at P14 also resulted in a significant reduction of Foxa2+ glands (Supplementary Fig. 5e–g). Immunostaining for K8, progesterone receptor (PR), and Ki67 demonstrated normal LE formation and proliferation status, suggesting that ablation of Lgr5+ cells selectively abrogates GE development (Fig. 5g). Together, these results identify endometrial Lgr5+ cells as being essential for uterine gland development during the prepuberty period.

**Wnt activity of Lgr5high cells in the developing endometrium.** To further characterize endometrial Lgr5+ cells, we performed comparative gene expression profiling of fluorescence-activated cell sorting (FACS)-sorted GFPhigh versus GFPneg populations from endometrial cells (EPCAM+ cells) of P14 Lgr5-2A-EGFP mice (Fig. 6a, Supplementary Fig. 6a). Microarray analysis revealed 179 differentially expressed genes between the two populations (Fig. 6b, c). Lgr5, Aldh1a1, and Prom1 were significantly higher in the GFPhigh population, whereas Spry1a and Zfp750 were enriched in the GFPneg cells. We further validated these results by qPCR and RNA ISH. Lgr5 expression was highly enriched in the sorted GFPhigh population (five-fold), validating our sorting strategy (Fig. 6d). Another Wnt target gene, Axin2, was also enriched in the GFPhigh population (5.5-fold), indicating robust Wnt-signaling activity in this epithelial stem/progenitor population (Supplementary Fig. 6d, e). As expected from the microarray analysis, Aldh1a1 and Prom1 were also markedly enriched in GFPhigh population (7.5-fold and 3-fold, respectively) in contrast to Spry1a and Zfp750, which were strongly down-regulated in the Lgr5high compartment (Fig. 6d, Supplementary Fig. 6b). Independent RNA co-ISH analyses showed that while Lgr5 expression colocalizes with that of Aldh1a1 or Prom1, it does not significantly overlap with Spry1 and Zfp750 (Fig. 6e, Supplementary Fig. 6c). Notably, the expression of Aldh1a1 has been reported to be upregulated in the glandular regions of neonatal endometrium [26,27], confirming that the Lgr5high endometrial population comprises predominantly GE cells.

Since various Wnt and Fzd genes are known to be important for uterus development, we next evaluated their relative expression levels in Lgr5-expressing cells. Analysis of microarray data showed distinct Wnt/Fzd expression patterns in GFPhigh and GFPneg populations (Fig. 6f). We confirmed the expression of essential Wnt/Fzd genes for uterus development by qPCR (Fig. 6g) [23-27]. Expression of Fzd10, a frizzled receptor expressed in developing uterine epithelium [23], was markedly higher in the GFPhigh population (3.1-fold). In contrast, Wnt7b, one of the Wnt ligands that potentially interact with Fzd10 to initiate Wnt signaling [23], was elevated in the GFPneg population (18-fold; Fig. 6g). Similarly, Wnt4, a well-established Wnt ligand essential for uterine development [24], was markedly higher in the GFPneg population. Wnt7a, another essential Wnt ligand that is expressed predominantly in the premature endometrium and is essential for uterine development [23], was expressed at similar levels between the two populations (Fig. 6g). Independent RNA co-ISH analyses showed that while Lgr5 colocalizes with Fzd10 expression, it does not significantly overlap with Wnt7b and Wnt4 (Fig. 6h), confirming the qPCR data. Of note, Wnt7a is expressed broadly in the endometrium and does not correlate with Lgr5 expression. Wnt5a, which is reportedly expressed in stroma [26], was selectively enriched in non-epithelial (EPCAM−) cells relative to both the GFPhigh and GFPneg epithelial populations (Supplementary Fig. 6f).

Lgr5 functions as a receptor for secreted R-spondins to modulate Wnt signal strength on Wnt-responsive stem cells in multiple tissues [28]. RNA ISH analysis of Rspo1 expression revealed localized expression of Rspo1 in stromal cells adjacent to the developing endometrium, including nascent glands harboring the Lgr5+ stem cells (Supplementary Fig. 6g). Rspo3 expression was confined to the muscle layer, whilst Rspo2 and 4 were not detected.

These expression analyses indicate that epithelial Lgr5high cells in developing endometrium exhibit robust Wnt signaling that is potentially facilitated by receptors, such as Fzd10 and Wnt/R-spondin ligands supplied by Lgr5neg cells in the adjacent endometrium or stroma.
These results strongly indicate that only the EPCAM\textsuperscript{+}GFPh\textsubscript{high} Lgr5\textsuperscript{+} endometrial cells function as a self-renewing stem/progenitor population in Rspo1/Wnt3a-supplemented ex vivo culture.

We next sought to ablate endogenous Lgr5\textsuperscript{+} cells in uterine organoids to evaluate their contribution to ex vivo organoid growth and maintenance. Uterine organoids were cultured for 3 days from EPCAM\textsuperscript{+} cells isolated from P14 Lgr5-DTR-EGFP
mouse uterus and DT was then added to ablate the resident Lgr5\(^+\) cells (Fig. 7g). We observed a ~50% reduction in outgrowth efficacy and an associated major decrease in organoid size in the DT-treated organoids compared to control (Fig. 7h, i). To evaluate whether the residual organoid growth observed using the Lgr5-DTR-EGFP model was due to incomplete Lgr5\(^+\) cell ablation, we repeated the experiment using our new, highly efficient Lgr5-2A-DTR mouse model (Supplementary Fig. 7a, b). Here, we observed a complete inhibition of organoid outgrowth following DT treatment, indicating that Lgr5\(^+\) cells are completely indispensable for organoid growth (Supplementary Fig. 7c, d). Of note, the high efficacy of this new Lgr5-2A-DTR ablation model precluded its use for in vivo ablation experiments in young mice due to rapid lethality resulting from systemic ablation of Lgr5\(^+\) stem cells essential for postnatal development.

Proliferating, progenitor-enriched organoids derived from Lgr5\(^+\) stem cells in other organs can be directed to differentiate by manipulating canonical Wnt-signaling pathways\(^{31}\). To determine whether this is also true for immature uterus organoids, we established Lgr5\(^+\) cell-derived organoids in media supplemented with 80% less Wnt3a. We observed a marked phenotypic change in the organoids, with low Wnt conditions suppressing the conversion of round-type organoids into vacuolated-type organoids (Fig. 7, k). Pharmacological activation of canonical Wnt signaling by addition of CHIR99021, a GSK inhibitor, to the low Wnt3a condition media resulted in ~80% of the Lgr5\(^+\) cell-derived organoids adopting a round-type morphology (Fig. 7, k). This suggested that the round-type organoids are more stem-like, while the vacuolated organoids are more differentiated. To better characterize the behavior of these two types of organoids, we first performed marker expression analysis. The round-type organoids exhibited a markedly higher proportion of Klf6-expressing cells than the vacuolated-type, indicative of their highly proliferative status (Fig. 7o). Expression of Prss28, a pre-differentiation marker, was expressed at similar levels in both organoid types (Fig. 7m). The vacuolated-type organoids displayed elevated expression of glandular differentiation markers Foxa2 and Lif\(^{32}\), reflecting their relatively differentiated status (Fig. 7n). As expected, qPCR analysis of organoids cultured with CHIR99021 revealed upregulation of the Wnt-signaling-associated components Lgr5 and Fzd10, whilst the differentiation markers Foxa2 and Lif were suppressed (Fig. 7l, m). In contrast, the LE markers Wnt7a and Scnn1a were expressed at similar levels in both organoid types while another LE marker Lrg1 was significantly higher in vacuolated-type organoids. However, expression of the various LE markers was markedly lower than in mature adult tissue, suggesting sub-physiological levels (Supplementary Fig. 7e).

Supplementing Wnt4 or Wnt7b in place of Wnt3a also activated Axin2 and selectively promoted the generation of round-type organoids, as expected from their ability to upregulate Lgr5 and Fzd10 expression (Supplementary Fig. 7f–h). We then knocked down Fzd10 in organoids to evaluate the functional relevance of Fzd10 (Supplementary Fig. 7i, j). This resulted in a major induction of apoptosis throughout the organoids, consistent with a role for Fzd10 as an essential Wnt receptor in maintaining organoid growth ex vivo.

These observations highlight the role of Wnt signaling in regulating the balance between stemness/proliferation and glandular differentiation in immature endometrium derived from Wnt-dependent Lgr5\(^{high}\) progenitor/stem cells.

Lgr5\(^{neg}\) endometrial epithelial cells are a Wnt niche. Lgr5\(^+\) epithelial stem cells typically reside in close proximity to stromal/epithelial niche cells supplying secreted Wnt/R-spondin ligands and other key growth factors\(^{32}\). In the developing uterus, various essential Wnt ligands\(^{5}\) and Rspo1 (Supplementary Fig. 6g) are expressed in the stromal compartment, and could act as a Wnt/R-spondin source for the Lgr5\(^{high}\) stem cells. However, the robust Wnt4/7b expression we identified in the Lgr5\(^{neg}\) epithelial cells also qualifies this endometrial population as a potential niche component for the Lgr5\(^{high}\) stem cells during development. To explore this possibility, we first evaluated the impact of exogenous Wnt ligand reduction on organoid formation efficiency in vitro. EPCAM\(^+\)GFPh\(^{high}\) cells sorted from P14 Lgr5-2A-EGFP mice could be developed into organoids under Wnt3a-reduced conditions (‘Low Wnt3a’) but the formation rate was markedly lower than in normal Wnt3a conditions (5% and 8%, respectively; Figs. 7e, 8c). We then co-cultured EPCAM\(^+\)GFPh\(^{high}\) cells with EPCAM\(^+\)GFPh\(^{neg}\) cells in the low Wnt3a condition (Fig. 8a). The organoid formation efficiency returned to similar levels observed using the normal Wnt3a condition (7.8%, Fig. 8b, c) and the mean size of generated organoids was greater under co-culture conditions (+GFPlac) (Fig. 8d). Moreover, compared to the EPCAM\(^+\)GFPh\(^{high}\) cell-derived organoids generated in low Wnt3a condition (Low Wnt3a), a significantly larger proportion (73% vs. 22%) of the organoids grown under co-culture conditions (+GFPlac) adopted the round-type morphology characteristic of a highly proliferative, undifferentiated state (Fig. 8b, e). When ETC159\(^{33}\), a porcine inhibitor, was initially added to the co-culture condition (+GFPlac) to inhibit any endogenous Wnt ligand secretion (+GFPlac +ETC159), the organoids predominantly appeared as vacuolated type (70%), and both the mean size and the organoid outgrowth efficacy were reduced (Fig. 8b–e). These results suggest that Lgr5\(^{neg}\) epithelial cells expressing robust levels of Wnt4/7b can function as an endogenous Wnt source for Lgr5\(^{high}\) stem cell-derived organoids in vitro.

To corroborate this finding in vivo, we evaluated neonatal uterine gland development following selective inhibition of paracrine Wnt signaling from epithelial cells by treatment of P7 wild-type mice with ETC159. After 7 days of daily i.p. injection, we evaluated glandular development by H&E (Fig. 8f). No Foxa2\(^+\) glands were observed in ETC159-treated mice, indicating that glandular development was significantly impaired by suppressing...
Wnt signaling in immature uterine epithelium (Fig. 8g–i). Notably, both Lgr5 and Axin2 expression in the ETC159-treated epithelium was markedly decreased and there was an accompanying major reduction in proliferation within the developing GE, confirming the efficient inhibition of Wnt signaling in vivo (Supplementary Fig. 8a–c). In contrast, LE development was largely unaffected by ETC159 treatment. Progesterone has been shown to be an inhibitor of gland development34. To evaluate whether Lgr5 expression is impacted when gland development is perturbed, we treated neonatal mice...
with progesterone from P2 to P10. At P14, there was a significant reduction of Lgr5 expression at developing gland buds (Supplementary Fig. 8d) indicating that the impairment in gland development by progesterone may be due to the reduction in Lgr5 expression.

**Discussion**

Here, we report the identification of mid-gestation embryonic Lgr5 stem/progenitor cells contributing to the development of the female reproductive tract epithelia, and a population of neonatal Lgr5 stem/progenitor cells supported by an epithelial Wnt source that are responsible for uterine gland development. Employing a new, non-variegated Lgr5-2A-EGFP reporter mouse model and highly sensitive RNA ISH techniques, we show that Lgr5 expression in the developing female reproductive tract first originates in the E11.5 coelomic epithelium, the precursor of the Müllerian duct. In vivo lineage tracing with our non-variegated Lgr5-2A-CreERT2; R26-tdTomato mouse model identifies this coelomic Lgr5 population as the origin of the epithelial lining of the upper vagina, uterus, and oviduct. We also observe robust contribution of embryonic Lgr5 population to the ovary surface epithelium and granulosa lineages as previously reported. Consistent with our previous report, Lgr5 expression was detected in oviduct and upper vagina around birth, but rapidly declines to undetectable levels by P7. In contrast, Lgr5 expression in the developing uterus exhibits a robust and dynamic expression pattern during pre-puberty. Prior to the onset of gland formation, Lgr5 expression is uniformly distributed throughout the developing endometrium. However, coincident with the onset of adenogenesis around P7, Lgr5 expression converts to a gradient, with highest levels present at the tips of developing antimesometrial glands. Fate mapping identifies these P7 Lgr5+ cells as stem/progenitor cells contributing to the development and maintenance of both the LE and GE compartments of the adult endometrium. Lgr5 expression within the developing LE continues to decrease over the next 2–3 weeks, until it becomes confined to the tips of the maturing endometrial glands. Induction of Lgr5+ cell-driven lineage tracing at this stage identifies the gland-resident Lgr5high population as stem/progenitor cells contributing exclusively to the development and maintenance of the adult GE (Fig. 9). A previous study using a PgrCre conditional knockout mouse model to evaluate Lgr5 gene function in the neonatal endometrium demonstrated that Lgr5 was dispensable for normal uterine development. Although Lgr5 function may be non-essential during these early stages, likely due to functional compensation by Lgr4, ablation of the Lgr5-expressing cells in our DT model demonstrates the significance and impact of the Lgr5+ progenitor cell population during uterine development. Somewhat surprisingly, LE development was largely unaffected by loss of the endogenous Lgr5+ cells, implying the existence of an Lgr5− stem/progenitor cells in this endometrial compartment at this stage. Collectively, these observations indicate that during pre-pubertal development of the uterine epithelium, separate stem/progenitor pools are established for the GE and LE compartments. Lgr5+ cells serve as dedicated GE stem/progenitor cells in the maturing endometrium, whilst the identity of the LE stem/progenitor cells remains to be established. In the adult endometrium, Lgr5 expression is confined to the LE, where it is hormonally regulated during the estrus cycle and appears to mark short-lived, non-stem/progenitor populations. It was recently reported that bipotent stem cells in adult endometrium reside in the zone between GE and LE, but further investigation is needed to better characterize them. Given that the Lgr5− cells are found in the developing GE residing proximally to Foxa2+ GE cells, it would be interesting to investigate whether the Lgr5+ postnatal progenitors can be the source of bipotent stem cells in the adult.

Lgr5+ stem cell populations in many tissues are regulated by canonical Wnt signaling, which is also known to be critical for uterine development in mice. Comparative expression profiling of Lgr5 stem/progenitor cells and their progeny in the developing endometrium revealed robust Wnt-signaling activity in the GE stem/progenitor cell compartment, likely transmitted via Fzd10 through interaction with several endogenous Wnt ligands, including Wnt4 and Wnt7b, which were expressed in adjacent Lgr5− cells. Given that Aldh1a1 has been reported as a stem/progenitor marker in developing endometrium based on single RNA sequence analysis and also Prom1 is an established stem cell marker in small intestine, the upregulation of these genes in Lgr5+ cells further supports their stem cell identity. Lgr5 is a facultative component of the Wnt receptor complex, functioning to amplify canonical Wnt signaling on stem cell populations through interaction with its family of ligands, the R-spondins. Using sensitive ISH analyses, we document Rspo1 expression within the stroma surrounding the Lgr5high stem/progenitor zone at the base of developing glands, highlighting a potential source of Wnt-modulating Lgr5 ligands in the pre-pubertal uterus.

Lgr5high cells FACS-sorted from developing endometrium supported Rspo/Wnt-dependent outgrowth into epithelial organoids ex vivo, further underscoring the identity of the Lgr5high cells as a Wnt-regulated stem/progenitor population. Using this assay, we further show that canonical Wnt-signaling levels are instrumental in regulating the balance between stemness/proliferation and differentiation in immature endometrial organoids. Pharmacological inhibition of endogenous paracrine Wnt secretion in neonate mice using porcupine inhibitor established a similar dependence of uterine gland development on endogenous Wnt signaling, in agreement with similar phenotypes reported for various Wnt KO mouse models. Conditional Poreknockout mice exhibit variable uterus phenotypes, with complete ablation compromising only adult endometrial gland maintenance, in contrast to partial knockout, which suppresses proper gland formation. The major impairment of endometrial gland formation we observe using pharmacological inhibition of Porcupine function, which targets the epithelial...
compartment, supports opposing roles for Wnt signaling within the stromal and epithelial compartments during neonatal gland formation. Importantly, we also identify Lgr5–endometrial cells as an epithelial Wnt source capable of substituting for exogenous Wnt ligands in supporting efficient organoid formation and expansion from Lgr5high cells ex vivo. Paneth cells have similarly been identified as an epithelial Wnt source for Lgr5+ stem cells in the intestine. In future, it will be interesting to investigate the potential existence of functional redundancy between the epithelial and stromal Wnt sources in the developing uterus. Progesterone is known to suppress endometrial development. We show Lgr5 expression is down-regulated in progesterone-.
treated mice, indicating that inhibition of glandular development by progesterone treatment also affects Lgr5 expression. This is compatible with the findings of Sun et al., where progesterone stimulation resulted in Lgr5 down-regulation. However, it is not clear whether loss of Lgr5 expression is a cause or consequence of impaired gland development and it is not possible to directly correlate this with the attenuated gland formation caused by loss of Lgr5+ cells in our own study. Given that we now show that Wnt signaling is required for gland development, it is possible that downregulation of Lgr5 expression by progesterone imparts Wnt-signaling initiation at the membrane of the gland progenitor cells, impacting their endogenous function.

The early establishment of cellular hierarchies is thought to be crucial for optimal neonatal development, particularly in tissues undergoing branching morphogenesis, such as the lungs or pancreas. The model we propose here for endometrial gland formation is similar to that of pancreas development, where a cellular hierarchy harboring Lgr5+ stem/progenitor cells at its apex is formed within nascent glands to ensure the development and maintenance of the adult glands.

The findings reported here deliver a major advance in our understanding of Wnt-driven gland formation in the developing uterus, identifying dedicated Wnt-regulated, gland-resident Lgr5+ stem/progenitor cells and supporting epithelial niche cells as critical orchestrators of endometrial development to ensure successful pregnancy in adults.

Methods

Mouse models. Lgr5-2A-EGFP and Lgr5-2A-DTR mice were generated by homologous recombination in embryonic stem cells targeting the 2A-EGFP and 2A-DTR cassette, respectively, to the stop codon of Lgr5. Lgr5-2A-CreERT2 mice were described previously. The Rosa26 iDTomato mice were purchased from Jackson Labs. Lgr5-2A-EGFP mouse model has been previously described. Vaginal smears were taken from adult females to determine the estrous cycle stages based on the presence and proportion of leukocytes, nucleated cells, and cornified cells. Sex genotyping was performed when analyzing embryos. Genotyping primers are described in Supplementary Table 1. All animal experiments were approved by the Institutional Animal Care and Use Committee of Singapore. The experiments were not randomized, and there was no blinded allocation during experiments and outcome assessment.

Animal experiments. 40HT (Sigma, H7904) and tamoxifen (Sigma, T5648) were dissolved in sunflower oil and injected i.p. at a single dose. 40HT was injected to P7 mice at 0.5 μg/g body weight and tamoxifen was injected to P14 and older mice at 0.15 mg/g body weight. For induction of lineage tracing from embryonic stages, 0.2 mg/g body weight of tamoxifen was injected to the pregnant female mice at E11.5. Timed pregnancies were staged relative to a vaginal plug that represents day 0.5 (E0.5). For DT injections, P7 Lgr5-2A-DTR or WT mice were i.p. injected with a single dose of 16.6 μg/kg body weight DT (Sigma, D0564) dissolved in PBS. For porcine inhibitor experiments, ETC159 (gift from Prof. David M. Virshup, Duke NUS Medical School, Singapore) was dissolved in 50% PEG400 (v/v) in water and was injected s.c. daily at a dose of 10 mg/kg body weight. Progesterone (Sigma, P491142) was dissolved in sesame oil (Sigma, S3547) and injected s.c. daily at 50 μg/g body weight from P2 to P10.

Cell dissociation and flow cytometry. Uterine horns were harvested from mice and finely chopped using scalpel blades. Minced tissue was then incubated in chelation buffer (5.6 mM sodium phosphate, 8 mM potassium phosphate, 96.2 mM sodium chloride, 1.6 mM potassium chloride, 43.4 mM sucrose, 54.5 mM d-sorbitol, 1 mM dithiothreitol) with 5 mM EDTA, 2 mg/ml collagenase I (Worthington, LS04196) and 1 mM DTT at 37 °C for 1 h. Chelation buffer containing tissue was filtered through 100 μm filter mesh, and centrifuged at 720×g for 4 min. The pellet was resuspended in 1X TrypLE (Life Technologies, 12604) with DNasel (0.8 U/μl) (Sigma, D4513) and incubated at 37 °C for 10 min with intermittent trituration for digestion into single cells. Digestion was quenched by dilution with cold HBSS buffer. The suspension was centrifuged at 720×g for 4 min. The pellet was resuspended in HBSS with 5% fetal bovine serum (FBS, Hyclone) and filtered through a 40 μm strainer. Cells were then stained with EPCAM antibody (APC-conjugated, Biologend, 118214) at 1:200 dilution and sorted on a BD Influx Cell Sorter (BD Biosciences) after adding 1 μg/ml propidium iodide (Life Technologies). Cells were collected in RLT Plus buffer (Qiagen) for RNA extraction or HBSS with 2% FBS and 1% PenStrep (Gibco) for organoid culture.

RNA isolation and qPCR. RNA was isolated from tissue, organoids, or sorted cells using Trizol (Qiagen) or RNeasy Universal Plus Kit (Qiagen) according to the manufacturer’s instructions. CDNA was generated using Superscript III (Life Technologies) according to the manufacturer’s instructions. qPCR was performed in triplicate for gene of at least three biological replicates using GoTaq SYBR Green dye (Promega, A6002) according to the manufacturer’s instructions. Relative quantification of gene expression was analyzed with Step One Software v2.1 (Applied Biosystems) using the ΔΔCT method with Gapdh as an endogenous reference. The qPCR primers are described in Supplementary Table 1.

Microarray and analysis. Hybridization and washing protocols were performed according to Orgene instructions. RNA quality was first determined by assessing the integrity of the 28s and 18s ribosomal RNA bands on Agilent RNA 6000 Pico LabChips in an Agilent 2100 Bioanalyzer (Agilent Technologies). A minimum of 2 μg of RNA was used to generate SPIA-amplified cDNA using the Ovation Pico WTA system (Nugen Technologies). Five micrograms of SPIA-amplified purified cDNA was then fragmented and biotin-labeled using the Nugen Encore Bio module (Nugen Technologies). Microarray was performed using the Affymetrix Mouse ST v2.0 GeneChips (Affymetrix). The individual microarrays were washed.
and stained in an Affymetrix Fluidics Station 450, and hybridized probe fluorescence was detected using the Affymetrix G3000 GeneArray Scanner. CEL files were generated for each array and used for gene expression analysis. The CEL files were then processed in R (v3.6.0) with the Bioconductor (v3.9) libraries oligo (v1.48.0), mogene20stranscriptcluster.db (v8.7.0), and limma (v3.40.2). We used robust multi-array average (RMA) to perform background correction and normalization with the rma function implemented in the oligo package. The experimental design was stored as two factors with two levels each: batch (1 and 2) and Lgr5-EGFP level (high and low). Linear models were fitted to the expression data with the function lmFit, and differential expression was tested with eBayes (default parameters). P-values were adjusted using the Benjamini and Hochberg method. An adjusted P-value < 0.05 was considered as significant.
**Fig. 8** Defining an epithelial Wnt source for neonatal Lgr5\(^{\text{GFPhigh}}\) stem/progenitor cells in the developing uterus. a Experimental design of co-culturing EPCAM\(^{+}\)GFP\(^{\text{GFPhigh}}\) and EPCAM\(^{+}\)GFP\(^{\text{GFPneg}}\) cells sorted from P14 Lgr5-2A-EGFP mouse. b Organoid cultures obtained using the indicated conditions (image representative of five replicates). Scale bars, 200 μm. c The efficiency of organoid formation from single EPCAM\(^{+}\)GFP\(^{\text{GFPhigh}}\) cells using the indicated culture conditions. Data from five independent experiments are presented as mean ± s.e.m. Data were tested for significance using unpaired two-tailed t-test (Low Wnt3a vs. +GFP neg; \(P = 0.025\), +GFP neg vs. +GFP neg + ETC159; \(P = 0.002\)). d Quantitation of organoid size under each culture condition. Three independent fields from each condition were analyzed. Data from five independent experiments are presented as mean ± s.e.m. Data were tested for significance using unpaired two-tailed t-test (Low Wnt3a vs. +GFP neg; \(P = 0.036\), +GFP neg vs. +GFP neg + ETC159; \(P = 0.006\)). e The average proportions of organoid types generated using the indicated culture conditions from five independent experiments. Data were tested for significance by a chi-square test (Low Wnt3a vs. +GFP neg; \(P = 9E^{-13}\), +GFP neg vs. +GFP neg + ETC159; \(P = 8E^{-10}\)). f Experimental strategy of in vivo ETC159 treatment to suppress Wnt secretion in neonatal uterus. g Immunostaining for Foxa2 on ETC159 treated (\(n = 5\)) and vehicle-treated control mouse (\(n = 5\)) at P14. The insets with solid lines indicate developing GE. Scale bars, 200 μm. h Quantification of the number of Foxa2\(^{+}\) glands in ETC159- treated and vehicle-treated control mouse uterus at P14. Three independent fields from each mouse were analyzed. Data from five independent mice are presented. Red bars represent median. Data were tested for significance using unpaired two-tailed t-test (\(P = 2E^{-10}\)). i 3D images of P14 vehicle and ETC159-treated WT mouse, stained for K8. The insets show glandular areas. Scale bars, 100 μm. All images are representative of five independent mice per treatment. \(* * * P < 0.001\), \(* P < 0.01\), \(* P < 0.05\).

**Fig. 9** Summary cartoon depicting the dynamic expression pattern and function of Wnt-regulated Lgr5\(^{+}\) cells in the immature uterus. At P7, Lgr5\(^{+}\) cells are uniformly distributed throughout the endometrium and contribute to the development of both the LE and GE. After P14, Lgr5\(^{+}\) cells become restricted to the tips of developing glands and exclusively contribute to the development of GE. Wnt signaling is instrumental in regulating the activity of the Lgr5\(^{+}\) stem/progenitor cells at P14, likely mediated by interaction of Fzd10 with Wnt ligands supplied by both epithelial and stromal populations.

**Organoid culture.** FACS-sorted single endometrial cells were seeded in growth factor-reduced Matrigel (Corning, 356231) and cultured in Advanced DMEM/F-12 media supplemented with 10 mM HEPES, 2 mM Glutamax, 1x N2, 1x B27 (all Invitrogen), N-acetyl-cysteine (Sigma, A9165), Primocin (Invitrogen, anti-pm1), 50 ng/ml EGF (Invitrogen, PHG0311), 100 ng/ml FGF10 (Peprotech, 100-188), 100 ng/ml Noggin (Peprotech, 250-38-50), 100 ng/ml Wnt3a (Millipore, GF-160), 1 μg/ml R-spondin (Peprotech, 120-38-500), 50 ng/ml HGF (Peprotech, 100-39H), 2 μM A83-01 (Tocris, 2939) and 10 mM nicotinamide (Sigma, N0636). 10 μM Rock-inhibitor Y27632 (Sigma, Y0503) and 10 mM nicotinamide (Sigma, N0636). 10 μM Rock-inhibitor Y27632 (Sigma, Y0503) was also added to single-cell cultures. Culture medium was changed every other day. After 7–10 days of culture, organoids were harvested for analysis. DT was added at a concentration of 0.25 ng/μl to the culture medium to induce Lgr5\(^{+}\) cell ablation at the indicated time point.
representative immunostainings were included in the manuscript. Tissue sections were repeated on at least three tissue sections per tissue block. Only representative immunohistochemistry and immunofluorescence

H&E staining was performed on deparaffinised and rehydrated 6 μm tissue sections which were stained with Haematoxylin 2 (Richard-Allan Scientific, 7231L) followed by Scott’s blue reagent (0.2% NaHCO₃ (w/v), 2% MgSO₄ (w/v) in water), then Eosin (Sigma, HT110132). Stained sections were dehydrated and mounted using DPX (Sigma 1.07979,0500). Whole-mount imaging and immunostaining. For whole-mount experiments, tissues were fixed in 4% PFA overnight at 4 °C, followed by permeabilization in 0.1% TritonX-100/0.3% H₂O₂ in 50% Sucrose/PBS before cryoprotecting and embedding into: 1) Tissue Freezer (Leica) for cryo-sectioning or 2) Osmium Tetroxide (Sigma, 24219-01) and 50% Sucrose/PBS for dehydration and mounting on glass slides. Tissues were then cleared with RapiClear (Sigma, 650–601–110) and then Eosin (Sigma, HT110132). Stained sections were dehydrated and mounted using DPX (Sigma 1.07979,0500).

Whole-mount imaging and immunostaining. For whole-mount experiments, tissues were fixed in 4% PFA overnight at 4 °C, followed by permeabilization in 0.1% TritonX-100/0.3% H₂O₂ in 50% Sucrose/PBS before cryoprotecting and embedding into: 1) Tissue Freezer (Leica) for cryo-sectioning or 2) Osmium Tetroxide (Sigma, 24219-01) and 50% Sucrose/PBS for dehydration and mounting on glass slides. Tissues were then cleared with RapiClear 1.52 (Sunjin Lab, Hsinchu City, Taiwan) according to the manufacturer’s instructions. Hoechst dye was used as nuclear counterstain. For immunostaining of whole-mount tissues for 3D imaging, tissues were permeabilized and labeled as described above followed by transferring into blocking buffer (5% NGS and 1% BSA in PBS) at 4 °C for 2 h. After washing tissues with washing buffer (0.3% NaCl solution in 1% PBS, pH 7.4) at 4 °C for 30 min, tissues were washed with washing buffer followed by employing secondary antibodies (anti-rabbit/mouse/rat Alexa 488/568/647 IgG, 1:500; Invitrogen) for FITC. FITC-labeled slides were mounted using Hycamtin solution (National Diagnostics, HS-106) with Hoechst as nuclear counterstain. Immunostainings were repeated on at least three tissue sections per tissue block. Only representative immunostainings were included in the manuscript.

H&E staining was performed on deparaffinized and rehydrated 6 μm tissue sections which were stained with Haematoxylin 2 (Richard-Allan Scientific, 7231L) followed by Scott’s blue reagent (0.2% NaHCO₃ (w/v), 2% MgSO₄ (w/v) in water), then Eosin (Sigma, HT110132). Stained sections were dehydrated and mounted using DPX (Sigma 1.07979,0500).

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RNA ISH. RNA ISH and co-ISH was performed using RNAscope 2.5 High Definition Brown Assay and Duplex Reagent Assay (Advanced Cell Diagnostics), respectively. Consecutive sections hybridised for Dapβ as negative control were included for each RNAscope experiment.

Microscopy imaging. The following microscopes were used for image acquisition: Nikon Ni-E microscope/DS-R2 camera for IHC, H&E and RNAscope slides and an Olympus FX3000RS upright confocal microscope for IF slides and whole-mount images. Cultured organoids were imaged with Olympus DP-27 camera on Olympus IX53-inverted microscope.

Image processing and analysis. All IF and 2D whole mount images were processed using ImageJ (NIH). Quantification of Foxa2-positive glands was performed by counting them in cross-section images taken from the middle of ureteric horns in line with previous publications. The surface area of overlapping Foxa2 and tdTomato stainings was measured by ImageJ (NIH). Whole-mount 3D rendering images were processed using Imaris 9.2.0 software (BITPLANE).

Statistical analysis. Statistical analyses were performed using GraphPad Prism, STA1A5, or Microsoft Excel function. Data were expressed as mean ± SE. Statistical differences were determined using unpaired two-tailed t-test, one-way ANOVA or chi-square test. P-values of statistical significance are represented as ***P<0.001, **P<0.01,*P<0.05.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Microarray data that support the findings of this study have been deposited in the Gene Expression Omnibus (GEO) under accession code GSE137974.

Received: 21 December 2018; Accepted: 5 November 2019; Published online: 26 November 2019

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Acknowledgements
We thank IMB-AMP and SBIC-Nikon Imaging Centre for imaging assistance, A*STAR SgN for FACS sorting. We thank Prof. D.M. Virshup, Duke NUS Medical School, Singapore for providing ETC159 and P. Sauvage, Development of Molecular Biology, Genetech, South San Francisco, CA 94080, USA for providing the Lgr5-DTR-EGFP mice. N.B. is supported by the Agency for Science, Technology and Research (A*STAR) and the National Research Foundation (NRF) NRF2017-03.

Author contributions
R.S. designed, performed experiments, analyzed data, and wrote the manuscript. K.B.A.M. performed histology experiments. Y.S. generated Lgr5-2A-EGFP and Lgr5-2A-DTR mouse lines and performed FACS experiments. C.L., L.T.T., A.H., and S.H.T. performed mouse experiments. Y.I. and S.N. performed microarray analysis. H.I. constructed 3D images. K.M. and Y.Y. performed organoid experiments. E.W. generated Lgr5-2A-EGFP and Lgr5-2A-DTR mouse lines. N.B. supervised the project, generated Lgr5-2A-EGFP and Lgr5-2A-DTR mouse lines and wrote the manuscript. All figures are created by the author and the co-authors.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41467-019-13363-3.

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Peer review information Nature Communications thanks the anonymous reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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