# Single Cell Transcriptomics identifies a WNT7A-FZD5 Signaling Axis that maintains Fallopian Tube Stem Cells in Patient-derived Organoids

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# ABSTRACT

Despite its significance to reproduction, fertility, sexually transmitted infections and various pathologies, the Fallopian tube (FT) is relatively understudied. Strong evidence points to the FT as the tissue-of-origin of high grade serous ovarian cancer (HGSOC); the most fatal gynaecological malignancy. HGSOC precursor lesions arise specifically in the distal FT (fimbria) which is reported to be enriched in stem-like cells. The role of FT stem cells in HGSOC initiation could not be investigated as these cells have not been identified or functionally characterized due to lack of physiologically relevant models. Here, we surmount technical challenges to re-construct the FT stem cell niche *in vitro.* Using functional approaches as well as bulk and single cell expression analyses, we show that a WNT7A-FZD5 signaling axis is critical for self-renewal of FT stem cells and is regulated by female sex hormones. Single cell expression profiling of reporter organoids identified Wnt/β-catenin Active cells (WβA cells) as a unique cluster of proliferative cells that is enriched in HGSOC markers, particularly *CA125*; a clinical marker of HGSOC progression and response to therapy. Remarkably, we find that the WNT7A-FZD5 signaling axis is dispensable for mouse oviduct regeneration. Overall, we propose a first basic description of the nature of FT stem cells and their molecular requirements for self-renewal, paving the way for mechanistic work investigating the role of stem cells in FT health and disease.

## ***KEY WORDS***

Fallopian tube; wnt; ovarian cancer; stem cells; organoids; Estrogen; single cell RNA sequencing

## ***ABBREVIATIONS***

| | |
|---|---|
| hFT | human Fallopian tube |
| mOV | mouse oviduct |
| WβS | Wnt/β-catenin Signaling |
| WβA | Wnt/β-catenin Active |
| SCT | Single Cell Transcriptomics |
| KO | Knockout |
| HGSOC | High Grade Serous Ovarian Cancer |

# INTRODUCTION

The human Fallopian tube (hFT) is lined with pseudo-stratified columnar epithelium composed of PAX8+ MYC+ secretory cells that nourish released eggs / zygotes, as well as TUBB4+ FOXJ1+ ciliated cells that beat rhythmically to facilitate ovum/zygote transport to the uterine cavity. Despite its significance to fertility, reproduction and women's health and disease, little is known about the FT's biology, cellular hierarchy and homeostasis which are critical for understanding infertility disorders, ectopic pregnancy, sexually transmitted infections and FT-derived cancers. A number of studies have attempted to bridge this knowledge gap, and reports indicate that the distal hFT/mouse oviduct (mOV) is enriched in stem-like cells possessing longevity and multipotency (Erickson *et al*., 2013; Ghosh *et al*., 2017; Xie *et al*., 2018; Wang *et al*., 2012; Zhu *et al*., 2020). In mice, studies identified a population of label-retaining cells (LRCs) at the distal mOV (Snegovskikh *et al*., 2014) that are enhanced for differentiated spheroid formation (Wang *et al*., 2012; Xie *et al*., 2018). Strong evidence for the existence of mOV stem cells came from *in vivo* lineage tracing that employed doxycycline-inducible labelling of secretory cells using a Pax8$^{rtTA}$ TetO$^{Cre}$ YFP$^{fl/fl}$ mouse model. Here, ciliated cells were shown to emerge from secretory cells (Ghosh *et al*., 2017). Similarly, in the hFT putative stem cells were shown to be secretory in nature, using spheroid (Paik *et al*., 2012), air-liquid interface (Yamamoto *et al*., 2016) and organoid-based approaches (Kessler *et al*., 2015).

However, using single cell transcriptomic (SCT) profiling on fresh hFT tissue, we and others have recently uncovered a previously unappreciated heterogeneity within the FT secretory compartment (Hu *et al*., 2020; Dinh *et al*., 2021). Therefore, although *in vivo* studies and human-based *in vitro* models refined the search for hFT/mOV stem cells, no studies have successfully pinpointed the secretory cell type driving FT renewal, due to limitations in model tractability and difficulty in cell biomarking and isolation. Wnt/β-catenin signaling (hereinafter WβS) is thought to be involved in hFT/mOV self-renewal (Kessler *et al*., 2015; Ghosh *et al*., 2017), but the precise molecular mechanisms remain unknown. Furthermore, humans and great apes possess FTs whereas the equivalent in mice is the oviduct. Due to major anatomical differences, biological differences are likely to exist, and no studies have scrutinized whether mOV biology is representative to the hFT.

In addition to being a site where stem-like cells concentrate, mounting evidence from several clinical and *in vivo* studies point to the distal FT fimbria as the site of origin of high grade serous ovarian cancer or HGSOC (Labidi-Galy *et al*., 2017; Kuhn *et al*., 2012; Kim *et al*., 2018). HGSOC is the most fatal gynaecological malignancy with a dismal 5-year survival of 25% that remains persistently low for over four decades (Kroeger & Drapkin, 2017). Understanding FT renewal may shed light on HGSOC initiation mechanisms, which remain poorly understood, further reinforcing the urgency of this investigation. Here, we harness three-dimensional patient-derived FT organoids to reconstruct the FT stem cell niche *in vitro*. We genetically label, isolate and

characterize putative FT stem cells using functional approaches as well as bulk and SCT analyses, identifying a hormonally regulated WNT7A-FZD5 signaling axis that is critical for FT stem cell maintenance.

# RESULTS

## *A serum-free culture method for robust regeneration of hFT organoids from single cells*

Multiple models of FT biology were previously reported but each model suffers from drawbacks limiting its utility in characterizing FT stem cells (Figure S1A). In particular, conventional 2D cultures of primary FT cells (Figure S1 B-D) were found to lack ciliated cells (Hu *et al*., 2020) and lose epithelial markers within 5-6 weeks of culture (Figure S1E). Organoid cultures were shown to be robust hFT/mOV models incorporating PAX8+ secretory and TUBB4+ ciliated cells (Kessler *et al*., 2015; Xie *et al*., 2018; Figure 1A), but a major limitation of the human organoid culture conditions is the lack of organoid regeneration after single cell dissociation of patient tissue or organoids; a pre-requisite for isolating and characterizing putative progenitor cells. TGF-β pathway promotes stem cell differentiation (Sakaki-Yumoto *et al*., 2013) and its inhibition is critical for regeneration of organoids from various organs (Sato *et al*., 2009; Bartfeld *et al*., 2015; Karthaus *et al*., 2014; Turco *et al*., 2017). We reasoned NOGGIN, which inhibits BMP-activated SMAD1/5/8-mediated signaling, and SB431542, a TGFβR1 / ALK5 inhibitor which blocks SMAD 2/3 TGFβ-mediated signaling, do not sufficiently inhibit TGF- β signaling to enable organoid regeneration. Thus, we employed A83.01 which has 10-fold higher potency compared to SB431542 (Tojo *et al*., 2005) and found it to restore organoid formation efficiency or OFE (Figure 1 B-C). Interestingly, we found that TGF-β suppression was not necessary for regeneration of mOV organoids from single cells (Figure S2 A-C).

In addition, we excluded WNT3A (Figure S3A) as a WNT source which we show is not needed and has no activity in hFT organoids (see below). Undefined serum factors contained within Wnt3a conditioned medium (CM) negatively interfere with organoid regeneration (Figure S3B) and confound the analysis of stem cell self-renewal requirements *in vitro*. Using this optimized medium, we confirmed that organoids emerge only from epithelial cells (Figure S3C), display invaginations characteristic of hFT tissue (Figure S3D), possess a spherical tube-like structure with a hollow interior (Supplementary Movie 1) and contain a rare population of KI-67+ proliferative cells (Figure S3E). Overall, the data above indicate that we have optimized serum-free culture conditions that robustly support the regeneration of hFT organoids from single cells.

## *Wnt/β-catenin signaling is essential for renewal of hFT stem cells*

FT organoids were previously shown to be monoclonal (Kessler *et al*., 2015). Using our optimised culture conditions, we sought to directly test organoid monoclonality by single cell culture. A small proportion of single cells cultured in single cells formed organoids containing differentiated progeny (Figure 1D), further confirming FT organoids as monoclonal structures emerging from single multipotent stem cells. We set out to characterize these putative stem cells using different approaches (Figure 1E). Our functional analyses above

indicate WβS activation is essential for stem cell renewal in organoids (shown earlier, Figure 1 B-C). To confirm this, we treated organoids with the tankyrase 1/2 inhibitor XAV-939 (Huang *et al*., 2009) and β-catenin / CBP inhibitor PRI-724 (Okazaki *et al*., 2019); both inhibit WβS at midstream (cytoplasmic) and downstream (nuclear) nodes, respectively (Figure S3F). Both treatments completely abolish organoid regeneration (Figure S3G). Furthermore, in our optimized serum-free setting, RSPO1 is the only component that augments WβS (Figure S3H). RSPO1 withdrawal reduces OFE by more than 90% (shown earlier, Figure 1 B-C). Since our culture medium does not contain a WNT source, we reasoned that WβS is activated via an endogenously secreted WNT. Indeed, blocking endogenous WNT secretion in organoids using Porcupine inhibitors (Figure S3I) reduced OFE by over 90% (Figure 1 F-G), phenocopying the effect seen upon RSPO1 withdrawal (show earlier, Figure 1 B-C). This suggested that RSPO1 cooperates with an endogenous WNT to drive hFT organoid regeneration.

WNT3A is a widely employed WNT source in various tissue-derived organoid culture systems, including hFT/mOV organoids (Hoffmann *et al*., 2020; Kessler *et al*., 2015, 2019; Kopper *et al*., 2019; Lõhmussaar *et al*., 2020). We expressed Wnt3a CM using L-Wnt3a cells and validated its activity using the Wnt reporter (TOPFlash) assay (shown earlier, Figure S3A). Contrary to the rescue of organoid regeneration seen in WNT-blocked intestinal organoids (Sato *et al*., 2011), Wnt3a failed to rescue the regeneration of WNT-blocked hFT organoids (Figure 1 H-I), suggesting it cannot substitute for the endogenously secreted WNT ligand. In an unexpected contrast to hFT organoids, the regeneration of mOV organoids was unaffected by blocking endogenous WNTs (Figures 1 J-K), suggesting mouse organoids renew using other mechanisms.

## ***WβS-active cells drive organoid regeneration***

The results above indicate that Wnt/β-catenin active (WβA) cells drive FT organoid regeneration.
To characterize WβA cells, we transduced organoids from benign non-HGSOC patients with the 7TGC lenti-construct (Figure 2A). We expanded FACS-selected transduced cells using our optimized culture conditions, to generate stable WβS-reporter organoids (Figures 2B & S4A) from multiple patients. Confocal imaging of fixed (Figure 2C) and live (Figure S4B) organoids confirmed localized activation of WβS in organoids. Immunofluorescence staining confirmed that WβA cells are PAX8+ / secretory in lineage (Figure 2D) and constitute 1.5-5% of all cells (Figure 2E and Figure S4A for mOV and hFT organoids, respectively), mirroring the proportion of organoid-forming units in hFT organoids. Crucially, blocking endogenous WNT secretion abolished EGFP+ cells (Figure 2E) while WβS activation increased the proportion of EGFP+ cells over 10-fold (Figures 2E and S4C), suggesting EGFP faithfully marked WβA cells. AXIN2, a reliable marker of WβS activation in several organoid systems (Boonekamp *et al*., 2021) including hFT organoids (Figure S4D) was elevated in EGFP+ cells (Figure 2F), further validating EGFP+ cells as WβA cells in this setting.

FACS-purified WβA cells displayed enhanced OFE relative to non-WβA cells (Figure 2 G-H). Whilst WβA cells were detected in all organoids within hFT organoid cultures, mOV organoids may or may not contain WβA cells (Figure 2I), and mOV WβA cells do not display enhanced OFE (Figure 2J) under a range of experimental

conditions (Figure S4 F-I), consistent with WβS-independence seen in mOV organoids (shown earlier, Figure 1 J-K).

## ***WNT7A is the driver of WβS and FT stem cell renewal***

To identify the WNT ligand driving stem cell-mediated expansion of hFT organoids, we isolated WβA (EGFP+) and non-WβA (EGFP-) cells from WβS-reporter organoids and profiled their transcriptomes at the single cell level using the SMART-seq2 protocol (Picelli *et al*., 2014). This identified *WNT7A* as the highest expressed WNT ligand and the only expressed canonical WNT in hFT organoids (Figure 3A). *WNT7A* expression was further validated using RNAScope FISH staining in hFT organoids (Figure 3B), hFT tissue (Figure S5 A-C), mOV organoids (Figure 3C) and mOV tissue (Figure S5 D-G), showing that *WNT7A*+ cells may or may not co-express WβS activation markers *AXIN2/Axin2* and *LGR5/Lgr5*. Therefore, it is difficult to conclusively say whether WNT7A signals in an autocrine or paracrine manner. However, this data implicates WNT7A as the target of Porcupine inhibitors IWP-2 and LGK-974 which abolish organoid regeneration (shown earlier, Figure 1 F-I). In contrast, *WNT5A* is lowly expressed and is known to be a classic activator of non-canonical Wnt signaling that is β-catenin and LRP5/6-independent (Qian *et al*., 2007). Of note, *WNT3A* expression is not detected in organoids (Figure 3A), suggesting hFT cells are not naturally primed to respond to WNT3A or activate WβS through it. This consistent with WNT3A's failure to rescue growth of WNT-blocked organoids (shown earlier, Figure 1 H-I).

While our data suggests endogenous *WNT7A* provides hFT organoids with independence from exogenously supplied WNTs, we find hFT tissue and organoids express *RSPO1* (Figure 3D) which does not substitute for exogenously supplied RSPO1 in promoting organoid renewal (shown earlier, Figures 1 B-C & F-I). We reasoned another source of RSPOs could be involved *in vivo*. To address this, we surveyed our tissue SCT dataset, which showed that 10% of stromal cells robustly express *RSPO3* (Figure 3E) which biochemical studies indicate is over 20-fold more potent in augmenting WβS compared to RSPO1 (Park *et al*., 2018). Furthermore, 25% of stromal cells express *WNT2* and 7.5% express *WNT9A* (Figure 3E), implying epithelial WNT7A may be a redundant WNT source *in vivo*. Notably, the *WNT2+*, *WNT9A+* and *RSPO3+* stromal cells are largely distinct, and we do not see robust WNT or R-Spondin contribution from the limited number of immune cells profiled (Figure 3E).

Next, we sought to functionally confirm whether WNT7A is essential for renewal of FT stem cells in organoids. Direct genetic knockdown of *WNT7A* mRNA was unsuccessfully attempted using 10 lentiviral shRNA constructs from two commercial sources (data not shown). We also aimed to test whether WNT7A protein rescues OFE of WNT-blocked organoids. In line with the reported difficulties in generating functional WNTs for *in vitro* assays (Tüysüz *et al*., 2017), all generated WNT7A protein reagents were not functional (Figure S6H), including WNT7A CM derived from *WNT7A*-cDNA construct-transfected HEK293 cells (Figure S6 A-B), WNT7A CM from primary 2D-cultured FT cells (Figure S6C) which we find endogenously overexpress WNT7A (Figure S6 D-E), native WNT7A protein (Figure S6G) and recombinant WNT7A protein from two commercial sources (one shown in Figure S6H). Interestingly, the *WNT7A* cDNA expression construct robustly activates WβS but the CM

derived from the same construct-transfected cells fails to activate WβS (Figure S6H), despite containing abundant WNT7A protein (shown earlier, Figure S6 A-B). We reasoned this could be due to a short signaling range as shown by biochemical and *in vivo* approaches for other WNT ligands (Farin *et al*., 2016; Goldstein *et al*., 2006; Alexandre *et al*., 2013). We confirmed this using a simple co-culture assay (Figure S6 I-J); 4E), which explained why our protein-based WNT7A reagents were not functional.

## ***FZD5 mediates WNT7A-driven maintenance of FT stem cells***

To overcome the above limitations and test the functional significance of WNT7A to organoid renewal, we attempted to identify and biochemically perturb the WNT7A receptor in organoids. SCT profiling identifies *FZD3*, *FZD5* and *FZD6* as the major FZDs expressed in hFT organoids (Figure 4A). This is also the case for hFT tissue (Figure S7A). The FZD family of receptors categorize into 4 subfamilies based on sequence and structural similarity: FZD5/8 subfamily, FZD4/9/10 subfamily, FZD1/2/7 subfamily and FZD3/6 subfamily. The FZD3/6 subfamily is the divergent one from this receptor family (Figure S7B) and participates in non-canonical Wnt signaling (Dong *et al*., 2018). FZD5, and not FZDs 3/6, was reported to bind WNT7A and activate WβS as shown in *in vitro* WNT-FZD pair screens (Yu *et al*., 2012; Voloshanenko *et al*., 2017). To validate these findings, we utilized HEK293 cells which endogenously express these FZDs (Figure S7C). siRNA knockdown (Figure S7D) of *FZD5*, but not *FZD3* or *FZD6*, inhibited *WNT7A*-induced TOPFlash (Figure S7E) while WNT7A induced the highest WβS/TOPFlash signal in a background of FZD5 overexpression compared to FZD3 or FZD6 overexpression (Figure S7F).

Although this data confirms WNT7A activates WβS through FZD5, direct functional evidence from hFT organoids is lacking. To address this, we utilized IgG-2919 and IgG-2921; two novel, selective, antibody-based inhibitors of FZD5 generated using an antibody phage-display system (Steinhart *et al*., 2017). Anti-FZD5 IgGs reduce TOPFlash signal in WNT7A and FZD5 overexpressing cells by more than 60% (Figure S7G). Consistent with WNT inhibition's dual effect on abolishing WβA cells (shown earlier, Figure 2E) and OFE (shown earlier, Figure 1 F-I), FZD5 inhibition abolishes WβA cells (Figure 4B) and OFE (Figure 4C) by over 90% (Figure 4D). This phenocopies the effect of RSPO1 withdrawal (shown earlier, Figure 1 B-C & 1 F-I). The only other FZD that shows cross-reactivity with anti-FZD5 antibodies is FZD8 (Figures 3C & S5 in Steinhart *et al*., 2017) which is not expressed in hFT organoids (shown earlier, Figure 4A). Altogether, these data strongly suggest that FZD5 is the cognate receptor for WNT7A in the FT, and that a WNT7A-FZD5 signaling axis drives WβS activation and renewal of hFT organoids.

Regeneration of FZD5-inhibited or WNT-blocked organoids is rescued by WβS activation downstream of ligand-receptor interactions using the selective GSK-3β inhibitor CHIR99021 (Figure 4 C-D). Partial rescue is also observed when WβS is activated at the ligand-receptor level using Surrogate Wnt (Figure 4 C-D), which competes with anti-FZD5 IgG's for binding to FZD's cysteine-rich domain (Janda *et al*., 2017; Steinhart *et al*., 2017). Surrogate Wnt has broad-spectrum activity against FZDs 1/2/5/7/8 but not FZDs 3/6 (Janda *et al*.,

2017), further demonstrating that FZD5 is the FZD receptor associated with WβS and organoid regeneration. FZD receptors are subject to constant turnover and proteasomal degradation by the action of the RNF43/ZNRF3 ubiquitin ligases (Hao *et al*., 2012), which are inhibited by R-Spondins (Carmon *et al*., 2011; Glinka *et al*., 2011; de Lau *et al*., 2011). As such, withdrawal of RSPO1 from Surrogate Wnt-treated organoids reduces OFE by 75-90% (Figure 4E), indicating that the reduced OFE seen upon RSPO1 withdrawal (shown earlier, Figures 1 B-C & 1 F-I) is due to FZD5 turnover.

Intriguingly, while organoids from all tested patients in this study showed sensitivity to WβS inhibition, organoids from one patient were resistant to WNT and FZD5 inhibition (Figure S8 A-B) as well as to RSPO1 withdrawal (Figure S8C). Patient 5 organoids were, however, sensitive to downstream WβS inhibition (Figure S8 A-B) as seen in other patients (shown earlier, Figure S3G). Tested under selective conditions of WNT-blocking for four passages, Patient 5 organoids show ectopic and robust growth that leads to large organoid sizes not typical of normal FT organoids (Figure S8D). Although Patient 5 was diagnosed with serous ovarian cancer, which is thought to derive from the FT (Kuhn *et al*., 2012; Kim *et al*., 2018), Patient 5 organoids do not carry TP53 mutations, which are known to be the initiating event of serous ovarian cancer (Labidi-Galy *et al*., 2017). While further characterization and more patient replicates are required to reproduce this finding, these WNT/FZD5-resistant organoids may represent purified mutant clones with early genetic changes that confer selective growth advantage and independence from stem cell niche requirements.

Finally, FZD5 inhibition has no effect on mOV organoids (Figure 4 F-G) consistent with the lack of effect seen upon blocking WNT secretion (shown earlier, Figure 1 J-K) and lack of OFE enrichment in isolated WβA mOV cells (shown earlier, Figure 2J). This is despite WNT blocking and FZD5 inhibition abolishing WβA cells in mOV WβS-reporter organoids (shown earlier, Figure 4B), suggesting that a WNT-FZD5 signaling axis also regulates WβS in mOV organoids, but unlike in hFT organoids, WβS is not essential for stem cell renewal in the mOV organoids.

## ***Estrogen downregulates WNT7A and triggers ciliogenesis***

Female Reproductive Tract (FRT) organs, including the hFT/mOV, are subject to cyclic hormonal influences. Estrogen acts as a ligand that dimerizes Estrogen receptors (ER) expressed in cells within hormone-responsive tissues (Björnström & Sjöberg, 2005). Little is known about Estrogen's influence on WβS in the hFT, and studies indicate Estrogen activates WβS in the FRT (Hideyuki *et al*., 1999; Kouzmenko *et al*., 2004; Hou *et al*., 2004) although other reports document Estrogen to exert an inhibitory influence on WNT7A (McLachlan *et al*., 1980; Couse *et al*., 2001; Wagner & Lehmann, 2006). Based on this, it can be hypothesized that Estrogen may uncouple the synonymous and proportional relationship we see between WβS and WNT7A in the FT.

To evaluate this hypothesis, Estrogen's and Progesterone's effects on WβS were tested in the presence of Estrogen Receptor (ERα) and Progesterone Receptor (PRβ), respectively. Interestingly, Estrogen robustly

activates transcription from WβS Reporter Elements (or WβS-RE) in the TOPFlash assay (Figure S9A), independently of WNT ligands (Figure S9B). Estrogen's activation of transcription at WβS-RE elements is unaffected by WβS inhibitors at midstream (XAV-939) and downstream (PRI-724) levels (Figure S9B). Estrogen also attenuates ligand-dependent and ligand-independent hyperactivation of WβS (Figure S9C) to levels seen in Estrogen-treated samples (Figure S9A), indicating that the E2-ERα complex may compete or cooperate with β-catenin for binding and activation at WβS-REs. To test this, we investigated the effect of Estrogen on TOPFlash signal upon β-catenin knockdown. β-catenin siRNAs were validated to effectively abolish β-catenin protein levels (Figure S9D) and activity (Figure S9 E-F). In this setting, liganded ERα activates transcription at WβS-RE elements independently of β-catenin (Figure S9G) and induces WβS target gene transcription 45-88% higher in the absence of β-catenin, suggesting the pair compete for binding at WβS-RE promoter elements. Next, we sought to delineate the effect of Estrogen on WNT7A. Interestingly, Estrogen (as well as Progesterone) causes degradation of intracellular and secreted WNT7A protein (Figure 5A), and dramatically reduces WNT7A activity (Figure 5B) without independently activating WβS (as shown earlier; Figure S9A). Overall, these data suggest that Estrogen can activate WβS target gene expression in hormone-responsive tissues, but specifically suppresses WNT7A and WNT7A-induced WβS in the FT setting.

Estrogen's differential effects on WβS and WNT7A are interesting. They were confirmed here using the TOPFlash assay which, although useful, is not physiologically relevant to the FT. Organoids offer a superior model to understand hormonal control of WβS and WNT7A, and SCT data confirms that our optimized organoid culture conditions successfully maintain hormone receptor-expressing cells (Figure 5C) as seen in human tissue (Figure 5D). In both settings, ERα is the predominant hormone receptor expressed, and we harnessed this model to understand Estrogen's influence on the FT. Estrogen treatment of hFT organoids triggers a phenotype of crippled, deeply staining organoids with extensive internal folding and invaginations (Figure 5 E-F), reminiscent of differentiation signs that appear in long-term cultured organoids (shown earlier, Figure 1D) and in differentiated organoids of other tissues (Yin *et al*., 2013). However, Estrogen triggers these changes within 72 hrs, and characterisation of Estrogen-treated organoids reveals Estrogen downregulates *WNT7A* expression, WβS and the proliferation marker Ki-67 (Figure 5 G-H). Furthermore, we detect upregulation of *FOXJ1*, an established master regulator and marker of ciliated cells (You *et al*., 2004), as well as upregulation of *CAPS*, *CCDC17*, *CCDC78* (Figure 5I) and *CCDC78* (Figure S9H); all novel ciliated cell markers we previously identified in a tissue-based SCT study (Hu *et al*., 2020).

Finally, WβS was recently shown to regulate the expression of DNA double-strand break repair genes, including *BRCA1*, *BRCA2*, *RAD51* and the *FANC* gene family, in various tissues (Kaur *et al*., 2021; Angers, 2021). Along with inhibiting WβS, we note Estrogen suppresses *BRCA1* and *BRCA2* expression in hFT organoids, possibly through *MYBL2* (Figure S9I) as reported (Kaur *et al*., 2021). This is phenocopied by Estrogen-independent WNT inhibition (Figure S9I), suggesting Estrogen regulates *BRCA1/2* expression, at least in part,

through regulating WβS. Collectively, the data indicates that Estrogen suppresses *WNT7A* and WβS, and robustly induces a transcriptional ciliogenesis programme in hFT organoids.

## ***Transcriptomic characterization of WβA cells***

Our data thus far reveal a hormonally-regulated WNT7A-FZD5 signaling axis that activates WβA cells which drive FT organoid regeneration. WβA cells are therefore candidate FT stem cells, which we sought to further characterize using our expression dataset which profiled WβA and non-WβA cells at the single cell level. Among the four transcriptomic clusters identified (Figure 6A) WβA cells concentrate in cluster 1 (Figure 6B; p-values for pairwise WβA cell enrichment in cluster 1 relative other clusters is < 0.0002; see Figure 6C). Cluster 1 cells are enriched in the expression of cell cycle and mitosis-related genes (Figure 6D), and a full list of cluster-based DEGs is available (Table S1). We also compared WβA with non-WβA cells irrespective of cluster position. Table S2 provides a full list of WβS status-based DEGs. Interestingly, *WNT7A* expression is enriched in the proliferative WβA cells (Figure 6E; p-value < $10^{-6}$). In hFT tissue, we also find *WNT7A*+ cells to be proliferative (data not shown) and transcriptionally unique (Figure 6F). Next, we aimed to identify the RSPO1 receptor which is responsible for the obligate RSPO1 requirement for hFT organoid regeneration (shown earlier, Figure 1 B-C & F-I). Since our organoid SCT data does not capture the expression of R-Spondin receptors (LGRs 4-6) possibly due to dropout of low abundance mRNAs, we carried out bulk RNA-seq of WβS-reporter organoids. This further confirmed upregulation of WβS in WβA cells (Table S3) and identified *LGR6* as the only R-Spondin receptor enriched in WβA cells (Figure 6G).

Further analysis of our organoid SCT data revealed Dipeptidyl peptidase 4 (*DPP4*) expression is enriched in WβA cells (Figure 6H; p-value < $10^{-9}$). DPP4 is a transmembrane enzyme involved in glucose and insulin metabolism (Ghorpade *et al*., 2018; Röhrborn *et al*., 2015) and was recently shown to mark WβA cells *in vivo* in the endometrium (Syed *et al*., 2020), which shares an embryonic origin and a developmental continuum with the mOV, corroborating our findings. Interestingly, WβA cells are enriched in *MUC16* expression (Figure 6H; p-value 0.0017). MUC16, also called CA125, is highly overexpressed in HGSOC tumours (Bast *et al*., 1983) and is a clinically established biomarker for patient diagnosis, disease progression and response to therapy (Partridge *et al*., 2009). WβA cells are also marked by *GPX3* (Figure 6H, p-value < $10^{-8}$), an extracellular glutathione peroxidase that protects cells against oxidative damage and was shown to support ovarian cancer invasion and survival (Worley *et al*., 2019). Finally, Claudin-3, a tight junction protein highly overexpressed in ovarian cancer (Rangel *et al*., 2003) and proposed to better diagnose CA125$^{low}$ ovarian cancers (Rosen *et al*., 2005) also marks the WβA cluster (Figure 6H; p-value < $10^{-7}$). Collectively, our organoid SCT data suggests that WβA cells are unique, and their proliferative capacity is consistent with progenitor cell character. The observation that WβA cells are marked by *CA125* and other HGSOC markers may implicate these cells in HGSOC initiation, representing an attractive future research direction that warrants investigation.

# DISCUSSION

The first organotypic culture techniques for human Fallopian tube (hFT) and mouse oviduct (mOV) tissues were described relatively recently (Xie *et al*., 2018; Kessler *et al*., 2015), and a number of studies have utilized this to make important progress in understanding FT and HGSOC biology (Zhang *et al*., 2019; Lõhmussaar *et al*., 2020; Kessler *et al*., 2019; Kopper *et al*., 2019; Hoffmann *et al*., 2020; Hill *et al*., 2018; de Witte *et al*., 2020). Organoids emerge from single multipotent cells in bulk and single cell culture, and we find ciliated cells appear several days after organoid passage (data not shown), consistent with *in vitro* (Kessler *et al*., 2015) and *in vivo* (Ghosh *et al*., 2017) studies showing ciliated cells emerge from secretory cells. Although this strongly points to the presence of a stem cell, no studies have thus far harnessed hFT organoids for stem cell expansion and characterization. This is due to limitations in published protocols in which culture conditions are poorly defined and in their current format are not amenable to single cell isolation and culture.

In this study, we develop serum-free culture conditions enabling stem cell expansion from single cells. We confirm that organoids grown under these conditions recapitulate basic features of hFT tissue, and have recently extended this to detect, in organoids, novel cell types and markers identified by the first single cell transcriptomic (SCT) study on hFT tissue (Hu *et al*., 2020). We harness our optimized culture conditions to conduct functional analyses on molecular determinants of stem cell renewal, and devise a strategy for marking, isolation and transcriptomic characterization of these cells, generating the first SCT dataset of hFT organoids.

**WNT7A, FZD5 and RSPOs in the hFT**: Our work implicates WNT7A in FT homeostasis and maintenance. This is consistent with *in vivo* studies that reported *Wnt7a* knockout (KO) mice to contain no or severely compromised mOV with diminished invaginations, as well as global abnormalities in the correct patterning of the neonatal female reproductive tract or FRT (Miller & Sassoon, 1998). Although viable, *Wnt7a* KO mice are reproductively sterile and the Mullerian duct fails to regress in male mice (Parr & McMahon, 1998). WNT7A is known to play an intimate role in patterning and differentiation of other tissue types, including limb, cardiac and neuronal synapses (Bond *et al*., 2003; Ingaramo *et al*., 2016; Hwang *et al*., 2004; Hall *et al*., 2000).

Our data shows strict concordance between the proportion of Wnt/β-catenin Active (WβA) cells and organoid renewal capacity, both in the range of 1.5-5% of cells. We note non-WβA cells may also generate differentiated organoids, consistent with regenerative plasticity seen in organoids derived from FRT organs (Ali *et al*., 2020; Syed *et al*., 2020) and other tissues (Serra *et al*., 2019; Huch, Bonfanti, *et al*., 2013; Sato *et al*., 2009; Cao *et al*., 2020). Our data shows that LGR6, and not FZD5, is enriched in WβA cells, and we hypothesize that LGR6 is the limiting factor in dictating multipotency of WβA *FZD5*+ *LGR6*+ cells. A limitation of our work is

the lack of functional analyses to evidence this. Furthermore, our tissue SCT data indicates epithelial WNT7A could be made redundant by mesenchymal-derived WNTs, offering an explanation as to why conditional *Wnt7a* loss does not disrupt mOV homeostasis *in vivo* (Dunlap *et al*., 2011). As FZDs 3/6 are largely non-canonical Wnt signaling mediators (Dong *et al*., 2018), we propose mesenchymal-derived WNT2 also signals through FZD5, consistent with *in vitro* WNT-FZD pair screens showing WNT2 activates WβS through FZD5 but not FZDs 3/6 (Yu *et al*., 2012). Unlike epithelial-derived WNT7A, we hypothesize epithelial-derived RSPO1 is not sufficient for hFT homeostasis, and our patient stroma SCT data hints at the possibility that the FT epithelium relies on the mesenchyme as an essential source of RSPO3 necessary for epithelial homeostasis. Another limitation of our work is the absence of WNT2 and RSPO3 in our functional analyses.

Overall, we propose a first description of the FT stem cell niche (Figure 7A). Our transcriptomic and RNA FISH data suggest *WNT7A* expression overlaps (but not completely) with WβA cells, so it is possible that WNT7A signals in an autocrine manner (Figure 7B). Further biochemical work is required to conclusively establish the mechanism of WNT7A signaling in the FT.

**WβA cells and mouse models**: WβS is recognized as a major driver of tissue regeneration under homeostatic and injury conditions (Clevers, 2006). Amongst various WβS reporters, AXIN2 and LGR5 are the most faithful (Boonekamp *et al*., 2021) and as such, AXIN2 and LGR5-based mouse models have been employed in biomarking and lineage tracing of WβA stem cells in various tissues (Clevers, Loh & Nusse, 2014; Van Camp *et al*., 2014). To this end, *Lgr5*+ cells were shown to contribute to mOV development, but *Lgr5* expression disappears by day 7 after birth (Seishima *et al*., 2019) and in adult mice lineage tracing studies have shown that *Lgr5*+ cells do not contribute to mOV homeostasis (Ng *et al*., 2014). This is consistent with our bulk transcriptomic analysis showing that WβA cells are not enriched in *LGR5* expression.

Although *Lgr5* specifically markers homeostatic WβA-ASCs in various tissues (Barker & Clevers, 2010), including the pyloric stomach (Barker *et al*., 2010) in which *Axin2*+ *Lgr5*- cells are facultative ASCs mobilized upon injury (Sigal *et al*., 2017), the opposite trend is seen in other tissue systems in which AXIN2 is a better marker of homeostatic WβA-ASCs. This was shown in the liver (Wang *et al*., 2015; Huch *et al*., 2013), stomach (corpus) region (Stange *et al*., 2013; Leushacke *et al*., 2017) and adult vagina (Ali *et al*., 2020; Ng *et al*., 2014). In the endometrium, which shares embryonic origins and a developmental continuum with the mOV, *Lgr5*+ cells contribute to development but not adult tissue homeostasis (Seishima *et al*., 2019), and *in vivo* lineage tracing has demonstrated *Axin2*+ cells to be long-lived multipotent stem cells that replenish glandular and luminal epithelia in the adult endometrium (Syed *et al*., 2020). This, coupled with our transcriptomic analyses showing *AXIN2* and *LGR6* are preferentially enriched in WβA cells, as well as our human and mouse RNA FISH data suggesting that *AXIN2*+/*Axin2*+ and *LGR5*+/*Lgr5*+ cells are largely distinct, suggests that alternative WβA tracing strategies should be investigated before conclusively ruling out a role for WβA cells in hFT/mOV

homeostasis. In this regard, we propose *Axin2* as a strong candidate for marking putative multipotent mOV progenitors, as well as *Lgr6* which we find to be the only RSPO receptor enriched in WβA cells.

These proposed *in vivo* investigations are conditional on resolving inter-species differences highlighted in this study. It was previously shown that regeneration of mOV organoids is WβS-independent (Lõhmussaar *et al*., 2020). We extend this observation to show that a WNT-FZD5 signaling axis also regulates WβS in mOV organoids, and RNA FISH staining confirms presence of *Wnt7a+* cells in mOV tissue and organoids. However, unlike in the human setting, inhibition of this signaling axis (which depletes the WβA cell compartment) has no effect on mOV organoid regeneration, and isolated WβA cells do not display enhanced OFE under a range of tested conditions. Similarly, TGF-β signaling inhibition, which is essential for hFT organoid renewal, is dispensable for mOV organoid regeneration. We find these differences to be striking and warrant further investigation, particularly to address whether these deviations represent a biological inter-species difference or are the result of *in vitro* culture conditions.

**Hormonal regulation of the FT stem cell niche:** Estrogen has been reported to promote WβS and proliferation in FRT tissues (Kouzmenko *et al*., 2004; Hou *et al*., 2004; Gunin *et al*., 2004; Polotsky *et al*., 2009) but the molecular details of this mechanism remain poorly defined. We report novel findings that suggest Estrogen-ERα surpasses the cellular WβS machinery to activate transcription of WβS target genes. In the presence of WNT7A, however, we note Estrogen signaling negatively regulates WNT7A protein and WNT7A-induced WβS, and in hFT organoids Estrogen downregulates *WNT7A* at the mRNA level. This is consistent with reports showing that W*NT7A/Wnt7a* expression drops in the endometrium during the Estrogenic phase of the menstrual cycle in humans (Bui *et al*., 1997) and mice (Miller *et al*., 1998). Our data is also consistent with *in vivo* studies in which treatment of mice with Diethylstilboestrol (DES; an Estrogen analogue) phenocopies the above-described *Wnt7a* KO phenotypes (McLachlan *et al*., 1980) and downregulates *Wnt7a* mRNA levels in the neonatal FRTs (Miller *et al*., 1998). This downregulation is absent in ERα KO mice (Couse *et al*., 2001) further confirming this is an estrogenic effect. Clinically, human patients exposed *in utero* to DES showed similar phenotypes to *Wnt7a* KO mice, including FT abnormalities, lack of tubal coiling, withered Fimbria and infertility (DeCherney *et al*., 1981).

The molecular mechanism of Estrogen's suppression of WNT7A remains unclear. However, *in vivo* studies of the endometrium suggest mesenchymal WNT5A, activated by ER+ fibroblasts, mediate Estrogen's suppression of *Wnt7a*, either by DES treatment or during the Estrogen-dominated phase of the estrous cycle (Mericskay, Kitajewski, & Sassoon, 2004). As WNT5A is a documented inhibitor of WβS via non-canonical Wnt signaling (He *et al*., 2008; Olson & Gibo, 1998; Topol *et al*., 2003), we propose it exerts its influence through epithelial FZDs 3/6. Although we do not detect ER+ WNT5A+ cells in our patient stroma SCT dataset, it is plausible that aged patients (which constitute the bulk of our patient cohort) do not activate mesenchymal WNT5A expression due to menopausal absence of Estrogen, and further investigation of samples from premenopausal hormone-

exposed cohorts is required to ascertain this. Overall, we propose a working model for hormonal regulation of the proposed FT stem cell niche (Figure 7C).

An inherent limitation of this model is the notion of modelling Estrogen's complex interactions *in vitro*. Cells are known to elicit differential responses to Estrogen depending on its local concentration, and the cyclic nature and physiological doses of Estrogen *in vivo* are difficult to recapitulate *in vitro*. The phenotypes observed in Estrogen-supplemented organoids, therefore, while providing a preliminary understanding of Estrogenic molecular changes, are likely not to capture the full spectrum of molecular and phenotypic changes induced by Estrogen *in vivo*. Another limitation of our work is the absence of the stroma in our organoid model, precluding the study of estrogenic stromal changes that regulate mesenchymal-to-epithelial signaling. To address this, we propose the optimization of complex FT organoid co-cultures methods incorporating stromal and other microenvironmental components to dissect these changes as well as study the effect of mesenchymal-derived factors such as WNT2, RSPO3 and TGF-β inhibitors, and their influence on the FT stem cell niche.

**WβA cells & HGSOC**: High Grade Serous Ovarian Cancer (HGSOC) is a public health burden and the main driver of high mortality in ovarian cancer. Serous Tubal Intraepithelial Carcinomas (or STICs) are thought to be HGSOC's precursor lesion, and were documented in sporadic HGSOC (Howitt *et al*., 2015; Chen *et al*., 2017) and germline BRCA-associated HGSOC (Howitt *et al*., 2015; Finch *et al*., 2006). STICs display an unusually specific localization to the distal FT (Crum, 2009); a region enriched in stem-like cells (Wang *et al*., 2012; Zhu *et al*., 2020; Erickson *et al*., 2013; Xie *et al*., 2018) and active WβS (Ghosh *et al*., 2017). Interrogation of the role of these cells in HGSOC initiation has been hampered by lack of knowledge about FT stem cells, and lack of reliable FT models.

In this work, we report that WβA FT cells from non-HGSOC patients possess unique transcriptomes featuring a number of established HGSOC markers. WβA FT cells are also marked by *WNT7A,* which was shown using orthotopic and subcutaneous xenograft models to drive HGSOC proliferation, invasion and metastatic dissemination throughout the peritoneal cavity (Yoshioka *et al*., 2012; King *et al*., 2015). High *WNT7A* expression was correlated with high *CA125* expression in HGSOC samples (Zhang *et al*., 2010), consistent with our transcriptomic analysis showing co-enrichment of *WNT7A* and *CA125* in WβA cells. In this regard, one hypothesis we propose is that WβA cells possess enhanced self-renewal capacity and may be primed to be the targets of early HGSOC-initiating mutations that produce precursor STIC lesions, which subsequently progress to advanced HGSOCs that retain the expression of these markers. Furthermore, recent studies show that WβS regulates expression of DNA double-strand break repair genes via a WβS/MYBL2 signaling axis (Kaur *et al*., 2021; Angers, 2021). This includes *BRCA1* and *BRCA2* genes, which are of clinical relevance to HGSOC initiation. Our work is a first report suggesting applicability of this regulation axis to the hFT setting. It is tempting to speculate, based on these findings, that WβA cells are enriched in *BRCA1/2* expression, and that *BRCA1/2* loss

preferentially targets WβA cells in HGSOC initiation. Notably, although we see robust reduction of *BRCA1/2* expression upon WNT inhibition, we do not observe enhanced *BRCA1/2* levels in WβA cells isolated from our WβS-reporter organoids. This could be due to endogenous WβS levels, which are higher *in vivo* compared to our organoid setting (Figure S4E), possibly due to contribution from mesenchymal WNTs and RSPOs *in vivo*. Overall, FT WβA cells may be preferential targets of *BRCA1/2* mutation and possibly HGSOC initiation, and more detailed molecular investigation is needed to conclusively establish this.

In conclusion, we propose a first basic description of the character of FT stem cells and their molecular niche requirements for homeostasis. Our work lays the foundation for subsequent functional and *in vivo* studies on FT homeostasis, disease, hormonal regulation and epithelial-mesenchymal crosstalk. Our findings provide a basis for mechanistic work investigating the role of FT stem cells in ovarian cancer initiation.

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# Single Cell Transcriptomics identifies a WNT7A-FZD5 Signaling Axis that maintains Fallopian Tube Stem Cells in Patient-derived Organoids

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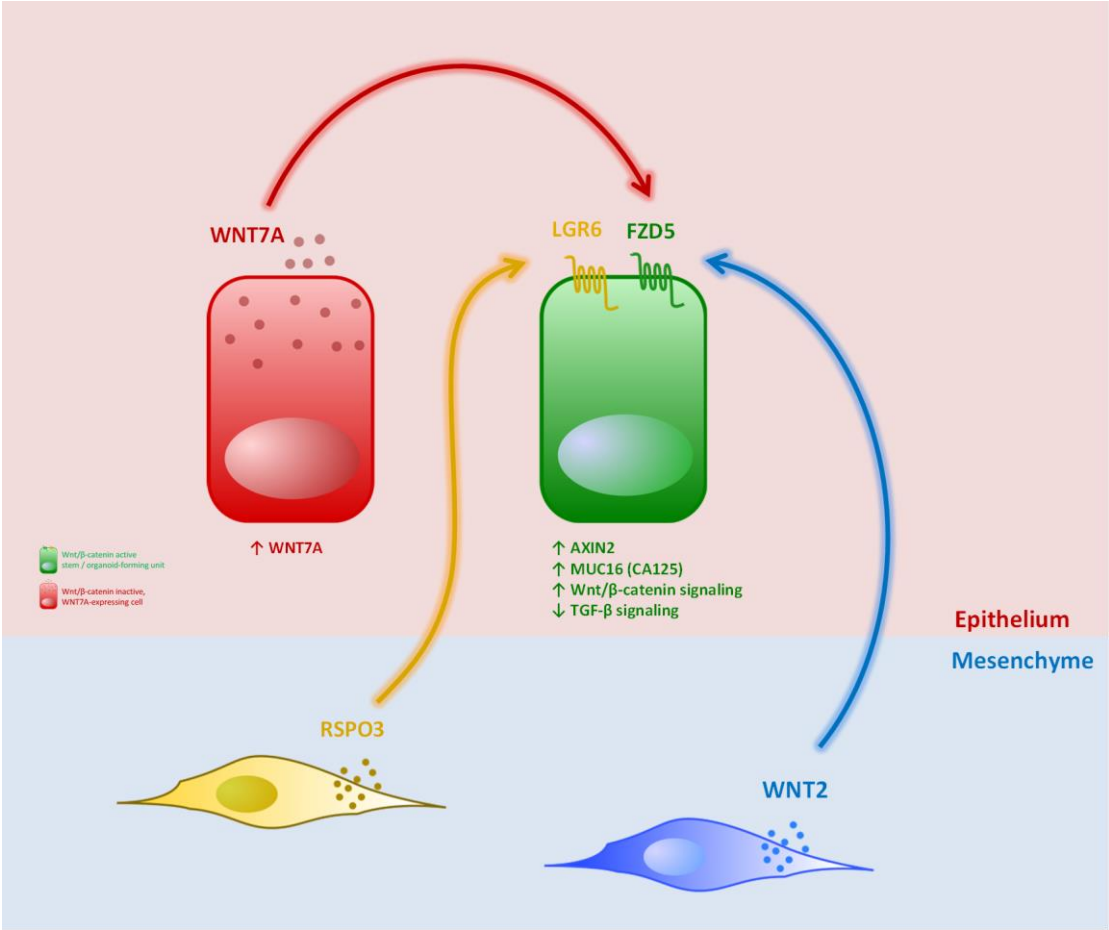
## MAIN FIGURES

# Graphical Abstract



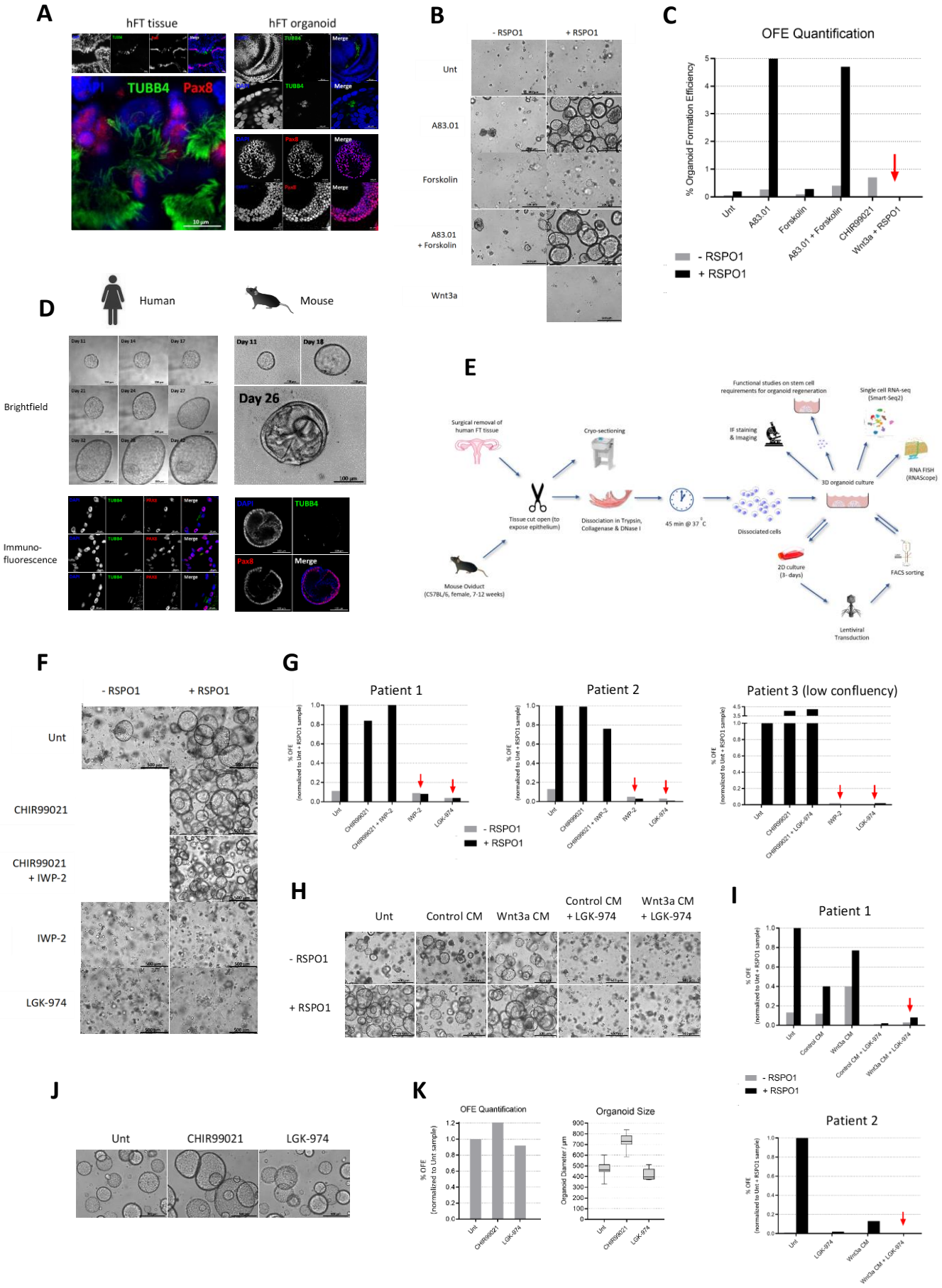



***Figure 1: WβS is essential for organoid regeneration and is driven by unidentified WNT(s).***

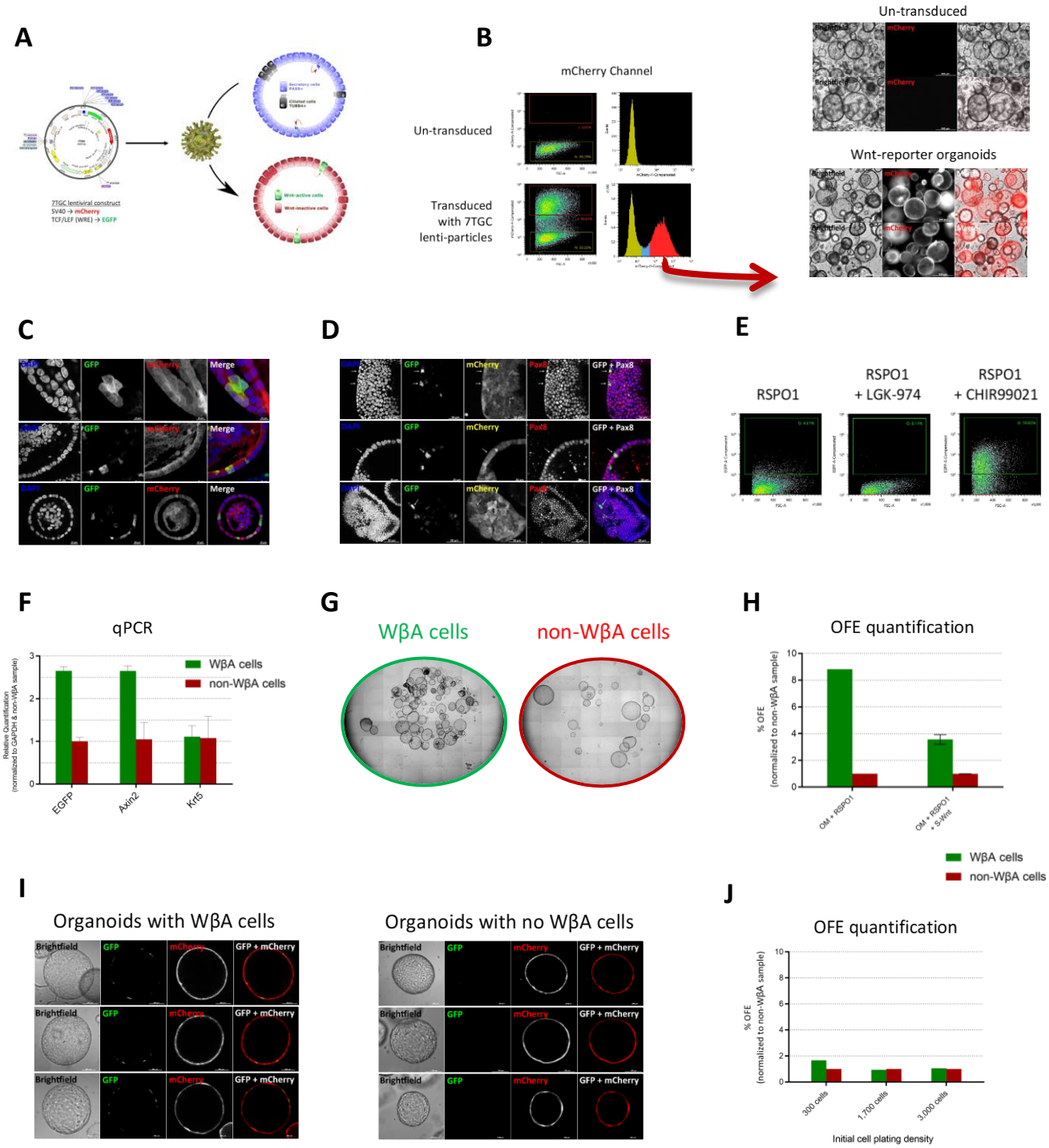



***Figure 2: WβA cells drive organoid regeneration.***

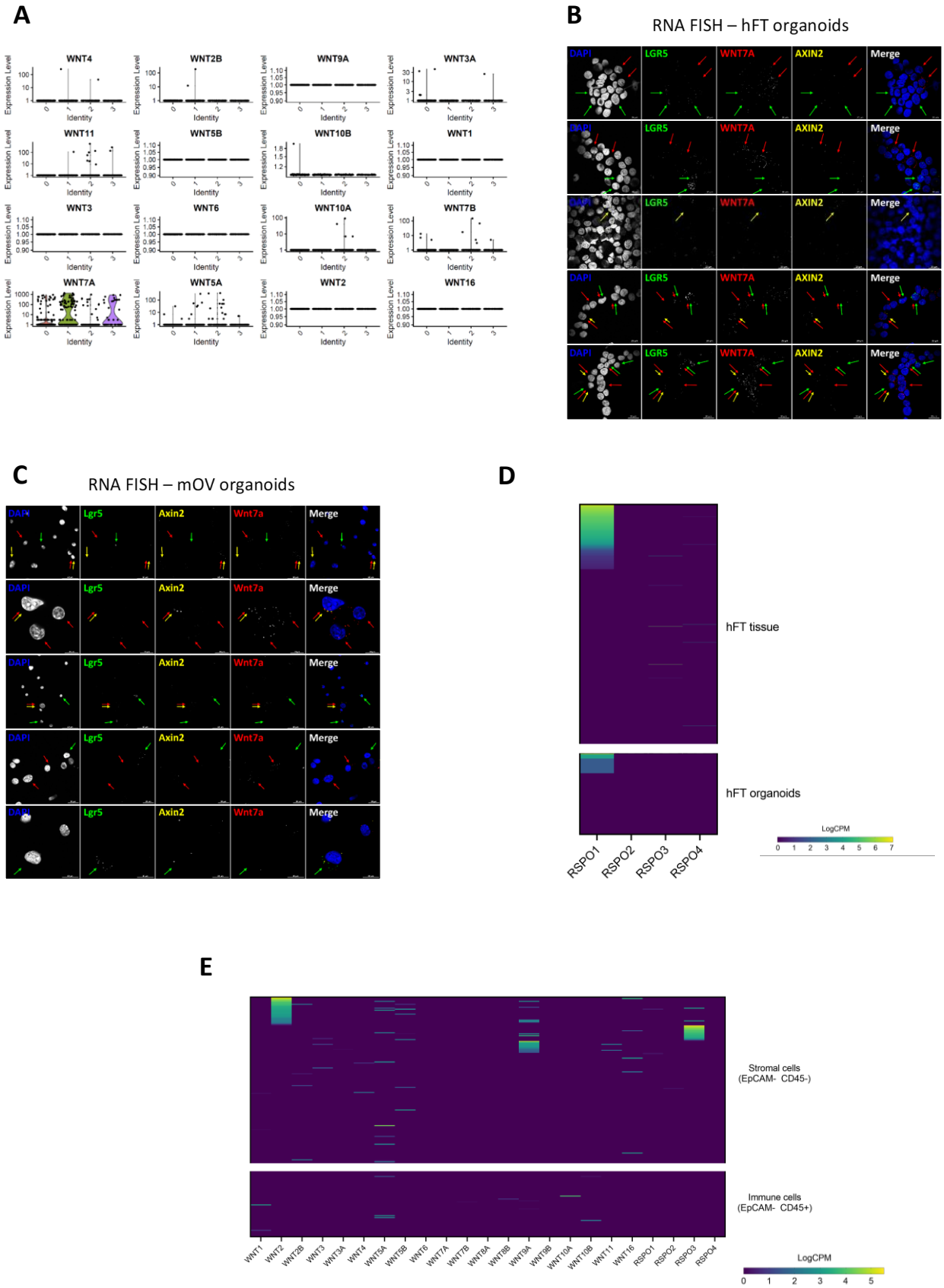



***Figure 3: WNT7A is the WNT ligand that cooperates with RSPO1 to drive renewal of hFT organoids.***

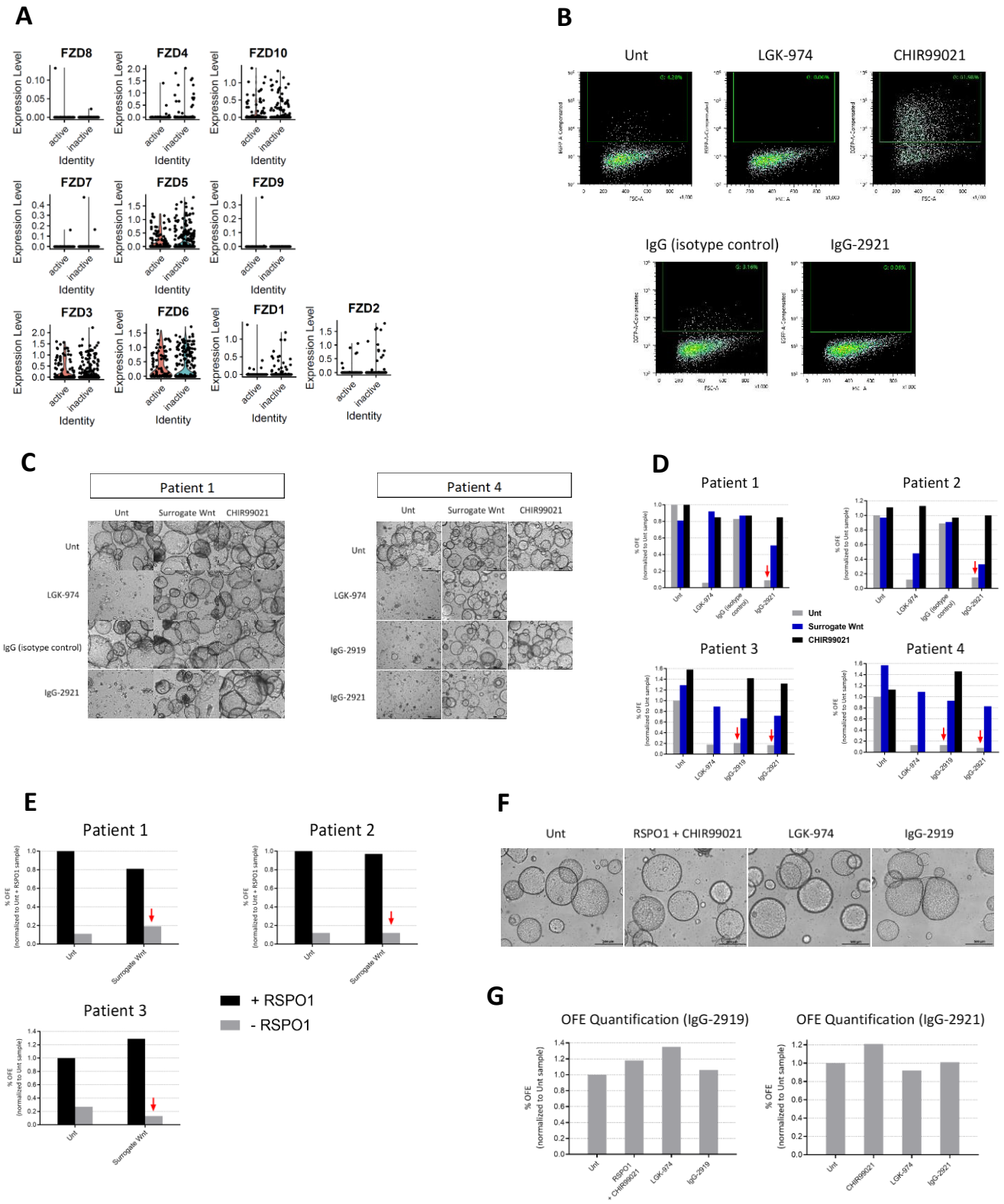



***Figure 4: FZD5 is the WNT7A receptor.***

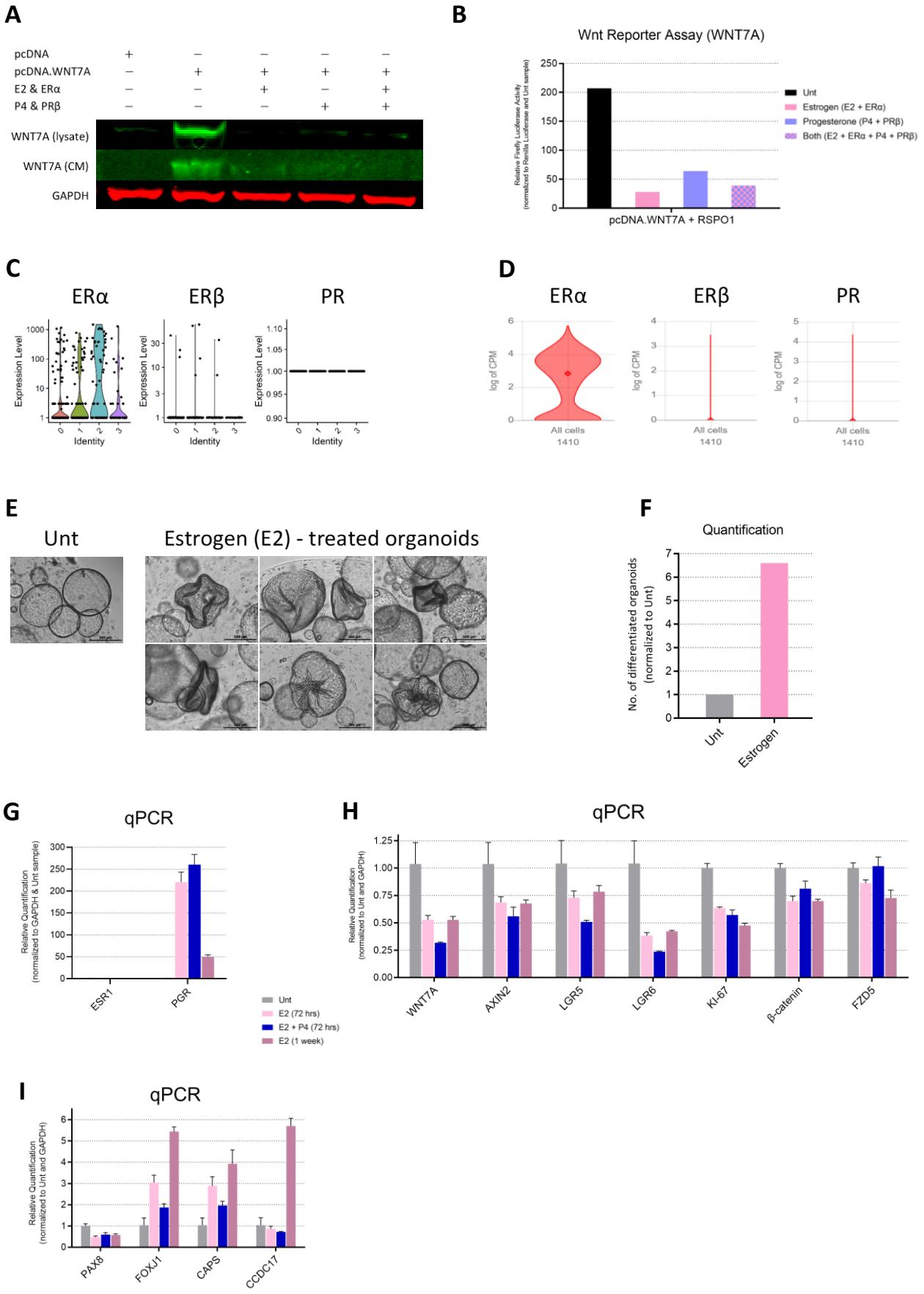
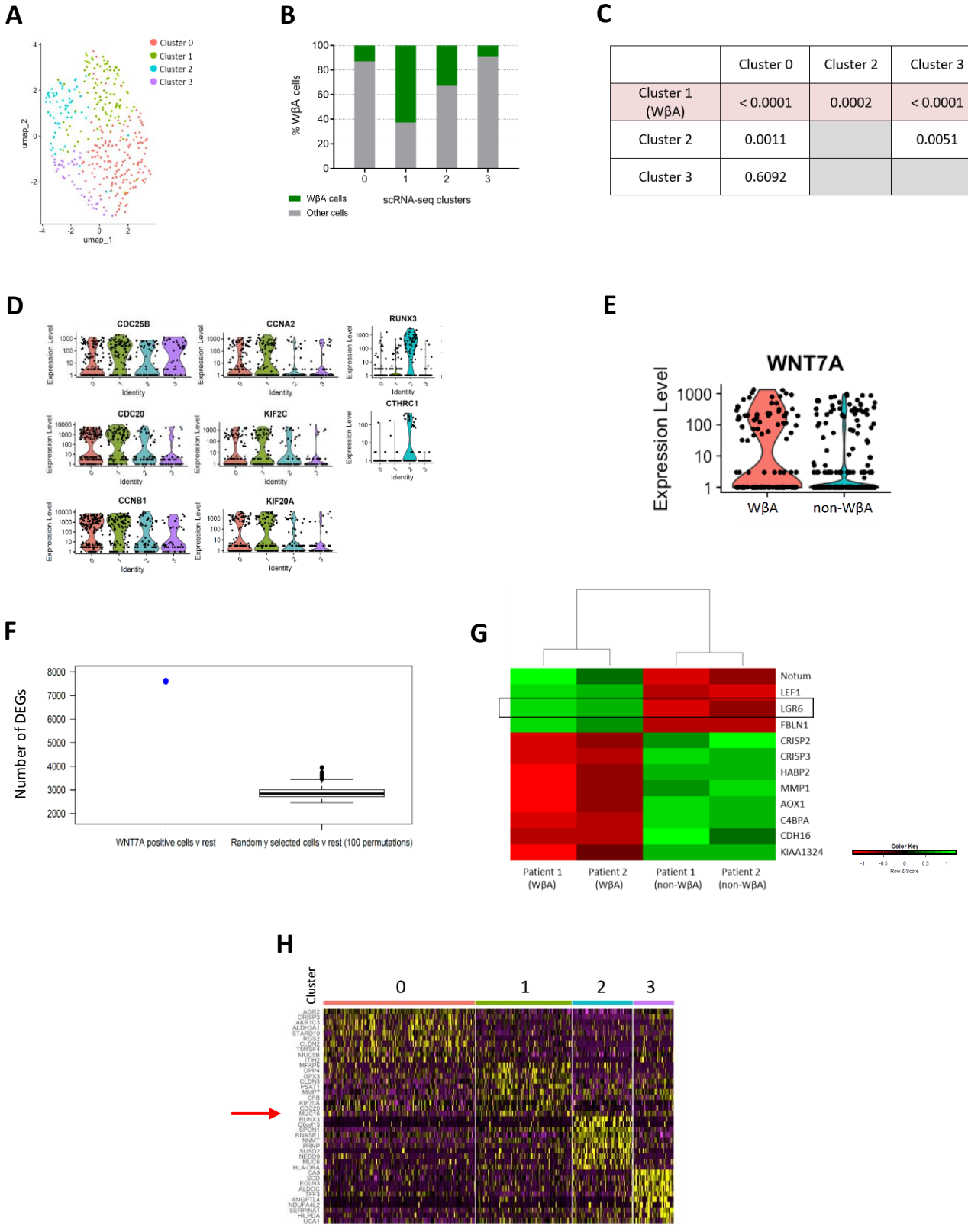



***Figure 5: Estrogen suppresses WNT7A and promotes differentiation of hFT organoids.***




| | Cluster 0 | Cluster 2 | Cluster 3 |
|---|---|---|---|
| Cluster 1 (WβA) | < 0.0001 | 0.0002 | < 0.0001 |
| Cluster 2 | 0.0011 | | 0.0051 |
| Cluster 3 | 0.6092 | | |

***Figure 6: Single cell transcriptomic analysis of WβA cells.***

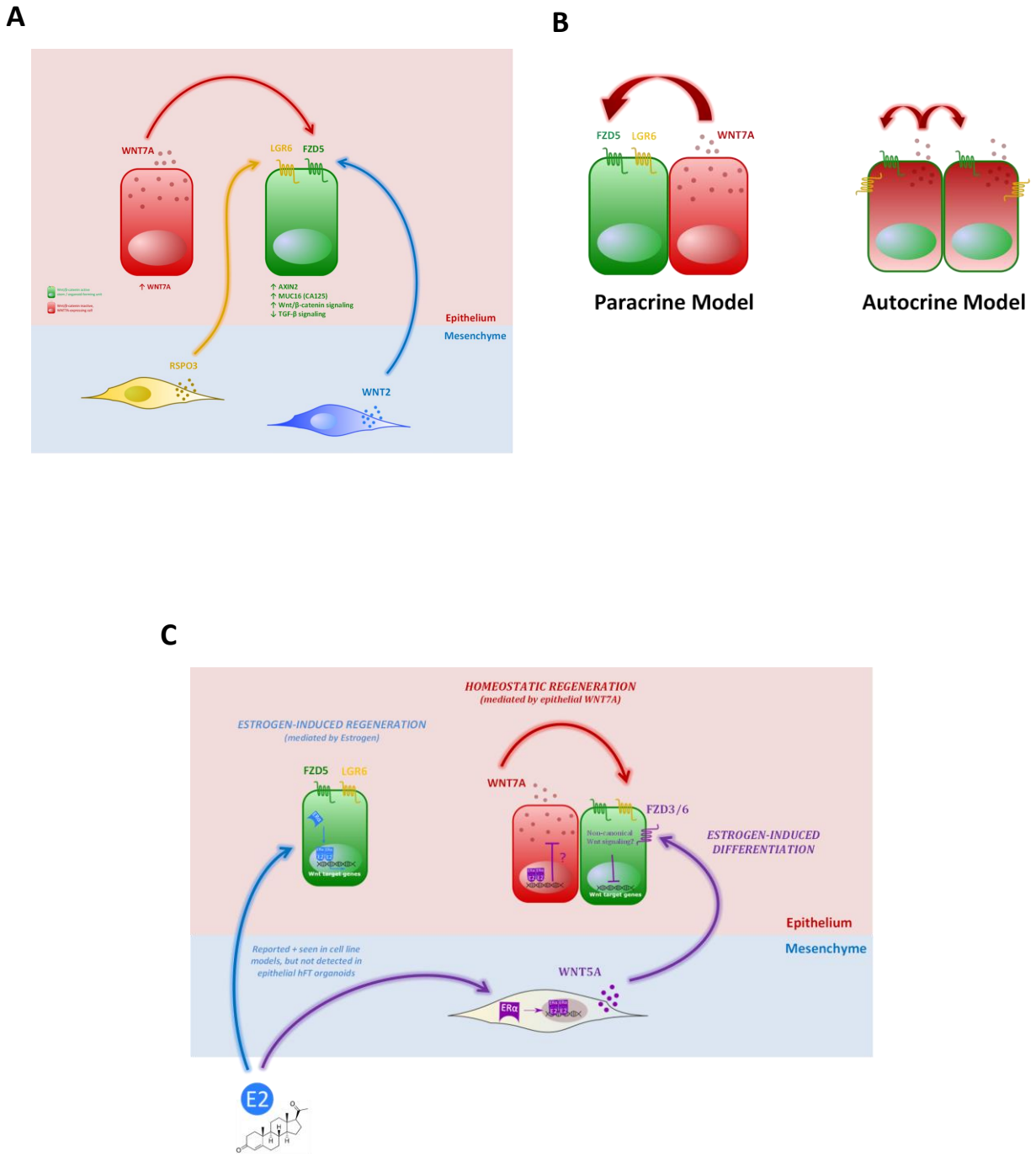



***Figure 7: Working model on the molecular maintenance and hormonal regulation of the FT stem cell niche.***

# Single Cell Transcriptomics identifies a WNT7A-FZD5 Signaling Axis that maintains Fallopian Tube Stem Cells in Patient-derived Organoids

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## FIGURE LEGENDS (MAIN & SUPPLEMENTARY FIGURES)

# LEGENDS FOR MAIN FIGURES

***Figure 1: WβS is essential for organoid regeneration and is driven by unidentified WNT(s).***

***(A)*** *Validation of hFT organoid culture and its relevance to hFT tissue.*

***(B)*** *WβS activation (RSPO1) and suppression of TGF-β signaling (A83.01) are essential for stem cell renewal in organoids. EpCAM+ CD45- cells were FACS isolated from dissociated patient-derived FT tissue and cultured under the conditions shown. Images taken on day 12.*

***(C)*** *Quantification of images in (B). Red arrow points to culture conditions of the reported method for culturing FT organoids.*

***(D)*** *hFT and mOV organoids emerge from a single multipotent stem cell. Sorted EpCAM+ CD45- cells were cultured as single cells in single wells of 96-well plates. 0.5-1% of cells formed organoids that gradually increased in size and complexity, as shown. Formed organoids contained PAX8+ secretory cells and TUBB4+ ciliated cells, evidencing multipotency of the organoid-forming cell.*

***(E)*** *Graphical summary of the experimental approaches employed to biomark and characterize putative FT stem cells.*

***(F)*** *RSPO1 cooperates with an endogenous WNT to drive organoid regeneration. Treatments done on passage 1 organoids from plating day. The CHIR99021 + IWP-2/LGK-974 samples show that lack of organoid regeneration in LGK-974 / IWP-2-treated samples is due to WNT inhibition and not inhibitor toxicity.*

***(G)*** *Quantification of images in (F), shown for three patient replicates. 19K cells, 12K cells or 7K cells were plated per sample for patients 1, 2 or 3, respectively.*

***(H)*** *WNT3A fails to rescue inhibition of WNT-blocked organoids. Treatments done on passage 1 organoids.*

***(I)*** *Quantification of images in (H), shown for two patient replicates. CM is conditioned medium. Activity of WNT3A CM was confirmed by the Wnt reporter (TOPFlash) assay (Figure S3A).*

***(J)*** *mOV organoids regenerate independently of mouse WNTs.*

***(K)*** *Quantification of OFE (left panel) and organoid size (right panel) of images shown in (J).*

***Figure 2: WβA cells drive organoid regeneration.***

***(A)*** *Organoids were transduced with viral lenti-particles generated using the 7TGC construct (see Methods for details), which enables mCherry expression under a general (SV40) promoter and EGFP under TCF/LEF elements, also called WβS-Reporter Elements (WβS-RE).*

***(B)*** *Transduced cells were selected by FACS and expanded using the optimised organoid culture conditions. Left panel shows FACS plots. Right panel shows images of organoids after 1 week of culture. Data representative of 4 patient replicates.*

***(C)*** *Confocal imaging of WβS-reporter organoids post-fixation, showing WβA (EGFP+) cells.*

***(D)*** *WβA (EGFP+) cells co-localize with PAX8, indicating WβA cells have a secretory cell lineage.*

***(E)*** *Proportion of WβA cells increases upon WβS activation (CHIR99021) and is abolished upon WβS inhibition (LGK-974).*

***(F)*** *WβA (EGFP+) cells express elevated levels of endogenous AXIN2, a marker of WβS activation.*

***(G)*** *WβA cells are enriched in organoid formation ability. EGFP+ and EGFP- cells were FACS isolated and cultured for 10-13 days.*

***(H)*** *Quantification of images in (I). OM + RSPO1 + SWNT treatments shown for two biological replicates. OFE is organoid formation efficiency.*

***(I)*** *Live imaging of mOV WβS-reporter organoids. In contrast to hFT WβS-reporter organoids, mouse organoid culture is composed of organoids that contain (left panel) or lack (right panel) WβA cells.*

***(J)*** *Quantification of OFE in mOV WβA vs non-WβA cells. mOV WβA cells are not enriched in organoid formation ability.*

***Figure 3: WNT7A is the WNT ligand that cooperates with RSPO1 to drive WβS and renewal of hFT organoids.***

***(A)*** *Single cell expression profile of the WNT family of ligands in hFT organoids. Based on SCT data, the frequency of WNT7A+ cells is ~ 5% in hFT tissue and ~20% in hFT organoids.*

***(B)*** *Confirmation of WNT7A+ cells in hFT organoids. WNT7A+ cells may or may not co-express markers of WβS activation AXIN2 and LGR5. AXIN2+ cells and LGR5+ cells are largely distinct. Organoids were dissociated, cytospinned and fixed prior to RNA FISH staining.*

***(C)*** *Confirmation of Wnt7a+ cells in mOV organoids. As in the human setting, Axin2+ cells here are distinct from Lgr5+ cells. Organoids were dissociated, cytospinned and fixed prior to RNA FISH staining.*

***(D)*** *Heatmap showing the single cell expression profile of the R-Spondin family of proteins in hFT tissue and organoids. Every row represents a single cell.*

***(E)*** *Heatmap showing the single cell expression profile of the WNT & R-Spondin family of ligands in the non-epithelial compartments of hFT tissue. Stromal and immune cells were FACS isolated using the antibodies shown.*

***Figure 4: FZD5 is the WNT7A receptor.***

***(A)*** *Single cell expression profile of the FZD family of receptors in hFT organoids.*

***(B)*** *Anti-FZD5 IgG-2921 depletes % of WβA cells in WβS-reporter organoids, phenocopying the effect of blocking WNT secretion. Organoids used here were mOV.*

***(C)*** *Anti-FZD5 antibodies (IgG-2921 and IgG-2919) reduce OFE by 80-90%, phenocopying the effect seen in WNT-blocked organoids (LGK-974). Treatments done on passage 1 organoids.. Images taken on day 12.*

***(D)*** *Quantification of images shown in (D) as well as for other patient replicates. Red arrows point to organoid inhibition due to FZD5 blocking.*

***(E)*** *Surrogate Wnt drives organoid regeneration in a FZD-specific, RSPO1-sensitive manner. Treatments done on passage 1 organoids.*

***(F)*** *mOV organoids are unaffected by blocking FZD5 receptor. Representative images shown on day 10.*

***(G)*** *Quantification of images shown in (F, left panel) as well as for another replicate using a different anti-FZD5 IgG (IgG-2921).*

***Figure 5: Estrogen suppresses WNT7A and promotes differentiation of hFT organoids.***

***(A)*** *Estrogen & Progesterone downregulate WNT7A protein. HEK293 cells were transfected with 1 μg of the indicated constructs. Cells were treated with 17β-estradiol (E2), progesterone (P4) or both for 72 hrs, at 100 nM (E2) or 1μM (P4). Western Blot shows cytoplasmic (lysate) and secreted (conditioned medium, CM) WNT7A protein levels.*

***(B)*** *Estrogen & Progesterone downregulate WNT7A activity. All samples were transfected with WNT7A construct. WNT7A-induced TOPFlash signal is reduced by 90% upon Estrogen treatment in HEK293 cells. ERα and PRβ were transfected at 200 ng per well. E2 and P4 were administered at the concentrations indicated in (A). Data is representative of two biological replicates.*

***(C)*** *Single cell transcriptomic profile of hormone receptor expression in hFT organoids. ERα is the predominant Estrogen receptor expressed. As expected, Progesterone Receptor (PR) is not expressed, as its expression is regulated by Estrogen. Organoids were not treated with Estrogen prior to scRNA sequencing.*

***(D)*** *Single cell transcriptomic profile of hormone receptor expression in hFT tissue. This mirrors expression seen in organoids.*

***(E)*** *Estrogen treatment of organoids triggers pronounced morphological changes within 72 hours, producing dark staining, crippled organoids with extensive internal folding and invaginations.*

***(F)*** *Quantification of images in (E).*

***(G)*** *Estrogen treatment of hFT organoids induces PR expression, a classic E2-ER target gene.*

***(H)*** *Estrogen suppresses WNT7A transcription in organoids. This is accompanied by a reduction in WβS activation marker AXIN2. Progesterone does not antagonize these changes.*

***(I)*** *Estrogen promotes differentiation of hFT organoids, triggering a reduction in secretory cell marker (PAX8) and an increase in ciliated cell markers. CCDC17, a ciliated cell marker recently identified using scRNA-seq of fresh hFT tissue (Hu et al., 2020), is activated in long-term but not short-term Estrogen-treated organoids. Data in (E-I) are for the same samples, and E2 (1 week) treatment is normalized to Unt (1 week) control sample not shown for simplicity.*

***Figure 6: Single cell transcriptomic analysis of WβA cells.***

***(A)*** *De novo clustering of cells after cell cycle correction, showing hFT organoids are composed of 4 transcriptomic clusters of cells.*

***(B)*** *Majority of WβA cells concentrate in Cluster 1.*

***(C)*** *P-values from pairwise Fisher's test comparisons between all clusters, showing enrichment of WβA cells in Cluster 1 is statistically significant.*

***(D)*** *The WβA cluster is proliferative and enriched in various G2-to-M cell cycle transition regulators.*

***(E)*** *WNT7A expression is enriched in WβA cells.*

***(F)*** *WNT7A+ cells have unique transcriptomes. The figure shows the number of differentially expressed genes (DEGs) obtained by comparing the transcriptomes of WNT7A+ cells versus WNT7A- cells. To ensure the number of DEG's between WNT7A+ and WNT7A- cells is not due to stochastic cell-to-cell differences, multiple control analyses were performed between two randomly selected groups of WNT7A- cells, each containing the same number of cells as the WNT7A+ group.*

***(G)*** *Selected DEGs from bulk transcriptomic analysis of WβA versus non-WβA cells.*

***(H)*** *Heatmap showing marker genes for each cluster. The WβA cluster is marked by CA125 (red arrow), a clinical marker of HGSOC diagnosis, progression and response to therapy.*

***Figure 7: Working model on the molecular maintenance and hormonal regulation of the FT stem cell niche.***

# LEGENDS FOR SUPPLEMENTARY FIGURES

***Figure S1: FT models.*** *(Supplementary to Figure 1A)*

***(A)*** *Approaches for modelling FT biology in vitro. 2D culture of primary FT cells suffers from the drawbacks shown. Spheroid cultures overcome the dimensionality barrier of 2D cultures, but generate multi-layered spheroids lacking cell-matrix adhesion, monolayer cell organization and long-term propagation. Air-liquid interface approaches produce well-differentiated cultures that can be effective in modelling stem cell renewal; however they are single-use cultures that cannot be expanded long-term and rely on a continuous supply of patient-derived or murine specimen (Levanon et al., 2010). Air-liquid interface cultures expose the epithelium to air and were originally optimized for airway epithelium (Lin et al., 2007; Pezzulo et al., 2011). In vivo lineage tracing methods are the gold standard for evidencing stem cell activity, but are expensive, highly technical, require protracted incubation times and are not amenable to high throughput approaches. Organoids are a miniaturized form of organs, capturing some of their in vivo complexities while maintaining the convenience of in vitro models.*

***(B)*** *Representative images of primary FT 2D culture on day 3. Red arrows point to emerging epithelial 'islands'.*

***(C-D)*** *2D-cultured primary FT cells express epithelial (Pan KRT & E-cadherin) and FT secretory (PAX8 & MYC) markers. Ciliated cell markers are absent (data not shown).*

***(E)*** *2D-cultured primary FT cells lose epithelial markers by Day 37.*

***Figure S2: Validation of mouse oviduct (mOV) organoids.***
*(Supplementary to Figure 1 J-K)*

***(A-C)*** *Similar to hFT organoids, mOV organoids are morphologically spherical* ***(A)****, epithelial* ***(B)*** *and express FT-specific markers* ***(C)****. mOV organoids here were grown after single cell dissociation.*

***(D)*** *Testing mOV regeneration under various conditions. Colour-coded key shows which growth factor is added in growth factor-free medium. EGF is important for regeneration of mOV organoids, as reported (Xie et al., 2018). FGF-10 can substitute for absence of EGF partially, or completely under conditions of WβS activation. In this work, mouse organoids were grown in complete culture medium containing EGF, FGF-10 and RSPO1, and lacking TGF-β (ALK4/5/7) inhibitors.*

***(E)*** *OFE of mOV organoids reduces upon Notch signaling inhibition using the γ-secretase inhibitor DAPT, but not upon WNT inhibition.*

***Figure S3: hFT organoids and validation of WβS reagents.*** *(Supplementary to Figure 1)*

***(A)*** *Activity of L-WNT3A CM was validated by the TOPFlash assay. WNT3A CM used at 25% here and in organoids as indicated. RSPO1 used at 500 ng/mL. (Supplementary to Figure 1 H-I)*

***(B)*** *Effect of serum-containing CM on organoid regeneration. (Supplementary to Figure 1 H-I)*

***(C)*** *Organoids emerge from epithelial FT cells and not fibroblasts.*

***(D)*** *Mature hFT organoids contain internal foldings and invaginations (red arrow) characteristic of FT tissue.*

***(E)*** *hFT organoids contain a small proportion of proliferative cells. (C-E are supplementary to Figure 1 B-C)*

***(F)*** *Validating XAV-939 and PRI-724's inhibitory activity on WβS. XAV-939 is a tankyrase inhibitor that stabilizes AXIN and the β-catenin Destruction Complex (Huang et al., 2009; Tian et al., 2013). PRI-724 disrupts β-catenin's interaction with its obligate transcriptional co-activator CBP (Okazaki et al., 2019).*

***(G)*** *WβS inhibition at midstream (XAV-939) or downstream (PRI-724) levels abolishes organoid regeneration, including that of CHIR99021-treated samples, showing that CHIR99021-induced organoid regeneration is due to WβS and not non-specific GSK-3β targets. (Supplementary to Figure 1G)*

***(H)*** *Testing effect of all reagents in the standard Organoid Medium (OM) on WβS. Apart from RSPO1, no growth factors, supplements or small molecule inhibitors activate WβS. CHIR99021 is used as positive control here and does not constitute part of the OM medium.*

***(I)*** *Validating inhibitory effect of IWP-2 and LGK-974 on WβS. (Supplementary to Figure 1 F-K)*

*IWP-2 and LGK-974 are selective Porcupine inhibitors. Porcupine is a membrane bound O-acyltransferase that facilitates palmitoylation of WNTs, which is essential for further WNT processing and secretion. Palmitoylation is the covalent addition of 16-carbon monounsaturated palmitic acid moiety to Serine, Threonine and less commonly Cysteine residues. Palmitoylation of WNTs takes place during protein synthesis in the endoplasmic reticulum (Mikels & Nusse, 2006).*

*In this study, CHIR99021 (3 μM), IWP-2 (3 μM), LGK-974 (2 μM), XAV-939 (5 μM) and PRI-724 (10 μM) were used at the indicated concentrations.*

***Figure S4: hFT and mOV WβS-reporter organoids.*** *(Supplementary to Figure 2)*

***(A)*** *FACS profile of hFT WβS-reporter organoids. (Supplementary to Figure 2E)*

***(B)*** *Live confocal imaging of WβS-reporter organoids. Note EGFP+ cells reporting localized WβS activation.*

***(C)*** *hFT WβS-reporter organoids were treated with WβS activators or none for 7 days. Notch activation using Valproic Acid (VPA) potentiates RSPO1 & CHIR99021-induced WβS activation. Quantification is done for 2-3 technical replicates. (Supplementary to Figure 2E)*

***(D)*** *AXIN2 and LGR5 are markers of WβS activation in hFT organoids. Treatments were done for 72 hrs. AXIN2 and LGR5 were also found to be faithful markers of WβS activation in organoids derived from human intestine, liver, colon, pancreas and stomach (Boonekamp et al., 2021). (Supplementary to Figure 2F)*

***(E)*** *Effect of culture conditions on expression of WβS target genes across patient-matched samples. WβS activation is more robust in fresh tissue, possibly due to mesenchymal sources of WNT and R-Spondin not present in organoid culture.*

***(F)*** *FACS isolation and organoid culture of mOV WβA or non-WβA cells. Both regenerate organoids at equivalent OFEs and give rise to the other cell state.*

***(G)*** *Effect of different culture conditions on OFE. BM1 is Basal Medium 1 (DMEM/F12 + HEPES + P/S). BET is BM1 + B27 supplement + EGF + SB431542. OM is complete organoid medium.*

***(H-I)*** *Effect of blocking endogenous WNT secretion on OFE* ***(F)*** *and size* ***(G)*** *of mOV organoids. (D-H are supplementary to Figure 2J)*

***Figure S5: RNA FISH staining.*** *Positive control probes targeted ubiquitously expressed housekeeping genes. Negative control probes targeted bacterial sequences. (Supplementary to Figure 3 B-C)*

***(A-C)*** *Fluorescent RNAScope FISH staining on fresh frozen hFT tissue sections. (Supplementary to Figure 3B)*

***(D-G)*** *Fluorescent RNAScope FISH staining on fresh frozen mOV tissue sections. Positive control signal is abolished by RNase treatment, confirming punctate dots detected are RNA. (Supplementary to Figure 3C)*

***Figure S6: Expression and validation of WNT7A protein reagents.***
*(Supplementary to Figure 3)*

***(A)*** *WNT7A conditioned medium (CM) was harvested from HEK293 cells transfected with WNT7A-cDNA construct. Left panel shows WNT7A immunoblot. V5 signal confirms specificity of the WNT7A antibody. Right panel is a quantification of band intensities.*

***(B)*** *Recombinant WNT7A protein (R&D) was used to quantify [WNT7A] harvested in Day 3 CM from (A). Left panel shows immunoblot; right panel is a quantification of band intensity to generate a calibration curve for estimating [WNT7A] in CM. This amounts to 3.4 µg/ml. WNT7A CM was used at 25% in downstream assays, so final [WNT7A] was 850 ng/ml.*

***(C)*** *Primary 2D-cultured FT cells secrete WNT7A protein readily detectable in CM, confirming the data below (D). CM was harvested after 4-day incubation of 1-week old culture. Control here is the same medium prior to incubation with cells.*

***(D)*** *WNT7A is strongly overexpressed in primary 2D-cultured FT cells. Upper panel shows expression of the WNT family of ligands in fresh versus FT 2D primary culture. The latter was done overnight or long-term (LT) for 2-6 days. Bottom left panel is an enlargement of the circled plot above. Bottom right panel is a qPCR assay showing that WNT7A mRNA levels are elevated in 2D-cultured cells relative to fresh or organoid cells in a patient-matched sample. qPCR shown with three distinct WNT7A qPCR primers.*

***(E)*** *WNT7A protein is detectable in lysates of primary 2D-cultured FT cells, and is almost completely abolished upon WβS pathway activation, hinting that WβS activation and WNT7A expression are mutually exclusive.*

***(F)*** *Confirmation that CHIR99021 treatment shown in (E) increases intracellular levels of stabilized β-catenin and WβS. Active β-catenin antibody (Merck Millipore) specifically recognizes β-catenin unphosphorylated in Ser 33, 37 and 41. Phosphorylation of these serine residues by GSK-3β and CK1α targets β-catenin for degradation. Unt sample was run concurrently on a different gel in the same SDS-PAGE run.*

***(G)*** *Isolation of native WNT7A protein using non-denaturing non-ionic detergent (Passive Lysis Buffer, Promega). The blot shown here is after SDS-PAGE, confirming presence of WNT7A in lysate extract.*

***(H)*** *WNT7A protein reagents are not functional. WNT7A-cDNA construct induces robust WβS activation as measured by the TOPFlash assay, but WNT7A CM obtained from the same construct-transfected cells does not induce WβS activation. All other WNT7A protein reagents failed to activate WβS. Some samples in the figure are normalized to internal controls not shown for simplicity.*

***(I)*** *WNT7A signals in a short-range gradient. Co-culture assay designed as shown.*

***(J)*** *WβS is activated in iRFP+ cells upon co-culture with WNT7A-transfected but not control-transfected cells.*

***Figure S7: FZD5 is a receptor for WNT7A.***
*(Supplementary to Figure 4)*

***(A)*** *Single cell expression profile of the FZD family of receptors in hFT tissue. (Supplementary to Figure 4A)*

***(B)*** *Protein sequence similarities between hFT-expressed FZDs, obtained by pairwise sequence alignments of RefSeq sequences.*

***(C)*** *FZD3, FZD5 and FZD6 are endogenously expressed in HEK293 cells. $C_T$ values for the 3 FZDs, as well as GAPDH, shown for comparison. FZD expression was confirmed using two independent qPCR primers for each FZD gene.*

***(D)*** *qPCR confirmation of FZD 3/5/6 siRNA knockdown.*

***(E)*** *WNT7A-induced TOPFlash signal reduces upon knockdown of FZD5, but not FZD3 or FZD6. All samples contained RSPO1, except Unt. WNT7A denotes pcDNA.WNT7A construct. Data from (C-E) are from the same experiment.*

***(F)*** *WNT7A induces highest TOPFlash signal in a background of FZD5 OE. IWP-2 treatment confirms this signal is due to WNT7A OE. Control is pcDNA construct. OE is overexpression.*

***(G)*** *Anti-FZD5 antibody shows activity against WNT7A-induced FZD5-mediated WβS activation in TOPFlash assay. Given the FZD5 OE construct encodes mouse FZD5, this also shows that anti-FZD5 IgG's also inhibit mouse FZD5. (Supplementary to Figure 4 C-D and F-G)*

***(H)*** *Surrogate Wnt potently activates WβS in TOPFlash assay. Surrogate Wnt-induced TOPFlash signal reduces with XAV-939 and PRI-724 treatment, further confirming that this activation happens through the canonical WβS machinery. (Supplementary to Figure 4 C-E and Figure S8)*

*Surrogate Wnt is a synthetic bivalent peptide containing an antibody epitope that binds the extracellular FZD cysteine-rich domain (CRD). Surrogate Wnt also contains a linker sequence and an LRP5/6-binding motif (Janda et al., 2017). The FZD-CRD epitope in Surrogate Wnt is the single chain variable fragment of OMP-18R5, while the LRP5/6-binding motif is the C-terminal portion of DKK1 (Janda et al., 2017). Overall, Surrogate Wnt selectively hetero-dimerizes FZDs with LRP6 co-receptor extracellularly, mimicking the action of endogenous WNTs.*

***Figure S8: Patient 5 organoids are resistant to inhibition of the WNT7A-FZD5 signaling axis.*** *(Supplementary to Figure 4 C-E)*

***(A)*** *Patient 5 organoids are resistant to blocking endogenous WNTs and FZD5, but sensitive to downstream WβS inhibitors (images with green outline) as seen in other patients (see Figure S3G). Images taken on day 12. Treatments done on passage 1 organoids. (Supplementary to Figure 4C)*

***(B)*** *Quantification of images shown in (A). Red arrows point to treatments that abolish organoid regeneration in all other tested patients. Green arrows point to treatments with downstream WβS inhibitors. (Supplementary to Figure 4D)*

***(C)*** *Unlike organoids from other patients, withdrawal of RSPO1 does not affect OFE of Patient 5 organoids. (Supplementary to Figure 4E)*

*Patient 5 organoids (A-C above) were tested in parallel with Patient 3 organoids on the same day using the same inhibitor medium master mixes. Patient 3 organoids showed sensitivity to all inhibitors of the WNT7A-FZD5 signaling axis (Figure 4 C-D).*

***(D)*** *Patient 5 organoids grown under the shown conditions for 4 passages. Image taken at passage 4 after allowing organoids to propagate for 3 weeks without medium change to replenish stem cell factors. This normally leads to differentiation or organoid death (Unt, left panel). Patient 5 organoids expanded under selective WNT-blocked (LGK-974) conditions and continued robust growth across passages, indicating growth advantage and ectopic independence from stem cell niche requirements. Targeted DNA sequencing showed Patient 5 organoids harbour no TP53 mutations.*

***Figure S9: Estrogen activates transcription at WβS-Response Elements (WβS-RE) independently of the WβS pathway.*** *(Supplementary to Figure 5)*

***(A)*** *Estrogen robustly activates WβS-RE elements. Progesterone slightly antagonizes this.*

***(B)*** *Estrogen activates WβS-RE elements in the presence of LGK-974, suggesting this activation is independent of WNT ligands. Estrogen-induced transcription of WβS-target genes is unaffected by midstream (XAV-939) or downstream (PRI-724) inhibition of WβS.*

***(C)*** *Estrogen counteracts elevated activation of WβS, reducing it to levels comparable with Estrogen-only treated samples.*

***(D-F)*** *β-catenin siRNAs effectively abolish β-catenin protein level (D) and activity (E-F). All samples in (E) contained CHIR99021. All samples in (F) contained Wnt3a and RSPO1.*

***(G)*** *Estrogen activates WβS-RE elements independently of β-catenin. This activation is higher in the absence of β-catenin, suggesting the pair compete for binding at WβS-RE promoter elements. (A-G are supplementary to Figure 5 A-B)*

***(H)*** *CCDC78, a ciliated cell marker (Hu et al., 2020), is strongly induced by long-term Estrogen treatment in hFT organoids. (Supplementary to Figure 5I)*

***(I)*** *Estrogen treatment reduces WβS and BRCA gene expression. This is phenocopied by WNT inhibition. 1 week treatment samples are normalized to an Unt (1 week) control sample not shown.*

*For all data in this Figure, Estrogen (E2, also called β-estradiol) was used at 100 nM. Progesterone (P4) used at 1 μM. ERα and PRβ constructs were transfected at 200 ng per well for TOPFlash assays.*

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