The Notch and Wnt pathways regulate stemness and differentiation in human fallopian tube organoids

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The epithelial lining of the fallopian tube is of critical importance for human reproduction and has been implicated as a site of origin of high-grade serous ovarian cancer. Here we report on the establishment of long-term, stable 3D organoid cultures from human fallopian tubes, indicative of the presence of adult stem cells. We show that single epithelial stem cells in vitro can give rise to differentiated organoids containing ciliated and secretory cells. Continuous growth and differentiation of organoids depend on both Wnt and Notch paracrine signalling. Microarray analysis reveals that inhibition of Notch signalling causes downregulation of stem cell-associated genes in parallel with decreased proliferation and increased numbers of ciliated cells and that organoids also respond to oestradiol and progesterone treatment in a physiological manner. Thus, our organoid model provides a much-needed basis for future investigations of signalling routes involved in health and disease of the fallopian tube.

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Adult stem cells of the human mucosa have been described in several tissues; however, the molecular mechanisms of renewal and differentiation remain obscure in many cases, including the fallopian tube, a central organ of human reproduction. It is lined by simple columnar epithelium containing secretory and ciliated cells, which produce tubular fluid and facilitate transport of gametes, respectively. In the fimbrium—the distal part that is in close contact with the ovary—this epithelium is organized into extensively branched mucosal folds. Since it is exposed to cyclical hormonal changes, mechanisms to ensure its long-term renewal and integrity are of critical importance. The presence of ‘stem cell-like’ cells has previously been postulated based on sphere-forming capacity and differentiation in vitro and evidence for the presence of label-retaining cells in the distal fallopian tube.

Here we demonstrate the existence of adult stem cells in the human fallopian tube epithelium, which gives rise to a monolayer of differentiated epithelial cells in a complex 3D organoid in vitro. They faithfully recapitulate the native tissue architecture, show robust growth and can be maintained long term in culture (>16 months so far). As in the intestinal tract, skin, liver and ovary, stemness is maintained by active Wnt signalling and is centred on a subfamily of leucine-rich repeat-containing G protein-coupled receptors Lgr4, 5 and 6 (refs 7–9), which are themselves Wnt target genes, and have the capacity to strongly amplify Wnt receptors Lgr4, 5 and 6 (refs 7–9), which are themselves Wnt subfamily of leucine-rich repeat-containing G protein-coupled receptors Lgr4, 5 and 6 (refs 7–9), which are themselves Wnt target genes, and have the capacity to strongly amplify Wnt signals, while the R-spondin family of proteins act as Lgr receptor agonists.

In congruence, the expansion capacity of organoids is modulated by Wnt3A and R-spondin-1 (RSPO1). Furthermore, Notch is responsible for the regulation of known stem cell factor genes. Our data reveal a significant overlap between Notch-dependent genes in the fallopian epithelium and the defined ‘stem cell signature’ of the mouse intestine, suggesting the existence of a conserved pathway that regulates tissue renewal and directly controls cell fate specification and differentiation in the organoid by inhibiting ciliogenesis.

Therefore, this new organoid model closely mimics the normal physiology and anatomy of the human fallopian tube epithelium and provides a starting point for future investigations into the regulatory mechanisms involved in its cellular renewal and pathology.

**Results**

**Adult fallopian tube organoids can be maintained long term.** To obtain insight into the renewal activity of the human fallopian tube mucosa, we carried out immunofluorescence analysis of healthy samples from gynaecological tissue specimens. Ki-67-positive cells were distributed along the epithelial folds, without restriction to a particular region with occasional clustering (Fig. 1a, asterisk). The proportion was higher in the distal compared with the proximal region (5.0 ± 0.3% versus 2.3 ± 1.3%, respectively) in line with previous reports.

To test whether these presumed stem cells can be maintained in vitro if given the appropriate paracrine environment, we isolated and seeded epithelial cells from patient samples in two-dimensional (2D) culture, followed by transfer to a three-dimensional (3D) Matrigel matrix, supplemented with a cocktail of growth factors to modulate Wnt, Notch, epidermal growth factor (EGF), fibroblast growth factor (FGF)-10 and transforming growth factor (TGF)-β signalling. Small round clusters of cells were visible after 24 h (Fig. 1b) and by 3 days post seeding developed into rapidly expanding spheres of a round, cystic phenotype, reaching up to 2.5 mm in diameter within 2 weeks. Each Matrigel drop seeded with 20,000 cells yielded an average of 5.0–7.0 organoids. The appearance of folds, invaginations of the epithelium, as a hallmark of mature organoid culture is detected during the second week of 3D growth (Fig. 1c, arrows). Overall growth rates during long-term culture remained constant, with passaging at a 1:3 ratio required every 2–3 weeks (Fig. 1b). The doubling time of cells in organoid cultures, quantified based on the number of progeny from a defined number of seeded cells over 2 weeks of cultivation, was 3.5–5 days (Supplementary Fig. 1a), and Hoechst 33342 labelling to visualize proliferation revealed several mitotic cleavage planes within a small region of the organoid surface (Supplementary Fig. 1b), suggestive of rapid growth. Between passages individual organoids greatly increased in diameter (Supplementary Fig. 1c). Live cell imaging of fragmented pieces immediately after passage 13 (~10 months) showed that they re-organized within 4–6 h, followed by rapid invagination of epithelial folds and spherical expansion (Supplementary Movie 1). Overall, this method has yielded expandable, stable organoid cultures in all healthy tissue samples from 50 donors tested so far, with only minor variations in sphere formation capacity and expansion rate between donors or between distal and proximal tubal regions (Supplementary Fig. 1d).

Freshly seeded epithelial isolates followed over 72 h using live cell imaging demonstrated that spheres do not form through the aggregation of cells but rather through the expansion of a subpopulation of individual cells (asterisks, Supplementary Movie 2). To investigate whether organoid formation is a monoclonal process, we labelled epithelial cells with either GFP- or mCherry-expressing lentivirus, and seeded them in Matrigel at equal ratio. The resulting organoids were almost exclusively either red or green (Fig. 1d), with less than 1% dual-colour organoids (from non-clonal origin). We also performed clonogenic assays by seeding FACS-isolated epithelial cell adhesion molecule (EpCAM) + cells at a ratio of one cell per 10 μl Matrigel drop in a 96-well format. Monoclonal organoids developed with a frequency of 0.5–1% (Fig. 1e), could be expanded long term (>3 months) and consisted of both secretory and ciliated cells, demonstrating the bipotency of the initiating progenitor cell (Fig. 1f). These stem cells are likely to be non-ciliated, since cilia were not seen in developing organoids until 2 weeks after initial cell seeding (Fig. 1g), despite the presence of ciliated cells in the initial cell isolate (Supplementary Fig. 1e). Hence, we conclude that organoid formation is vastly monoclonal and is driven by bipotent EpCAM + stem cells of the fallopian tube, which maintain long-term potency in our system.

Organoids faithfully recapitulate the mucosal fold architecture. Detailed characterization of the organoid epithelium using confocal microscopy revealed highly polarized columnar cells, exhibiting precisely aligned nuclei, a restricted location of the main epithelial adhesion molecule E-Cadherin (Cdh1) to the lateral membrane, and an orientation of the apical pole to the luminal side (Fig. 2a,b). Mature organoids contained PAX8-positive secretory cells as well as PAX8-negative, acetylated tubulin-positive ciliated cells. Occasional PAX8-positive ciliated cells (Fig. 2c), which were also observed in tissue sections (Supplementary Fig. 2a), suggest that loss of PAX8 represents a late event during differentiation to a ciliated phenotype, in line with our observation that both cell types derive from bipotent unciliated stem cells.

We further observed subapical localization of occludin in a nodal pattern, indicative of functional tight junctions (Fig. 2d, left). In addition, organoids contained clusters of cells expressing high levels of Ca125 protein (MUC16, Fig. 2d, centre), very similar to those observed in the tissue (Supplementary Fig. 2b). As previously shown for epithelial cells of the fallopian tube, organoids also exhibited basolateral accumulation of vimentin (Fig. 2d, right). Transmission electron microscopy analysis
(Fig. 2e) confirmed the high degree of overall similarity to fallopian tube tissue, including fully developed junctions between the cells consisting of desmosomes, zonula adherens and tight junctions (arrow, right panel), as well as fully assembled cilia (asterisks, right panel) and active secretion (double arrow, left panel), all of which indicate mature functional differentiation. Organoids also recapitulate the higher-order epithelial tissue architecture, as evidenced by frequent folding of epithelial sheets initially observed in phase-contrast images, is also prominent in confocal images of organoid sections, resembling the extensive folds seen in the authentic tissue (Supplementary Fig. 2b).

Comparative analysis of phenotypes at 1 and 4 months of culture showed long-term stability with no apparent changes in the epithelial structure and polarity over time, as organoids undergo a new cycle of growth after each passage (Fig. 3a). In agreement with the stable time window between passages, the percentage of actively proliferating cells also remained constant over time (Fig. 3b) as did the number of ciliated cells (Fig. 3c). We conclude that, in contrast to other organoid models, the presence of Wnt3A and RSPO1 in the organoid culture medium maintains a subpopulation of stem cells long term, while at the same time allowing for full differentiation of the complete fallopian tube phenotypes.

Active Wnt signalling preserves stemness in fallopian organoids. A requirement for active paracrine signalling to preserve functional stem cells has previously been described for gastrointestinal organoid models. On the basis of the presumptive conservation of core renewal mechanisms, fallopian tube organoids were supplemented with EGF, FGF10 and factors that modulate paracrine Wnt signalling (Wnt3a and RSPO1), suppress...
TGF-β receptor kinase (ALK4/5) and BMP signalling (Noggin) and inhibit anoikis (ROCK inhibitor). Organoids did form in basic medium but their number doubled in the presence of EGF, while addition of RSPO1 increased their size (Fig. 4a). EGF and Wnt activation was crucial for maintenance of stable growth over time, as seen in comparative images from cultures maintained for 3 months with different supplements (Fig. 4b).

Although organoids could be initiated without TGF-β RK inhibitor (Supplementary Fig. 3a), they exhibited slowdown of expansion and finally growth arrest by four to six passages (3–4 months). In the presence of TGF-β RK inhibitor, on the other hand, quasi-indefinite expansion (>1 year) was seen routinely.

To demonstrate conclusively that Wnt signalling is active in fallopian tube organoids, we introduced the 7×Tcf-eGFP reporter by lentiviral transduction, which contains an oligomerized TCF4-binding-site sequence, ensuring that expression is limited to cells in which this classical Wnt transcriptional response is initiated. Confocal live cell imaging over 40 h showed abundant green fluorescent protein (GFP)-positive cells (Fig. 4c),
increased expression levels of Indeed, in the presence of RSPO1, Wnt3A addition strongly presence of RSPO1 alone or in combination with Wnt3A. expression of several stem cell genes using qPCR in the 6,17,18). To examine this interdependency, we measured effects of Wnt3A and RSPO1 on the expression of stimulation, we used quantitative PCR (qPCR) to assess the lyt enhancer factor (LEF) DsRed (Supplementary Fig. 3b). To examine the importance of exogenous Wnt pathway dynamics of organoid growth remains stable over time as determined by quantification of the relative number of proliferative Ki-67-positive cells in confocal sections at 1- and 8-month culture. (c) The proportion of ciliated cells also remains unchanged as determined by quantification of ciliated cells. Error bars represent ± s.d. from a minimum of five independent fields of view. confirming activation of the canonical Wnt pathway. Notably, we detected several GFP-positive cells that were actively dividing (Supplementary Movie 3). In line with this, we independently confirmed transmission of the Wnt signal to the nuclei of early organoids with the lentiviral reporter T-cell factor (TCF)/lymphoid enhancer factor (LEF) DsRed (Supplementary Fig. 3b). To examine the importance of exogenous Wnt pathway stimulation, we used quantitative PCR (qPCR) to assess the effects of Wnt3A and RSPO1 on the expression of hTERT, a direct target of Wnt signalling14 reported to be highly expressed in intestinal stem cells15. Both Wnt ligands triggered strong upregulation of hTERT (Fig. 4d) and increased proliferation (Ki67), suggesting that this pathway is involved in maintaining fallopian tube stem cells. In addition, the Wnt target genes Axin2 and cMYC were upregulated.

Next, we investigated whether there is interdependency between Wnt3A and RSPO1, and how this contributes to sphere growth and differentiation. LGR6 appears to be a good candidate for mediating the effects of RSPO1, based on the high sequence conservation and functional homology with LGR5 (refs 6,18), which is not expressed in either the fallopian tube or the organoids, as indicated by the microarray data. Immunofluorescence labelling revealed a randomly distributed population of LGR6-positive cells in both tissue and organ sections (Supplementary Fig. 4). In the organoids these cells were often, but not exclusively, found in regions enriched in Ki67 (Supplementary Fig. 4a, asterisks).

On the basis of the hypothesis that the Wnt pathway plays a role in the maintenance of the fallopian stem cell niche, we used systematic Gene Set Enrichment Analysis (GSEA) to test whether the downregulated genes are part of a larger molecular programme related to stemness. For comparison, we used the human homologues of a set of 274 previously described adult mouse intestinal crypt 'stem cell signature' genes21. On the basis of the similarity of the paracrine signalling pathways involved, we wanted to examine whether the underlying conserved molecular mechanisms could be identified. GSEA revealed that the set of downregulated genes in DBZ-treated organoids is significantly enriched (GSEA permutation test, \( P < 0.001 \)) for the intestinal 'stem cell signature' genes (Fig. 5c). Of the 274 genes, 78 were significantly downregulated (log ratio error, \( P < 0.05 \)) after Notch inhibition (Fig. 5d) including EphA4, RNF43, SMO and the frizzled receptors FZD2 and FZD7 (Supplementary Table 1), all of which are known to play important roles in preservation of stemness22–24. Thus, conserved molecular mechanisms regulating adult stem cell niches are active in the fallopian tube, and expression of stemness-related genes in the epithelium is supported by active Notch signalling.

**Notch signalling sustains expression of stem cell genes.** As the Notch signalling pathway is known to play a pivotal role in regulation of the adult intestinal stem cell niche20, we investigated whether growth of organoids and cell fate determination towards secretory or ciliated phenotypes is affected by inhibition of Notch by adding the γ-secretase inhibitor DBZ. Phase contrast imaging indicated increased folding within the organoids after 7 days of treatment (Fig. 5a, asterisk), and microarray analysis on the Agilent platform (44 k array) showed that DBZ caused a strong shift in the global gene expression pattern, affecting nearly 4,000 genes (cutoff at >1.5-fold regulation; see data deposited under the accession number GSE60919). Among the downregulated genes we identified several associated with the maintenance of stemness (AXIN2, LGR6 and Olfactomedin4). This effect was confirmed using qPCR in samples from three independent patients (Fig. 5b).

Effectiveness of DBZ treatment was confirmed by downregulation of the Notch signalling target HES1. The observed downregulation of LGR6, the orphan transmembrane receptor belonging to the Wnt pathway, provides further evidence of the interplay between Wnt and Notch in the regulation of organoid growth and differentiation. LGR6 appears to be a good candidate for mediating the effects of RSPO1, based on the high sequence conservation and functional homology with LGR5 (refs 6,18), which is not expressed in either the fallopian tube or the organoids, as indicated by the microarray data. Immunofluorescence labelling revealed a randomly distributed population of LGR6-positive cells in both tissue and organ sections (Supplementary Fig. 4). In the organoids these cells were often, but not exclusively, found in regions enriched in Ki67 (Supplementary Fig. 4a, asterisks).

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**Regulation of differentiated cells in the organoid.** Down-regulation of stemness-promoting genes should be accompanied by reduced proliferation as well as increased differentiation. To obtain a systematic, unbiased overview, we used the online GOrilla platform25,26 to perform gene ontology enrichment analysis of the list of genes regulated by Notch that were identified with microarray. The programme identified cilia...
Accessory microtubuli as highly enriched primary cellular components (Supplementary Fig. 5), suggesting that the Notch pathway promotes a secretory cell phenotype and that inhibition triggers differentiation into ciliated cells. To validate the microarray data, the expression levels of several genes indispensable for ciliogenesis and/or cilia function were analysed using qPCR: ARMC4, DNAI1, FOXJ1 and LRRC6 (refs 27–30). Organoids from four different donors revealed a strong induction of all four genes after DBZ treatment for 7 days (Fig. 5e). Confocal analysis of organoids from three further patients confirmed that DBZ treatment reduced the proportion of Ki67-positive cells while simultaneously increasing the proportion of ciliated cells more than twofold (Fig. 6a–d).

Therefore, renewal of the tubal epithelium depends on the Notch pathway, and differentiation into different phenotypes can be modulated by signal strength. This might have significant implications for understanding tubal epithelium pathology, as most models of tubal carcinogenesis postulate ‘secretory cell outgrowth’31 as the initial step in transformation.

Since the fallopian tube is responsive to a hormonally active environment that plays a role in the aetiology of disease, we tested the responsiveness of organoids to stimulation with oestradiol (500 pmol l\(^{-1}\)) or progesterone (50 ng ml\(^{-1}\)) for 2 weeks. Global gene expression pattern analysis by microarray identified more than 1,500, and 450 genes regulated in response to oestradiol and progesterone treatments, respectively (Fig. 6e). Several of these genes were further validated using qPCR (Fig. 6f).
are known to be inversely regulated during different phases of the menstrual cycle, such as progesterone receptor. We also observed a strong oestradiol-induced upregulation of oviductal glycoprotein 1 (OVGP1) thought to play an important role during fertilization. Analysis of genes significantly regulated across three donors reveals clustering for both types of treatment (oestradiol or progesterone) and direction of regulation. Thus, our model should be valuable for studies on the role of hormones in the regulation of the fallopian tube epithelium.

**Discussion**

Here we report the establishment of human fallopian tube organoids that faithfully recapitulate the phenotype of *in vivo* tissue and can be stably expanded for more than a year without apparent changes in phenotype, essentially demonstrating the existence of fallopian tube stem cells. These are single bi-potent stem cells that give rise to whole organoids through long-term proliferation and differentiation into secretory and ciliated cells. Successful cultivation depends on supplementation with appropriate growth factors that support paracrine signalling pathways, in particular Wnt and Notch, to preserve the stem cell niche. The absence of these supplements is a likely reason for previous failures in expanding fallopian tube cells *in vitro* and their incomplete polarization, resulting in abundant multilayering and the absence of a central cavity. The effects of Notch inhibition strongly support a central role of the Wnt and Notch pathways in the regulation of stemness.
The overlap between Notch-dependent genes in the human fallopian tube and the stem cell signature network of the mouse intestine points to similar regulatory mechanisms in these two niches. LGR5-positive intestinal stem cells are known to exhibit high telomerase activity in vitro, which rapidly decreases in transit amplifying and differentiating cells. Thus, it is likely that the increase in expression of hTERT, Ki67, Olfactomedin4 and Notch1 that we observed when active Wnt signalling is maintained by RSPO1 and Wnt3A reflects an increase in the number of stem cells.

The requirement for RSPO1 further indicates a decisive role of its receptors for epithelial tissue renewal. Our microarray data reveal an insignificant expression of LGR5, the main receptor for RSPO1 in gastrointestinal tissues. In line with this, a recent report also showed only very limited expression in the human fimbrium and none in the adult mouse fallopian tube. In contrast, we detected strong expression of its homologue LGR6 in tissue sections as well as organoids, suggesting that it could be a functional RSPO1 receptor, and potentially also a stem cell marker in the fallopian tube.

Our findings have considerable implications for the understanding of fallopian epithelial renewal in vivo and its role in disease development. While cellular transformation of the ovarian surface epithelium is still a possibility, it is the epithelium of the fimbrium that is now widely considered as the most frequent tissue of origin of high-grade serous ovarian cancer, while...
intestine and uterus are likely to be additional tissues contributing to the aetiology of this peritoneal malignancy. While the exact contribution of each of these organs to this highly complex disease is yet to be determined, there is clearly a need for a better understanding of the biology of fallopian tube epithelium. In this context, our organoid model from patient material, exhibiting the polarized epithelium that readily responds to hormonal stimulation opens up exciting new possibilities for studying the aetiology of ovarian cancer. Deregalation of Notch signalling has been identified as one of the most frequent and significant changes in ovarian cancer patient. Thus, our finding that Notch is an important regulator of stemness and differentiation which have been defined as ‘outgrowths of secretory cells’ strongly suggests that the secretory phenotype preceeds organoids and the advancement of screening technology.

Existing primary cell models enabling numerous downstream applications, such as the generation of mutant organoids and the advancement of screening technology.

Methods

Antibodies and chemicals. The following primary antibodies were used: mouse monoclonal anti-E-Cadherin (BD Biosciences, Cat. No. 610181, 1:200), rabbit polyclonal anti-Ki 67 (Cell Signaling, Cat. No. 9027, 1:100), rabbit monoclonal anti-occludin (Invitrogen, Cat. No. 10336-1-AP, 1:50), mouse monoclonal anti-acetylated tubulin (Sigma, Cat. No. 6-11-B1, 1:100), rabbit polyclonal anti-LGR6 (Abcam, Cat. No. AB10112358, 1:100), mouse monoclonal anti-Ca 125 (Calbiochem, Cat. No. Ca1004, 1:100), mouse monoclonal anti-occcludin (Invitrogen, Cat. No. 33-1500, 1:100) and rabbit polyclonal anti-vimentin (Cell Signaling, Cat. No. 5741, 1:100). We further used the following antibodies: mouse anti-Dox5 (Thermofisher Scientific, Cat. No. 62531), γ-secretase Inhibitor XX (Calbiochem, Cat. No. CAS 209894-56-5), β-estradiol (Sigma, Cat. No. E2758) and progesterone (Acris Organics, Cat. No. Cas 57-83-0).

Epithelial progenitor isolation from fallopian tube samples. Human fallopian tube samples were provided by the Department of Gynecology, Charité University Hospital, Campus Virchow Clinic and Campus Charité, Berlin, Germany. Scientific usage of the material was approved by the institutional review board of the Charité-University of Medicine and by the Commission of the Charité, Berlin (EA/02/007), and all subjects gave informed consent to their tissue being used in scientific research. Fragments were sourced from standard surgical procedures for benign gynecological disease. Only anatomically normal fallopian tubes were used. Tubes were transported and dissected within 2–3 h of removal.

Samples were washed with Dulbecco’s PBS (DPBS), and excessive connective and vascular tissue was removed. Tubes were opened longitudinally to expose the mucosal folds, washed with DPBS and incubated with 0.5 mg ml−1 collagenase type I (Sigma) for 45 min at 37°C to dissociate the epithelial cells from the extracellular matrix. The mucosal cells were then scraped off with a pipette and collected in a Falcon tube. After centrifugation (3 min at 25°C, 300g) the cell pellet was resuspended in ADF (Invitrogen), 12 mM HEPES, 1% GluMAX (both from Invitrogen), 5% FCS (Biocron), supplemented with 10 ng ml−1 human EGF (Invitrogen), µM ROCK inhibitor (Y-27632, Sigma) and 1% penicillin/ streptomycin (Invitrogen), seeded in uncoated cell culture flasks and kept at 37°C, 5% CO2, in a humidified incubator.

Organoid culture. Once they reached 70% confluence, epithelial cells were detached with TrypLE Express (Gibco), centrifuged (7 min at 4°C, 300g) and seeded in 50 µl Matrigel (Corning) in pre-warmed 24-well plates at a density of 25,000 cells per well. The Matrigel was solidified for 20 min at 37°C and overlaid with 500 µl pre-warmed expansion medium (ADF, 25% conditioned human Wharton’s jelly medium) and further cultured in ref. 37 and at the 0.5% TGF-β Kinase Inhibitor IV (SB431542, Calbiochem). Cultures were kept at 37°C, 5% CO2 in a humidified incubator. A tissue fragment of ~ 5 cm yields a confluent monolayer of epithelial cells containing ~ 8 × 106 cells, within 5 days post isolation. These cells can be efficiently transferred to 3D, and could potentially yield ~ 400 wells seeded at 20,000 cells per Matrigel drop. With an average expansion rate of approximately ninefold between passages (Supplementary Fig. 1a), this would yield almost 106 cells within 3 weeks of isolation.

Basic medium (Fig. 3a) contained ADF supplemented with 12 mM HEPES, 1% GluMAX, 2% B27, 1% N2, 1 mM nicotinamide and 0.5 µM TGF-β Kinase Inhibitor IV.

For hormone stimulation experiments, organoids were treated for 2 weeks with beta-estradiol (500 pmol l−1), progesterone (50 ng ml−1) or mock-treated (alcohol) in standard 3D growth medium described above. Subsequently, RNA was isolated and microarray was performed.

FACS isolation of EpCAM+ cells and single-cell culture. Isolated epithelial cells at P0 were detached with TrypLE and labelled for 25 min on ice with fluor-escin isothiocyanate-conjugated anti-human CD326 (EpCAM) antibody (Millenyi Biotec, 130-080-301, 1:30) diluted in ADF medium supplemented with 1% N2, 2% B27, 1 mM nicotinamide, 1 mM acetylcysteine and 9 µM ROCK inhibitor, according to Jung et al.9 EP-CAM-positive single cells were sorted by FACS with exclusion of dead cells using propidium iodide (see Supplementary Fig. 6). Sorted single cells were embedded in Matrigel in a 96-well format at a dilution of one cell per well and cultured as described above.

Maintenance of long-term cultures. The medium was exchanged every 2–4 days, and spheroids were passaged every 14–21 days. For passaging, the Matrigel containing the spheroids was dissolved in 1 ml cold ADF. Mechanical fragmentation was achieved by vigorous pipetting (8–10 times) with a fire-polished glass Pasteur pipette in a 15-ml Falcon tube. Sheared spheroids were centrifuged for 5 min at 4°C, 300g. The resulting pellet was resuspended in cold Matrigel and replated at the desired density.

Lentiviral manipulation. Replication-deficient lentiviral particles were produced by transfection of 293T cells (ATCC CRL-11268) with 2 × HBS (50 mM HEPES, 280 mM NaCl and 1.5 mM Na2HPO4), 1 M CaCl2 and a mixture of the following vectors: packaging vector pCMV-Tag2B (Addgene plasmid 22205), envelope vector pMD2.G (Addgene plasmid 12295) and the 2G mCherry expression plasmid 24304, the pLVTHM-GFP or pLVTHM-mCherry plasmid (Addgene plasmid 12247) or the TGF/LEF-BAT red (Addgene plasmid 6674). Two days after transfection, the supernatant containing the lentiviral particles was aspirated, filtered (0.45 µm) and concentrated with Lenti-X Concentrator (Clontech). The lentiviral pellet was dissolved in the ADF medium.

For transduction, fallopian tube epithelial cells were seeded at early passage (P0 or P1) in six-well plates and grown to 40–50% confluence. The cell pellet of 10 × 106 lentiviral concentrated particles carrying the respective plasmid were taken up in 1 ml ADF medium supplemented with 12 mM HEPES, 1% GluMAX, 10 ng ml−1 human EGF, 9 µM ROCK inhibitor and 8 µg ml−1 polybrene (Sigma). The cells were incubated with the 1 × lentivirus-containing medium (1 ml per well) overnight. When the cells reached ~ 70% confluence, an organoid culture was prepared as described above.

Immunohistochemistry. Organoids were released from Matrigel with washing with PBS, fixed in 3.7% paraformaldehyde for 1 h at room temperature, dehydrated in an ascending series of alcohols, followed by isopropanol and acetone (all steps 20 min at room temperature). Finally, the dehydrated organoids were paraflin-embedded and cut into 5-µm sections on a microtome. Immunofluorescent labelling was carried out as described previously. In brief, sections were contrasted using tannic acid and dehydrated in an ethanol series. After critical
point drying, specimens were coated with 3-nm carbon/platinum and analysed using a LEOS906E transmission electron microscope.

**Transmission electron microscopy.** Human fallopian tube tissue as well as organoids derived thereof were fixed with 2.5% glutaraldehyde, postfixed with osmiumtetroxide, contrasted using tannic acid and uranyl acetate, dehydrated in an ethanol series and embedded in Poly/Bed 812. After polymerization, sections were cut at 70 nm using a diamond knife. Sections were contrasted with lead citrate and analysed in a LEOS906E transmission electron microscope.

**Image analysis and cell counts.** Image analysis was performed with an SP-8 confocal microscope (Leica). Numbers of ciliated and Ki67-positive cells were determined using wide angle (× 20) images covering ~200–700 cells per image in ImageJ with the ‘cell counter’ plugin. Seven fields of view were counted for control and DBZ-treated organoids, equivalent to a total of >2,000 cells per condition.

**RNA quantification and quality control.** Total RNA was isolated with TRIZol (Life Technologies) according to the supplier’s protocol, using glycogen as the carrier. Quality control and quantification of total RNA were assessed using an Agilent 2100 Bioanalyzer with an RNA Nano 6000 microfluidics kit (Agilent Technologies) and a NanoDrop 1000 UV-Vis spectrophotometer (Kisker). The Lgr5 intestinal stem cell signature: robust expression of mouse genes previously published as the stem cell signature genes of LGR5 + cells in intestinal crypts were identified using data from the Inparanoid data base. GSEA for that list was performed on genes pre-ranked by gene expression-t-score between DBZ-treated and non-treated organoids, using standard settings with 1,000 permutations.

**Gene ontology enrichment analysis.** The list of all upregulated target genes (cutoff > 1.5-fold) was uploaded to the GOrilla online platform, a tool for identifying and visualizing enriched GO terms in ranked lists of genes. An analysis was performed for the ontology term ‘Cellular Components’. Output is provided as a colour-coded diagram with all significantly enriched GO terms.

**References**

1. Puik, D. Y. et al. Stem-like epithelial cells are concentrated in the distal end of the fallopian tube: a site for injury and serious cancer initiation. Stem Cells 30, 2487–2497 (2012).
2. Wang, Y. et al. Identification of quiescent, stem-like cells in the distal female reproductive tract. PLoS ONE 7, e40691 (2012).
3. Fre, S. et al. Notch and Wnt signals cooperatively control cell proliferation and tumorigenesis in the intestine. Proc. Natl Acad. Sci. USA 106, 6309–6314 (2009).
4. Ihrie, R. A. et al. Persistent sonic hedgehog signalling in adult brain determines neural stem cell position identity. Nature 71, 250–262 (2011).
5. Oshimori, N. & Fuchs, E. Paracrine TGF-beta signalling balances BMP-mediated repression in hair follicle stem cell activation. Cell Stem Cell 10, 63–75 (2012).
6. Ng, A. et al. Lgr5 marks stem/progenitor cells in ovary and tubal epithelia. Nat. Cell Biol. 16, 745–757 (2014).
7. Barker, N. et al. Identification of stem cell markers in small intestine and colon by marker gene Lgr5. Nature 449, 1003–1007 (2007).
8. Snippert, H. J. et al. Lgr6 marks stem cells in the hair follicle that generate all cell lineages of the skin. Science 327, 1385–1389 (2010).
9. Luo, W. et al. Lgr4 is a key regulator of prostate development and prostate stem cell differentiation. Stem Cells 31, 2492–2505 (2013).
10. de Lau, W. et al. Lgr5 homologues associate with Wnt receptors and mediate R-spondin signalling. Nature 476, 293–297 (2011).
11. Anton, A. et al. Ki67 and p53 expression of the fallopian tube mucosa in breast cancer patients with hereditary risk. Arch. Gynecol. Obstet. 289, 1079–1085 (2014).
12. Lawson, K. et al. In vitro three-dimensional modeling of fallopian tube secretory epithelial cells. BMC Cell Biol. 14, 43 (2013).
13. Sato, T. et al. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett’s epithelium. Gastroenterology 141, 1762–1772 (2011).
14. Zhang, Y., Toh, L., Lau, P. & Wang, X. Human telomerase reverse transcriptase (hTERT) is a novel target of the Wnt/beta-catenin pathway in human cancer. J. Biol. Chem. 287, 32349–32351 (2012).
15. Schepers, A. G., Vries, R., van den Born, M., de Wetering, M. & Clevers, H. Lgr5 intestinal stem cells have high telomerase activity and randomly segregate their chromosomes. EMBO J. 30, 1104–1109 (2011).
16. Kamis, K. et al. LDL-receptor-related proteins in Wnt signal transduction. Nature 407, 530–533 (2000).
17. Carmon, K. S., Gong, X., Liu, Q., Thomas, A. & Liu, Q. R-spondin function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/beta-catenin signaling. Proc. Natl Acad. Sci. USA 108, 11452–11457 (2011).
18. Kessler, M. et al. Chlamydia trachomatis disturbs epithelial tissue homeostasis in fallopian tubes via paracrine Wnt signaling. Am. J. Pathol. 180, 186–198 (2012).
19. de Lau, W. et al. The Wnt agonist R-spondin1 regulates systemic graft-versus-host disease by protecting intestinal stem cells. J. Exp. Med. 208, 285–294 (2011).
20. van Es, J. H. et al. Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. Nature 435, 959–963 (2005).
21. Muzzo, I. et al. The Lgr5 intestinal stem cell signature: robust expression of proposed quiescent + 4’ cell markers. EMBO J. 31, 3079–3091 (2012).
22. de Lau, W., Peng, W. C., Gros, P. & Clevers, H. The R-spondin/Lgr5/Rnf43 module: regulator of Wnt signal strength. Genes Dev. 28, 305–316 (2014).
23. Khodosevich, K., Watanebe, Y. & Monyer, H. EphA4 preserves postnatal and adult neural stem cells in an undifferentiated state in vivo. *J. Cell Sci.* **124**, 1268–1279 (2011).

24. Balenci, L., Wonders, C., Coles, R. L., Clarke, L. & van der Kooy, D. Bone morphogenetic proteins and secreted frizzled related protein 2 maintain the quiescence of adult mammalian retinal stem cells. *Stem Cells* **31**, 2218–2230 (2013).

25. Eden, E., Lipson, D., Yogev, S. & Yakhini, Z. Discovering motifs in ranked lists of DNA sequences. *PLoS Comput. Biol.* **3**, e39 (2007).

26. Eden, E., Navon, R., Steinfeld, I., Lipson, D. & Yakhini, Z. GORilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists. *BMC Bioinformatics* **10**, 48 (2009).

27. Choksi, S. P., Lauter, G., Swoboda, P. & Roy, S. Switching on cilia: transcriptional networks regulating ciliogenesis. *Development* **141**, 1427–1441 (2014).

28. Noone, P. G. *et al.* Mutations in DNA1 (IC78) cause primary ciliary dyskinesia. *Chest* **121**, 97s (2002).

29. Onoufriadis, A. *et al.* Combined exome and whole-genome sequencing identifies mutations in ARMC4 as a cause of primary ciliary dyskinesia with defects in the outer dynein arm. *J. Med. Genet.* **51**, 61–67 (2014).

30. Zhao, L. *et al.* Reptin/Ruvbl2 is a Lrrc6/Seahorse interactor essential for cilia motility. *Proc. Natl Acad. Sci. USA* **110**, 12697–12702 (2013).

31. Chen, E. Y. *et al.* Secretory cell outgrowth, PAX2 and serous carcinogenesis in the Fallopian tube. *J. Pathol.* **222**, 110–116 (2010).

32. Crum, C. P. *et al.* Through the glass darkly: intraepithelial neoplasia, top-down differentiation, and the road to ovarian cancer. *J. Pathol.* **231**, 402–412 (2013).

33. Vaughan, S. *et al.* Rethinking ovarian cancer: recommendations for improving outcomes. *Nat. Rev. Cancer* **11**, 719–725 (2011).

34. Perets, R. *et al.* Transformation of the fallopian tube secretory epithelium leads to high-grade serous ovarian cancer in Brca1Tp53Pten models. *Cancer Cell* **24**, 751–765 (2013).

35. Cancer Genome Atlas Research Network. Integrated genomic analyses of ovarian carcinoma. *Nature* **474**, 609–615 (2011).

36. Willyard, C. Rise of the organoids. *Nature* **523**, 520–522 (2015).

37. Willert, K. *et al.* Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature* **423**, 448–452 (2003).

38. Farin, H. F., Van Es, J. H. & Clevers, H. Redundant sources of Wnt regulate intestinal stem cells and promote formation of Paneth cells. *Gastroenterology* **143**, 1518–1529 (2012).

39. Jung, P. *et al.* Isolation and in vitro expansion of human colonic stem cells. *Nat. Med.* **17**, 1225–1227 (2011).

40. Churchill, G. A. Fundamentals of experimental design for cDNA microarrays. *Nat. Genet.* **32**(Suppl): 490–495 (2002).

41. Ostlund, G. *et al.* InParanoid 7: new algorithms and tools for eukaryotic orthology analysis. *Nucleic Acids Res.* **38**, D196–D203 (2010).

42. Subramanian, A. *et al.* Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl Acad. Sci. USA* **102**, 15545–15550 (2005).

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Author contributions

M.K. and T.F.M. conceived the project and designed experiments, which were conducted by M.K., K.H., O.T. and S.J. V.B. performed electron microscopy experiments. V.B. and B.T. conducted live cell imaging, H.B. and H.-J.M. analysed microarray data, M.M., J.S. and C.F. selected the patients and provided human fallopian tube samples. M.K. and T.F.M. wrote the manuscript, and T.F.M. supervised the project.

Additional information

Accession codes: The microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) under accession code GSE60919.

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