

Identification of Fallopian Tube Stem Cells using Patient-derived Organoids



This thesis is submitted for the degree of Doctorate of Philosophy

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February, 2021*

Declaration

This thesis is submitted for the degree of Doctorate of Philosophy. The thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration, except where specifically indicated in the text. I would like to declare that most of the figures in Chapter 6.3 were published in:

Hu, Z., **Alsaadi, A.** *, Artibani, M. *, Wietek, N., Morotti, M., Shi, T., ... Ahmed, A. A. (2020). The Repertoire of Serous Ovarian Cancer Non-genetic Heterogeneity Revealed by Single-Cell Sequencing of Normal Fallopian Tube Epithelial Cells. *Cancer Cell*, 37(2), 226-242.e7.

<https://doi.org/10.1016/j.ccell.2020.01.003>

* these authors contributed equally to this work

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Finally, I confirm that this thesis adheres to the Oxford University Medical Sciences Division regulations on word limits on DPhil theses. The word count for this thesis is 49,879 words.

Dedication

I dedicate this thesis to my ever-loving parents, Ali Alsaadi and Fatima Alaw, for their continued love, support and motivation during my educational endeavours and beyond.

I would like to extend this dedication to my loving and supportive wife, Patricia Gelineck, for her continuous support (and patience!) during the ups and downs of this project.

Finally, I would also like to dedicate this thesis to my secondary school biology teacher, Dr Rakshana Zafar, who was the first to inspire my interest in Biology. I wish you all the best in life, and I hope you find the strength to win your battle against breast cancer.

Acknowledgements

I should like to express my gratitude to Prof Ahmed Ashour Ahmed, for providing me with the opportunity to conduct graduate studies in his laboratory. I thank him for all the research guidance and support he rendered me during the course of this project.

My gratitude extends to my laboratory colleagues at the Ovarian Cancer Cell Laboratory at the University of Oxford, for their technical help and valuable feedback during the course of my DPhil. Particular thanks go to Dr Matteo Morotti, Dr Nina Wietek and the Gynaecology Oncology Surgical team for help in recruiting and consenting patients for the study, as well as Dr Zhiyuan Hu for help in conducting the single cell experiments and the follow up bioinformatic analyses.

This project employed diverse experimental techniques, and I am thankful to the core facilities at the Weatherall Institute of Molecular medicine for help in training and guidance. In this regard, I would like to pay particular thanks to the Wolfson Imaging Centre, the Flow Cytometry Facility and the Single Cell Facility.

Finally, I would like to thank the funding bodies, without whom this work would not have seen fruition. This includes the Oxford University Press (Clarendon Scholarship), the Nuffield Department of Women's & Reproductive Health, Ovarian Cancer Action, the Diane Oxberry Trust and Mr Khalid Baeshen.

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Hilary Term 2021

ABSTRACT

Ovarian Cancer is the fifth most common cancer in women. High Grade Serous Ovarian Cancer (HGSOC) presents as the most aggressive histotype, accounting for over 70% of cases. Survival from HGSOC is amongst the lowest for any cancer. Unfortunately, over 70% of patients present at late stages, when 5-year survival verges at a dismal 10-25%. Although the tissue-of-origin of HGSOC is now known to be the Fallopian Tube (FT), the cell-of-origin remains obscure. HGSOC precursor lesions almost always arise in the distal FT, a site reported to be enriched in stem-like cells. Interrogation of HGSOC initiation has been hampered by the lack of appropriate, physiologically relevant models of the FT. Investigation of FT stem cells as the cell-of-origin of HGSOC could not be undertaken, as FT stem cells remain to be identified.

In this thesis, I show that widely-used FT-derived 2D culture is not a suitable FT model. I employ organoids as a superior model, and show that organoids emerge from a single cell to give rise to large FT-like structures composed of all expected cell types. I confirm novel cell subtypes identified by single cell RNA-seq in hFT tissue, and show that organoids impressively reproduce these findings in an *in vitro* setting, presenting an opportunity for further functional characterization.

Furthermore, I utilize organoids as a model to re-construct the stem cell niche *in vitro*. I show that organoids are propagated by rare self-renewing cells, for which Wnt activation and TGF- β inhibition are obligate requirements. For further characterization, I use lentiviral-based techniques to genetically integrate a fluorescent-based reporter of Wnt signaling into hFT organoids. Using functional and single cell transcriptomic approaches, I show that a WNT7A-FZD5 signaling axis is critical for renewal of stem cells, with WNT7A protein acting in very close range, possibly through direct physical interaction between donor and recipient cells. I find that RSPO signaling through LGR5 receptor is essential for maintenance of the WNT7A-FZD5 signaling axis. Single cell profiling shows that Wnt-active cells form a cluster of cells that are proliferative and marked by CA125, a clinical marker of HGSOC progression and response to therapy. Single cell data also show that organoids express Estrogen Receptor α . I note that Estrogen suppresses WNT7A in FT organoids, leading to pronounced differentiation and ciliogenesis. Finally, the thesis negates all of these findings for mouse oviduct stem cells, which regenerate using entirely different mechanisms, at least within the organoid setting.

Overall, this thesis proposes a first description of FT stem cells and a basic characterization of their molecular niche requirements. It lays the foundation for further studies and *in vivo* confirmation of FT stem cells, conditional on addressing inter-species differences. Findings in this thesis open the door to investigating the role of FT stem cells in HGSOC initiation.

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LIST OF ABBREVIATIONS

°C	degrees celcius
βDC	β-catenin destruction complex
μg	microgram
μl	microliter
μm	micrometer
μM	micro molar
2D culture	two-dimensional culture on plastic surface
3D	three-dimensional
ALI culture	Air-Liquid Interface culture
APC	adenomatous polyposis coli
ASC	Adult Stem Cells
bp	base pair
BSA	bovine serum albumin
CAPS	calcyphosin
CK1α	casein kinase 1α
CM	conditioned medium
CMV	cytomegalovirus
CSC	cancer stem cell
DIC	differential interference contrast
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphates
E. coli	escherichia coli
ECM	extracellular matrix (matrigel)
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EOC	epithelial ovarian cancer
ERα	Estrogen Receptor α (also called ESR1)
ESR1	Estrogen Receptor 1 (also called ERα)
FACS	fluorescence-activated cell sorting

LIST OF ABBREVIATIONS

FBS	fetal bovine serum
FF	follicular fluid
FGF-10	fibroblast growth factor-10
FISH	fluorescence <i>In situ</i> hybridization
FOXJ1	forkhead box protein j1
FRT	female reproductive tract
FSK	forskolin
FT	fallopian tube
FTE	fallopian tube epithelium
FZD	frizzled receptor
g	gravitation force
GFP	green fluorescent protein
GM	genetic modification
GSK-3 α	glycogen synthase kinase-3 α
GSK-3 β	glycogen synthase kinase-3 β
HGSOC	high-grade serous ovarian cancer
hr	hour
ICC	immunocytochemistry
IF	immunofluorescence
IgG	Immunoglobulin G
IHC	immunohistochemistry
IWP-2	inhibitor of wnt protein-2
kDa	kilo dalton
KO	knockout
KRT14	keratin 14
KRT17	keratin 17
KRT5	keratin 5
KRT7	keratin 7
L	liter
LGR5	leucine-rich repeat-containing g protein-coupled receptor 5
LGR6	leucine-rich repeat-containing g protein-coupled receptor 6
LRP5/6	low-density lipoprotein receptor-related protein 5/6

LIST OF ABBREVIATIONS

M	molar
MAPK	mitogen-activated protein kinase
Matrigel	trading name for extracellular matrix (ECM)
min	minute
miRNA	micro ribonucleic acid
ml	milliliter
Mw	molecular weight
nm	nanometer
nM	nanomolar
nmol	nanomoles
OE	over-expression
OFE	organoid-forming efficiency
OM	organoid medium
OSE	ovarian surface epithelium
P/S	penicillin / streptomycin
PAGE	polyacrylamide gel electrophoresis
PAX8	paired box gene 8
PCR	polymerase chain reaction
pmol	picomole
PR	progesterone receptor
PVDF	polyvinylidene fluoride
QPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
rpm	rounds per minute
RSPO	roof plate-specific spondin
scRNA-seq	single cell RNA sequencing
SDS	sodium dodecyl sulphate
sec	second
shRNA	small hairpin ribonucleic acid
siRNA	small interfering ribonucleic acid
STIC	serous tubal intraepithelial carcinoma
TGF- β	transforming growth factor- β

LIST OF ABBREVIATIONS

Tris	tris(hydroxymethyl)-aminomethane
U	unit
Unt	untreated
V	volts
WB	western blotting
WB	western blotting
Wnt	wingless + integrase
Wnt	wingless gene
WRE	Wnt Reporter Elements (also called TCF/LEF elements)

PREAMBLE

“Anatomy is to physiology as geography is to history” goes the saying by the French royal physician of King Henry II (Tubbs, 2015). Understanding the anatomy of an organ, or indeed the anatomical origin of a disease, is fundamental to understanding the disease’s pathophysiology and natural history. In light of this, there has been a recent emergence of scientific interest in the Fallopian tube (FT) as the tissue-of-origin of Ovarian Cancer, the 5th most common cancer in women. Ovarian cancer is one of the deadliest cancers and exhibits low survival rates, which have persisted for over four decades. Nonetheless, the FT is an organ of prime importance in its own right, owing to its role in human fertility, reproduction and the continuity of the human race. It is therefore rather peculiar that, in the 21st century molecular medicine, the Fallopian tube remains an understudied organ in comparison with other organs such as the intestines, skin and breast, which have witnessed remarkable progress in the understanding of their cellular and molecular biology. This might be attributable to the increasingly evident scientific and societal phenomenon of prioritising research into tissues from which the highest incidence or mortality diseases emerge, perhaps understandable in a world with finite resources.

Consequently, uncovering the cellular and molecular mechanisms of FT biology and homeostasis holds the key to addressing a number of important questions: How does the FT regenerate? Is there a dedicated cell type within the FT responsible for long-term homeostasis? What’s the nature of the niche supporting this cell type, and how is this cell type induced to self-renew or differentiate? Is this in response to short-range and local niche signals or long-range hormonal cues? Why do precursor lesions of Ovarian Cancer concentrate in FT regions enriched in stem-like cells? Can knowledge of FT homeostasis be exploited in personalized regenerative medicine and cell-based therapies? More generally, is FT regeneration compromised in patients with fertility disorders? How do bacterial infections, pelvic inflammatory diseases and sexually-transmitted diseases affect FT biology and homeostasis and lead to infertility? What can knowledge about FT regeneration tell us about embryonic origins and development of the FT from the Mullerian duct? The implications from understanding the mechanisms of homeostasis, regeneration and repair of the FT are manifest and render further interrogation.

In light of this, the thesis aims to shed light on the long sought-after inquiry on FT regeneration, in an attempt to better understand, at a cellular resolution, the initiation of the most common and aggressive type of ovarian cancer, termed High Grade Serous Ovarian Cancer (HGSOC). To conduct this investigation, I harness organoids as an experimental model throughout the thesis.

PREAMBLE

Organoids, or organotypic cultures, are patient-derived, three-dimensional and stem cell-driven cultures that are self-organizing and self-renewing. Organoids strikingly recapitulate *in vivo* tissue architecture and cellular diversity from many different tissue types isolated from several species (Willyard, 2015; Clevers, 2016). This is unprecedented. Never before in the history of science have we possessed the ability to re-engineer human organ-like structures in the lab, in a way that so convincingly mirrors the *in vivo* setting.

For the purpose of this thesis, which seeks to identify FT stem cells, organoids offer the major advantage of preserving stem cells, and allowing their expansion in a near native format, without the need for genetic manipulation or immortalization. Research on other tissues has demonstrated that traditional lab methods are unable to preserve and expand stem cells. In the Results chapter, I provide evidence to propose that this is also the case for the putative FT stem cell identified in this thesis. I therefore employ FT and other ASC-based organoids as an experimental model for *in vitro* reconstruction of the stem cell niche, as well as a tool for *in vitro* expansion and characterisation of FT stem cells.

Up to 90% of HGSOC patients can survive for more than 5 years, if their disease is detected at early stages. Unfortunately, over 70% of patients are diagnosed at late stages, when 5-year survival verges at a dismal 10-25%. Identification of FT stem cells may hold the key to understanding HGSOC initiation, which may help in devising a method for early detection of the disease. There is consensus in the field that early detection is currently the single most effective route towards improving survival from HGSOC, even with current treatment regimens.

However, before diving into details, it behoves us to understand the nature of adult tissue-resident stem cells, pathways that maintain them, their developmental origins and their relation to tumourigenesis. This will be the scope of the introductory chapters. The thesis is divided into chapters, each containing a number of sections and subsections. To avoid duplication of content, signposting has been used extensively to link different sections, wherever appropriate. Chapters 1, 2 and 3 are introductory and focus on the questions aforementioned. Briefly, Chapter 1 focuses on the signaling pathways that were identified (in Results) to be critical for maintenance of FT stem cells, with a section dedicated for identification of stem cells in other tissues and the lessons that can be drawn and applied to identification of FT stem cells. Chapter 2 explores the role of these stem cell pathways in embryonic development of the FT, and Results will later exploit this knowledge to formulate hypotheses and successfully identify parallels between FT development and adult stem cell biology. Chapter 3 focuses on Ovarian Cancer, the FT and relevance of the identified pathways to tumour initiation.

Chapter 4 sets out in detail the aims and objectives of the thesis. Chapter 5 is a descriptive outline of the techniques and approaches employed to test hypotheses in this project. Chapters 6, 7 and 8 constitute the Results chapters. Chapter 6 validates FT organoids as an *in vitro* model of FT biology. Chapter 7 identifies pathways necessary for maintenance of FT stem cells, and devises approaches for specific labelling, isolation and characterization of these cells. Chapter 8 interrogates the molecular signals that maintain stemness in identified putative stem cells. Finally, Chapter 9 concludes with a summary, limitations and proposed future directions for this work.

A manuscript is currently been collated for publication of this work. Excluding this and a number of local and international seminars, conference posters and contributions to grant applications, I submit that no part of this thesis has been published elsewhere.

CHAPTER 1

WNT SIGNALING, STEM CELLS
& CANCER

WNT SIGNALING, STEM CELLS & CANCER

Wnt is a portmanteau word for Wg and Int. Wg stands for Wingless, a segment polarity gene that gives rise to wingless flies when mutated in the fruit fly *Drosophila melanogaster* (Nüsslein-volhard & Wieschaus, 1980). Int-1 is the name of a genomic site called Integration-1. When Nusse & Varmus (1982) injected mice with the Mouse Mammary Tumour Virus, the virus caused breast tumourigenesis by preferentially integrating into the Int-1 locus. The nearest coding gene to Int-1 locus, which was overexpressed by virus promoters, was itself called Int-1 gene. A few years later, when Int-1 gene homologues were sought in *Drosophila*, it was realized to be the same as the Wingless gene (Rijsewijk *et al.*, 1987). Wg and Int-1 were thereafter combined in nomenclature, and the Int-1 gene became known as Wnt1. Subsequent genes of the same family were named Wnt2, Wnt3, etc. Soon after, mouse Wnt1 mRNA was documented to induce duplication of body axis in frog embryos, a property known to be due to Wnt signaling induction. This was the first indication of the highly conserved nature of Wnt signaling, even between vertebrates and invertebrates (McMahon & Moon, 1989). A few years later, APC was identified as the mutated gene in hereditary forms of colorectal cancer (Kinzler *et al.*, 1991), providing the first link between Wnt signaling and human disease.

It is observable from this brief historic perspective that Wnt signaling has associated with development and cancer almost instantly since its discovery. These two fields of scientific research continue to further and be furthered by our understanding of Wnt signaling. Today, Wnt signaling is the most important pathway in adult stem cell (ASC) biology, and arguably the most important in embryonic development of many organs. Wnt signaling plays a critical role in cell proliferation, cell fate decisions, morphogenesis, cell invasion, motility, apico-basal polarity, calcium signaling as well as in many human diseases. In this chapter, we attempt to capture a glimpse of how the pathway operates, its components, its biomarking of adult stem cells as well as its role in tumourigenesis.

1.1 Wnt Signaling

1. Canonical Wnt signaling

Like many other pathways, Wnt signaling is a ligand-activated, receptor-mediated pathway. Binding of a Wnt ligand to its cognate receptor activates a signaling cascade that culminates in β -catenin-mediated activation of Wnt target genes, which regulate proliferation, self-renewal and

differentiation. This is what is referred to as the canonical Wnt/ β -catenin signaling pathway (Figure 1).

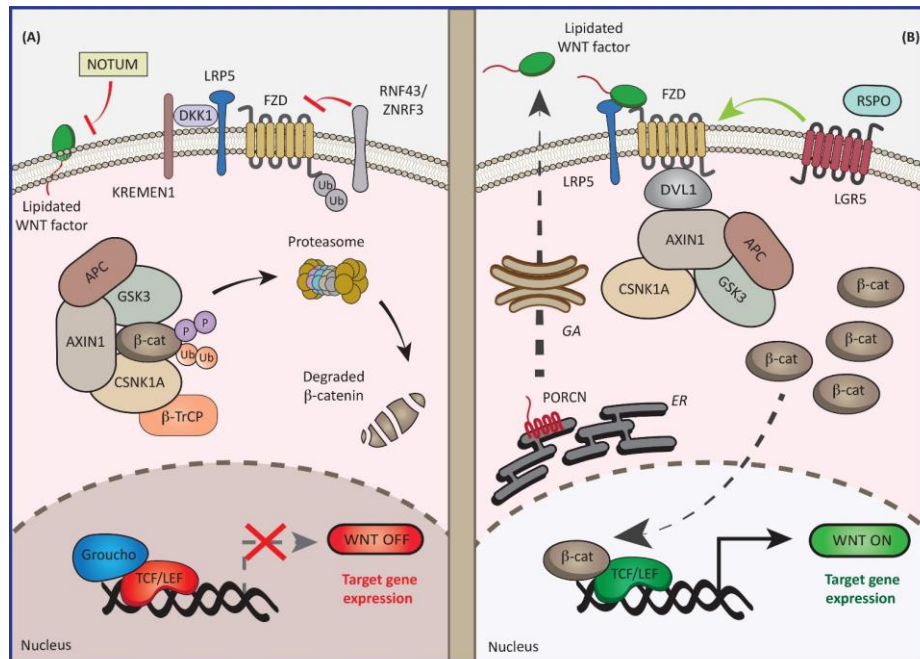


Figure 1: The Wnt/ β -catenin signaling pathway. (A) In the absence of a Wnt ligand, the β -catenin Destruction Complex (β DC) assembles efficiently to degrade cytoplasmic β -catenin, the effector of the Wnt/ β -catenin signaling pathway. At the receptor level, the E3 ubiquitin ligase RNF43 / ZNRF3 ubiquitinates FZD receptor and targets it for degradation. Dickopf-1 (DKK1) binds to and inhibits LRP5/6, an integral co-receptor in the transduction of Wnt signals. **(B)** Upon binding of a Wnt ligand to a FZD receptor, certain integral components of the pathway are recruited, preventing the β DC complex from assembling. This releases the negative regulation imposed on β -catenin, allowing its stabilization, nuclear translocation and transcriptional co-activation of Wnt target genes. R-Spondin (RSPO), another secreted protein, binds to LGR receptors (de Lau et al., 2011) and the pair inactivate the E3 ubiquitin ligase RNF43 / ZNRF3, allowing sustained FZD presence at the plasma membrane and potentiating Wnt signaling. [Image taken from: (Galluzzi et al., 2019)].

In the absence of a Wnt ligand, the Frizzled (FZD) receptor is constantly ubiquitinated and targeted for destruction. Intracellularly, APC, AXIN and the Ser/Thr kinases GSK3 and CSNK1A oligomerize to form a multiprotein scaffold (Figure 1). Termed the β -catenin Destruction Complex (or β DC for short), this scaffold facilitates the sequential phosphorylation of β -catenin, first by casein kinase alpha (CSNK1A, also called CK1 α) and then by GSK3. (Reya & Clevers, 2005). Phosphorylation of β -catenin targets a Serine / Threonine-rich conserved sequence at the amino terminal of β -catenin, generating a 3D motif called a phosphodegron. Presence of a phosphodegron motif (on any protein) confers regulation and ubiquitination by E3 Ubiquitin Ligases; in this instance this is β -TrCP (Stamos & Weis, 2013), which targets β -catenin for degradation. This leaves the TCF/LEF transcription factor complex bound to its transcriptional co-repressor Groucho, silencing expression of Wnt target genes.

Binding of a Wnt ligand to its FZD receptor and LRP5/6 co-receptor initiates Wnt signaling. The first intracellular event is the phosphorylation of Dishevelled (DVL) protein. DVL recruits GSK-3 β , followed by CK1 α , to the plasma membrane where the pair phosphorylate the cytoplasmic tail of LRP co-receptor. It is unclear how and in what sequence these kinases act, but phosphorylation of LRP creates a docking site for AXIN, which is also recruited to the plasma membrane. Cytoplasmic absence of AXIN, as well as physical sequestering of GSK-3 β and CK1 α away from the cytoplasm, essentially disintegrates the β DC complex. Cytoplasmic β -catenin is now unregulated and translocates to the nucleus, where it displaces Groucho. β -catenin acts as a transcriptional co-activator to the TCF/LEF transcription factors, and together the pair bind to TCF/LEF promoter elements located upstream of Wnt target genes, promoting their transcription. TCF/LEF promoter elements are also called Wnt Responsive Elements (or WRE for short).

Upon Wnt/ β -catenin activation, the set of genes expressed in a cell is highly context-dependent, temporally-restricted and tissue-specific, depending on the cell type and the presence of transcriptional partners of the β -catenin / TCF / LEF transcriptional machinery. However, one universal Wnt target gene is AXIN2 (Jho *et al.*, 2002). Other Wnt target genes include Cyclin D, Myc, LGR5, LGR6, hTERT and others (The Wnt Homepage, 2020). Activation of Wnt signaling is seen to equate to maintenance of stem cell potency, although strictly speaking the outcome of pathway activation can influence variable other processes and depends on other pathways. For example, Wnt signaling activation in the intestinal crypts maintains stem cells only if Notch signaling is co-activated. Upon Wnt activation and Notch inhibition, intestinal cells undergo terminal differentiation towards the Paneth cell lineage (Yin *et al.*, 2013).

2. Non-canonical Wnt signaling

The above description concerns the Wnt/ β -catenin signaling pathway, otherwise known as canonical Wnt signaling. Non-canonical Wnt signaling pathways exist, and include the Wnt/Ca²⁺ signaling pathway and the Wnt / Planar Cell Polarity (PCP) pathway. Both are independent of LRP5/6 and β -catenin. The Wnt/Ca²⁺ pathway increases intracellular calcium levels. It functions through Phospholipase C and activates Protein Kinase C and Cam kinase (Tu *et al.*, 2007), affecting cell adhesion. The Wnt/PCP pathway, on the other hand, remodels cytoskeletal networks in cells to allow coordinated and unidirectional polarization of growing epithelial cells (Katoh, 2005). Reported activators of non-canonical Wnt signaling include Wnt5a (D. Qian *et al.*, 2007) and Wnt11 (Widelitz, 2005), as well as FZD3 and FZD6 (Dong *et al.*, 2018). Unless otherwise stated, Wnt signaling in this thesis refers to the canonical Wnt/ β -catenin signaling pathway.

3. Crosstalk between canonical and non-canonical Wnt signaling

So much remains to be known about Wnt signaling, canonical and non-canonical, and how its biology operates in a tissue-specific context. It was shown, for example, that a single Wnt ligand can activate Wnt/ β -catenin signaling in one context and non-canonical Wnt signaling in another context. This is also documented for FZD receptors. Ror2, a non-canonical Wnt receptor that functions as a Receptor Tyrosine Kinase, was shown to inhibit Wnt/ β -catenin signaling upon binding to Wnt5a, but in other contexts, potentiates Wnt/ β -catenin signaling upon activation by Wnt1 or Wnt3a. Ror2 binding to Wnt5a activates proliferation (He *et al.*, 2008), which was thought to be an outcome exclusive to canonical Wnt signaling. In addition, recent data suggests that downstream targets of canonical and non-canonical Wnt signaling, which were thought to be exclusive, cross-talk and regulate each other (van Amerongen & Nusse, 2009).

Given the well-documented link between hyperactivation of Wnt/ β -catenin signaling and tumourigenesis, it is plausible to wonder if Wnt/PCP signaling can act as a tumour-suppressing pathway. Indeed, siRNA-mediated depletion of Wnt5a (Wnt/PCP activator) in mouse mammary cell lines mimics ectopic activation of Wnt/ β -catenin signaling and transformation (Olson & Gibo, 1998). Wnt5a overexpression in colon cancer cell line SW480 inhibits Wnt/ β -catenin signaling (Topol *et al.*, 2003). Wnt/PCP pathway may thus act as a tumour-suppressive pathway, inhibiting canonical Wnt signaling. Furthermore, although it's helpful to attempt to categorize the wider family of Wnt and Wnt receptors into general themes, in reality the textbook model of linear signaling nodes, and the categorization of different Wnts and FZDs into canonical versus non-canonical, may be too simplistic. In Chapter 2, we shall see an example of how Wnt7a, a classic Wnt/ β -catenin inducer, is suppressed by Wnt5a, possibly through non-canonical signaling, to govern Estrogen signaling and adenogenesis during female reproductive tract development.

1.2 Upstream Activators of Wnt Signaling

1. Wnt ligands

Wnt ligands are a 19-member family of secreted glycoproteins that function in cell-to-cell communication. At least 14 members of this family have been shown to activate Wnt/ β -catenin signaling in different contexts (Najdi *et al.*, 2012). Each Wnt ligand has a unique function, and this is exemplified by loss of functional studies. For example, Wnt1 knockout (KO) mice show developmental defects in midbrain while Wnt4 mutants display abnormalities in kidney and female reproductive tract (Nusse & Clevers, 2017).

Wnt proteins are hydrophobic in nature, contain a site for signal peptidase and a number of sites for N-linked glycosylation. Due to their essential functions, Wnt ligands are conserved across all metazoans, sufficiently enough for Wnt genes from mice to illicit pathway activation in frog embryos, or Wg gene from flies to activate Wnt signaling in mice. However, no single organism expresses the full complement of Wnt ligands. Wnts are typically 40 kDa in molecular weight and 350-400 amino acids in length, with over a quarter of residues conserved within the ligand family. On the genetic level, Wnt ligands contain 4 exons and 3 introns in roughly corresponding positions (Nusse & Varmus, 1992).

2. Wnt processing and secretion

Wnt ligands are inherently local short-range messengers, but can exhibit long-range signaling if complexed with chaperones or other carrier proteins. The best characterized Wnt ligand is mouse Wnt3a, the only Wnt ligand, in addition to *Xenopus* Wnt8, to be isolated in its active form containing important post-translational modifications. Wnt ligands possess glycosylation sites that were shown to be critical for Wnt secretion. In mouse Wnt3a, these N-linked glycosylation sites are at Asparagines 87 (MacDonald *et al.*, 2014) and 298 (Komekado *et al.*, 2007). Absence of glycosylation inhibits Wnt3a secretion. However, removal of glycosylation from already secreted Wnt protein does not affect its activity (Nusse & Varmus, 1992). Wnt7a is the only expressed Wnt ligand in oviduct embryonic development (Miller & Sassoon, 1998; see Figure 14) and FT organoids (this thesis), and the most robustly expressed Wnt ligand in fresh FT epithelial cells (Hu *et al.*, 2020). I document in this thesis that secreted Wnt7a is also heavily glycosylated (Chapter 8, Figure 61).

Another important Wnt post-translational modification is palmitoylation, which happens on a single Serine residue identified as Serine 209 in Wnt3a (MacDonald *et al.*, 2014). 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, and is catalysed by Porcupine (PORCN), a membrane bound O-acyltransferase (Mikels & Nusse, 2006). Wnts are thereafter carried to the plasma membrane by the carrier protein Wntless (WLS), which recognizes only palmitoylated Wnts (Najdi *et al.*, 2012). Mutation or knockdown of PORCN or WLS block secretion of endogenous Wnts (MacDonald *et al.*, 2014). In fruit flies, the wingless phenotype caused by loss of Wg (Wnt1 homologue) can be mimicked by loss of PORCN (Clevers, 2006).

It remains unknown why Wnt secretion is so dependent on palmitoylation. It is hypothesized that palmitoylation is required for tethering Wnt ligands to membranes, enabling further glycosylation and thereafter secretion. Wnt palmitoylation is also thought to be important in engaging FZD receptors at the biochemical level (Nusse & Clevers, 2017). In mice intestines and derived organoids, fluorescently-labelled Wnt3a was shown to tether to the plasma membrane and travel from Wnt-producing to Wnt-receiving cells in a membrane-bound manner, not by diffusion (Farin *et al.*, 2016). This membrane-based transport requires Wnt hydrophobicity, possibly conferred by palmitoylation. I show data in this thesis that suggests that WNT7A, responsible for maintaining FT stem cells, also signals through membranes, and is only functional if producing and receiving cells are in very close proximity, possibly through direct physical interaction (Chapter 8, Figure 60). I show that in human FT tissue, WNT7A-producing and Wnt-active cells are in close proximity. Furthermore, exogenously supplied WNT7A cannot rescue the inhibition of WNT-blocked FT organoids, indicating that FT organoids are primed to utilizing only membrane-bound Wnt ligands.

Removal of the palmitate moiety from Wnts after their secretion, using acyl protein thioesterase, abolishes protein activity (Schulte *et al.*, 2005). This can be rescued by overexpression (Noordermeer *et al.*, 1995), suggesting that high protein concentration can overcome lack of activity of unpalmitoylated Wnt. The palmitate moiety could therefore function by increasing Wnt's hydrophobicity and allowing its concentration in a hydrophilic environment. The role of PORCN palmitoylation of Wnt ligands and its interesting essentiality for Wnt secretion and activity remains a subject of investigation. In this thesis, I employ potent and selective inhibitors of PORCN as a means to block WNT secretion in hFT organoids, in which WNT7A is the only expressed canonical WNT ligand. I find that this compromises human FT organoid regeneration, but not regeneration of mouse oviduct organoids. Given that Wnts are oncogenes and are involved in initiation and metastasis of various cancers, blocking Wnt secretion is an opportune anti-cancer therapy (Xiangqian Zhang & Hao,

2015; Liu *et al.*, 2013) and PORCN inhibitors are currently under clinical trials (NIH, 2020). Although proving useful, *in vivo* studies show that PORCN inhibitors trigger a number of side-effects, particularly affecting osteogenesis (which is Wnt dependent) and leading to loss of bone density and strength (Funck-Brentano *et al.*, 2018)

3. Importance of Cysteine residues in Wnts

A striking feature of Wnt ligands is the presence of 24 conserved cysteine residues that are evenly spread out. These cysteine residues create 11 pairs of inter-cysteine disulphide bonds that are crucial for establishing the 3D structure of Wnt ligands (Figure 2B). With few exceptions, the last cysteine residue is generally 1-2 amino acids away from the last residue in Wnt ligands. When this 24th Cysteine (C24) residue is mutated to Tryptophan, Wnt1 protein loses its ability to transform cells, so this residue is important for Wnt ligand function (Nusse & Varmus, 1992). In fact, cysteine to alanine mutagenesis of each cysteine residue in the prototype Wnt ligand (mouse Wnt3a) was shown to either block Wnt secretion or inhibit Wnt activity, with variable phenotype severities. The third cysteine in Wnt ligands (C77 in mouse Wnt3a) was studied extensively, as it was originally thought to be the site of palmitoylation by PORCN, which is now known to be Serine 209. S209A mutagenesis blocks Wnt secretion, while C77A mutagenesis generates a mature Wnt ligand with significantly reduced activity (MacDonald *et al.*, 2014).

Structurally, Wnt ligands are composed of a number of domains, including an amino terminal, a linker domain, a cysteine knot domain and three hairpin domains (Figure 2B). Hairpin 2 (thumb) and hairpin 3 (index finger) domains create protruding 'thumb and finger' β -strands that engage FZD receptors and initiate Wnt signaling. Cysteine-to-alanine (C-to-A) mutagenesis in the Hairpin 1 domain are well-tolerated and have a mild effect on Wnt secretion and activity. C-to-A mutations in the index finger domain do not affect secretion, but produce Wnt proteins with reduced activity. Thumb domain and cysteine knot domain C-to-A mutagenesis, however, abolish Wnt secretion. In general, single cysteine mutations have a more severe phenotype than mutation in paired cysteines (MacDonald *et al.*, 2014), possibly due to unpaired cysteines acting in a dominant negative manner and generating ectopic disulphide bonds.

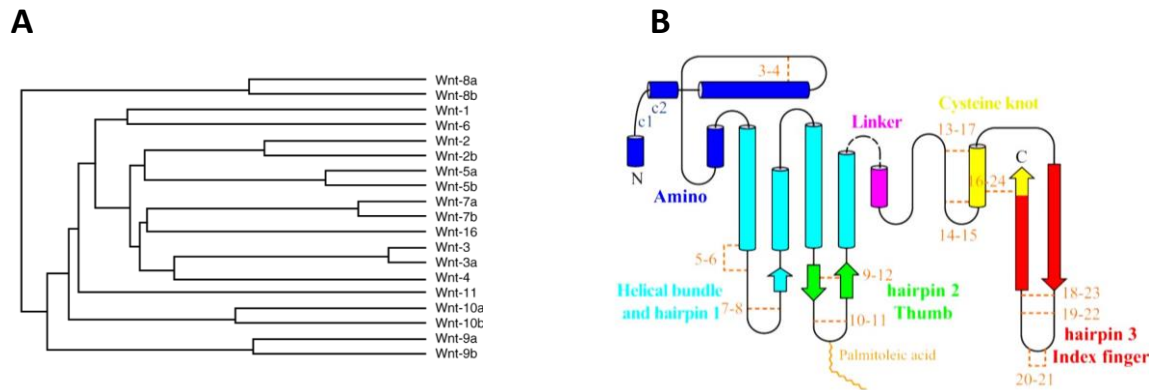


Figure 2: Structure and relatedness amongst Wnt ligands. (A) Phylogenetic tree showing evolutionary relatedness of mouse Wnts based on Clustal W multiple alignments of amino acid sequences. **(B)** Typical structure and domain organization of Wnts, showing distinctive α -helices (barrels) and β -sheets (lines). The structure shown here is that of *Xenopus* Wnt8. Orange numbers are cysteine residues; dotted lines indicate disulphide bonds between cysteine pairs [(A) adapted from (MacDonald *et al.*, 2014); (C) taken from (Qian *et al.*, 2003)].

An interesting observation on Wnt secretion is that, upon transfecting wild type Wnt3a construct into HEK cells, monomeric Wnt3a is detected in conditioned medium, but also a small proportion of high molecular weight oligomeric Wnt3a (Xinjun Zhang *et al.*, 2012). Cysteine-mutant Wnt3a constructs have an elevated level of oligomeric Wnt3a (MacDonald *et al.*, 2014). It is proposed that, prior to secretion, Wnt ligands undergo constant re-shuffling of cysteine disulphide bonds until the correct configuration is identified. This process may not be 100% efficient in wild type Wnts, and is much less efficient in cysteine mutant Wnts when there is a free unpaired cysteine that acts in a dominant negative fashion. Addition of an ectopic cysteine to an otherwise wild type sequence shows the same phenotype (Najdi *et al.*, 2012), corroborating this notion.

Wnt ligands may undergo longer-range transport and stabilization by tethering within transport vesicles containing lipoprotein particles, or by the action of GPI-anchored Heparan Sulfate Proteoglycans (Lin, 2004). However, the latter was shown only in *Drosophila*. Notum is a secreted deacylase that negatively regulates Wnt ligands by removing PORCN-attached lipid modification, effectively deactivating Wnt ligands (shown earlier, Figure 1A). Dickkopf-1 (DKK1) is another soluble factor that inhibits Wnt signaling, by binding and inhibiting the LRP5/6 co-receptor, not by interacting with Wnts directly (Najdi *et al.*, 2012).

4. Frizzleds (FZD) are receptors for Wnt ligands

Frizzleds (FZD) are the natural receptors for Wnt ligands. A ten-member family (FZD1-FZD10 in vertebrates), these receptors transduce canonical and noncanonical Wnt signaling. Structurally, FZDs are seven transmembrane domain G-protein coupled receptors. FZD-mediated G protein activity is only seen in noncanonical Wnt signaling, and evidence for this in canonical Wnt/ β -catenin signaling is seen in *Drosophila* (Katanaev *et al.*, 2005) but largely lacking in humans and mice.

FZDs contain a Cysteine-Rich Domain (CRD) at the N-terminal extracellular side of the receptor (MacDonald *et al.*, 2014; see Figure 3A). CRD domain is 120 amino acids long and contains 10 cysteine residues. Structural analysis of CRD-bound Wnt reveals multiple interaction points, most notably a deep hydrophobic pocket that binds to the palmitate modification on Wnt ligands, as well as an interaction point between Wnt's C-terminus and FZDs CRD (Janda *et al.*, 2012). FZD's CRD domain binds Wnt ligands with high affinity. Mutating these cysteines does not abolish FZD activity, suggesting the CRD domain functions to capture Wnt ligands, while other FZD domains relay signaling (Mikels & Nusse, 2006). A clear candidate for signal relay is the KTxxxW motif (Figure 3A), which is a binding site for PDZ domain-containing proteins such as Dishevelled. Mutation of the KTxxxW motif abolishes FZD function and Wnt/ β -catenin signaling (Cong *et al.*, 2004). The CRD domain exists in other receptors as well, for instance Ror2, which as discussed binds Wnt5a to inhibit Wnt/ β -catenin signaling. The CRD domain is also part of the secreted factor called Soluble Frizzled-Related Protein (or SFRP). As the name suggests, SFRP acts as a mock to compete with FZD receptors for Wnt binding, and as such is a negative regulator of the pathway (Nusse & Clevers, 2017). In this thesis, I identify FZD5 as the transducer of WNT7A-induced β -catenin signaling in FT stem cells. I employ novel, selective antibodies targeting FZD5-CRD domain as a means to inhibit FZD5 signaling (Steinhart *et al.*, 2017), and find that this abolishes FT organoid self-renewal.

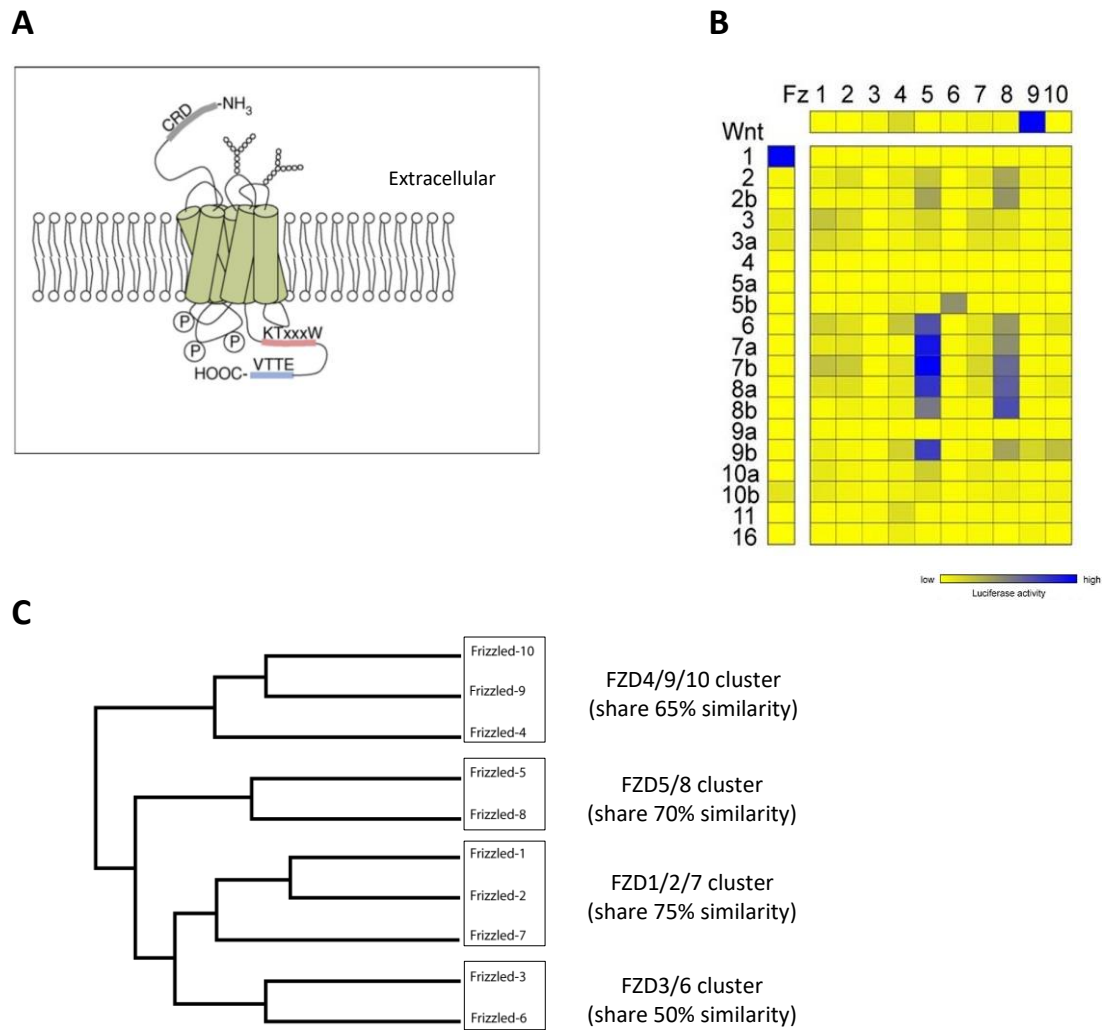


Figure 3: Frizzled (FZD) receptor structure and activation of Wnt/ β -catenin signaling. (A) Overall structure of FZD receptors. CRD is the Cysteine-Rich Domain to which Wnt ligands bind via their lipid moieties. Y-like structures on the extracellular side of FZD are putative glycosylation sites, thought to be important for FZD processing and maturation. Upon activation, FZD receptors heterodimerize with LRP5/6 co-receptors and recruit Dishevelled via the KTxxxW motif, shown in red. This leads to disassembly of the β DC complex, stabilization of β -catenin and activation of Wnt signaling. (B) Wnt activation as measured by pairwise co-expression of a Wnt ligand and a FZD receptor, as well as LRP co-receptor. This was done in technical triplicates, using HEK cells expressing Luciferase under WRE elements. Note the strong Wnt signal generated by WNT7A and FZD5 pair.

This data, taken from (Yu et al., 2012), normalizes Wnt+FZD+LRP signal relative to Wnt+LRP and FZD+LRP signal, introducing a bias against Wnts endogenously expressed in HEK cells. This bias can be surmounted by a similar analysis on HEK cells with a pan Wnt and FZD CRISPR knockout background. (C) Phylogenetic tree showing clusters of FZD receptors and overall intra-cluster similarity. [(A) adapted from: (Schulte & Bryja, 2007); (B) taken from (Yu et al., 2012)].

FZD KO mice display a multitude of developmental defects. Like Wnts, these are unique phenotypes highlighting the importance of the mutated FZD to the development of a specific tissue. Some redundancy in KO phenotypes is seen, particularly between FZDs with higher sequence similarity (Figure 3C). The fourth cluster is composed of FZD3 and FZD6, which share 50% similarity and are seen as the divergent FZDs from this receptor family. FZD3 and FZD6 participate in non-canonical Wnt signaling (Dong *et al.*, 2018), and their expression is detected in fresh FT tissue and organoids (this thesis). The only other FZD receptor expressed in FT fresh tissue and organoids is FZD5, widely reported to signal through the canonical Wnt/ β -catenin pathway and a reported receptor for Wnt7a (Yoshioka *et al.*, 2012; Najdi *et al.*, 2012). Using single cell analysis of patient-derived organoids, this thesis proposes that a WNT7A-FZD5 signaling axis drives Wnt signaling and stem cell self-renewal in the human FT.

However, it is fair to say there is some cross-reactivity between Wnt ligands and FZD receptors, and no one Wnt ligand can be seen to have a dedicated FZD receptor, and vice versa. In fact, some FZDs were shown to auto-activate, independently of Wnts, by dimerization via their CRD domains (Kaykas *et al.*, 2004). To add to this complexity, some Wnts have been documented to oligomerize and activate higher order complexes containing more than one FZD receptor (Tao *et al.*, 2019; Chen *et al.*, 2020). So in theory, a Wnt homodimer can bind to two different FZD receptors, one activating Wnt/ β -catenin-dependent proliferation while the other activating the Wnt/PCP pathway, to direct growth of an epithelial sheet towards a required direction. Once this is coupled with the potential cytoplasmic cross-talk between canonical and non-canonical Wnt signaling downstream of ligand-receptor interactions, one can begin to appreciate the complex nature of this pathway and the difficulty in predicting its outcome based solely on Wnt-FZD pair interactions.

5. R-Spondins potentiate Wnt signaling

FZD receptors are subject to constant turnover and proteasomal degradation by the action of the RNF43/ZNRF3 ubiquitin ligases, which multi-ubiquitinate lysine residues on the cytoplasmic loops of FZDs (Hao *et al.*, 2012). Loss of these ubiquitin ligases in mouse intestines leads to hyper-expansion of Wnt-dependent crypts (Koo *et al.*, 2012), due to sustained presence of FZD receptors augmenting Wnt signaling. Roof-plate specific Spondin (R-Spondin or RSPO for short) are secreted ligands that inhibit the action of RNF43/ZNRF3 ubiquitin ligases, and allow FZD receptors to persist on the plasma membrane and sustain Wnt signaling. RSPOs were so called due to their specific expression in the roof plate of the neural tube of developing mouse embryos (Jin & Yoon, 2012). RSPOs were first shown to augment Wnt signaling in *Xenopus* embryos (Kazanskaya *et al.*, 2004), and later shown to

phenocopy the loss of Rnf43/Znfr3 ubiquitin ligases in intestinal crypts, both in mice (K. A. Kim *et al.*, 2005a) and organoids (Barker *et al.*, 2010a).

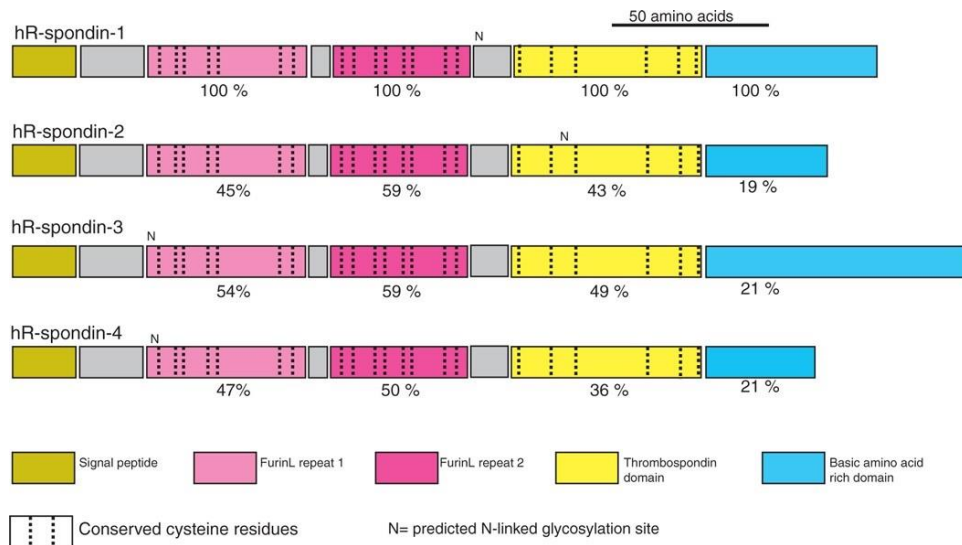


Figure 4: Domain organization of human R-Spondins. Each domain is encoded by a separate exon. Four R-Spondins (RSPO1-4) were detected in vertebrate genomes. RSPOs are composed of two N-terminal Furin domains and a thrombospondin domain. Furin 1 domain binds the extracellular side RNF43/ZNRF3 ligases (Hao *et al.*, 2016), while Furin 2 binds LGR4-6 (see below). Dotted lines indicate cysteine residues. Cysteine positions are largely conserved between RSPO family members. Structural analysis revealed that 5 cysteine residues, three in FurinL repeat 1 domain and two in FurinL repeat 2 domain, were free and not in disulphide bonds. Mutations in some of these cysteines block RSPO secretion or reduce its activity (Li *et al.*, 2009), implicating these residues in ligand-receptor interactions. Furin domains participate in Wnt/ β -catenin signaling. Domain sequence similarity is shown as a percentage compared to RSPO1. Pairwise sequence comparisons between RSPOs reveals an overall 40-60% similarity. [Taken from: (de Lau *et al.*, 2012)].

RSPOs contain five protein domains, each encoded by a separate exon (Figure 4). Of note, the Thrombospondin (TSR1) domain is what categorizes RSPOs under the TSR1 family of proteins, of which there are 41 members in the human genome. All TSR1 proteins are secreted ligands or transmembrane proteins with TSR1 domain located extracellularly (de Lau *et al.*, 2012). As key regulators of Wnt signaling, RSPOs have been identified as important players in embryonic development, ASC biology and many of the processes that Wnt signaling regulates. Furin repeats of RSPOs were shown to be critical and sufficient to potentiate Wnt signaling (Li *et al.*, 2009). The mouse mammary tumour virus screens, mentioned at the outset of this chapter, were shown to integrate near and overexpress RSPOs, leading to mammary tumours in mice (Lowther *et al.*, 2005) similar to the ones documented for Wnt1. As we shall see in chapter 2, RSPO1 cooperates with Wnt4 to initiate female reproductive tract specification during development.

6. LGRs are R-Spondin receptors

For some time, it has remained elusive which receptor mediates the RSPO-driven potentiation of Wnt signaling. Several studies have claimed that RSPOs activate FZD receptors, LRP co-receptors or interact directly with Wnts. Conclusively, a number of reports have shown, by direct biochemical and *in vivo* evidence, that LGRs are the receptors for RSPOs (Carmon et al., 2011; Glinka et al., 2011). LGR stands for Leucine-rich repeat-containing G protein-coupled Receptor. This family of receptors are characterized by a large ectodomain and seven transmembrane domains (Figure 5). Members of this family are categorized into three subfamilies. The first includes receptors for Follicle stimulating hormone (FSH), Thyroid stimulating hormone (TSH) and Luteinizing hormone (LH), the second includes LGR4-6 and the last represented by LGR7 and LGR8 (Hsu et al., 2000). In this thesis, LGRs refer to LGR4-LGR6 subfamily.

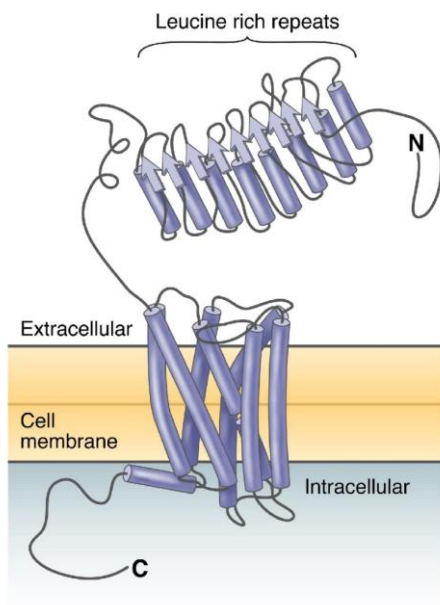


Figure 5: General structure of LGR4-6 receptors. LGR4-6 were identified as RSPO receptors. They are seven transmembrane pass receptors. LGR4-6 are characterized by a large ectodomain composed of Leucine-rich repeats (18 in LGR4 and LGR5; 13 in LGR6). Each repeat is 24 amino acids long. The ectodomain is flanked by N-terminal and C-terminal Cysteine-rich sequences. [Taken from: (Barker & Clevers, 2010)].

Using the Wnt-reporter TOPFlash assay (section 5.11), it was shown that RSPO's enhancement of Wnt signaling could be abolished by LGR4 knockdown using siRNAs. LGR4 is the only LGR endogenously expressed in HEK cells. This knockdown can be rescued by ectopic expression of LGR4, LGR5 or LGR6, but not other family members (de Lau et al., 2012). Like FZD receptors, even though LGRs are G protein-coupled receptors, there is no involvement of G-proteins in this signaling axis (Carmon et al., 2011). RSPO is thought to bind LGRs using one of its Furin repeats, with the other Furin repeat recruiting E3 ubiquitin ligases RNF43 and ZNRF3. This forms a complex that is internalized and therefore physically sequesters ubiquitin ligases away from FZD receptors. As

aforementioned, this allows sustained presence of FZD receptors and increases Wnt signaling intensity and duration (Nusse & Clevers, 2017).

Interestingly, although RSPO/LGR signaling is theoretically an augmenter and not activator of Wnt signaling, its absence inactivates Wnt signaling due to the high turnover of FZD receptors. This was shown for mouse intestines, in which Wnt/FZD signaling alone is not sufficient for self-renewal of LGR5+ stem cells (Yan *et al.*, 2017). This is also the case for intestinal organoids (Sato *et al.*, 2009). The emerging understanding is that Wnt ligands prime stem cells to express LGRs. LGRs then potentiate the Wnt signal to allow stem cell expansion and self-renewal. I document in this thesis that endogenous WNT7A, the only canonical WNT ligand expressed in hFT organoids, is necessary but not sufficient for expansion of putative FT stem cells in organoids. RSPO1 supplementation is essential, and withdrawal of RSPO1 abolishes organoid regeneration. On the other hand, RSPO1-supplemented organoids fail to expand if endogenous WNT7A signaling is blocked by the pan-WNT inhibitor LGK-974 (this thesis). This illustrates the general concept that cooperativity between Wnt ligands and RSPOs is essential for driving Wnt signaling and stem cell renewal.

The discovery of the LGR family, particularly LGR5 and LGR6, revolutionized our understanding of gut and ASC biology at large. Over the last decade, LGR5 and LGR6 were shown, using *in vivo* lineage tracing and mouse KO studies, as well as organoid-based research, to be highly specific markers of tissue-resident stem cells in several organs. This will be the subject of discussion in section 1.4. So far, we have considered positive regulators of the Wnt signaling pathway. In the next section, we will explore few of the most important intracellular regulators of the pathway.

1.3 Downstream Regulators of Wnt Signaling

1. APC is a classic tumour-suppressor

APC stands for Adenomatous Polyposis Coli. APC is one of the best characterised tumour-suppressor genes. As a critical member of the β DC complex, APC is a negative regulator of the Wnt/ β -catenin signaling pathway. It was initially identified by genetic linkage analysis in patients suffering from Familial Adenomatous Polyposis or FAP (Bodmer *et al.*, 1987). Germline or sporadic inactivation of the APC gene is now recognized to be the cause of colorectal cancer, the second most common cancer worldwide.

APC is functionally pleiotropic and promiscuous in its interactions with other proteins (Figure 6). Inactivating APC mutations tend to be non-sense, producing a truncated APC protein devoid of the β -catenin, AXIN2 and microtubule binding domains. Truncated APC is unable to negatively regulate β -catenin by direct binding or through the β DC complex. The result is stabilization, accumulation and nuclear translocation of β -catenin, hyperactivating Wnt signaling and initiating tumourigenesis.

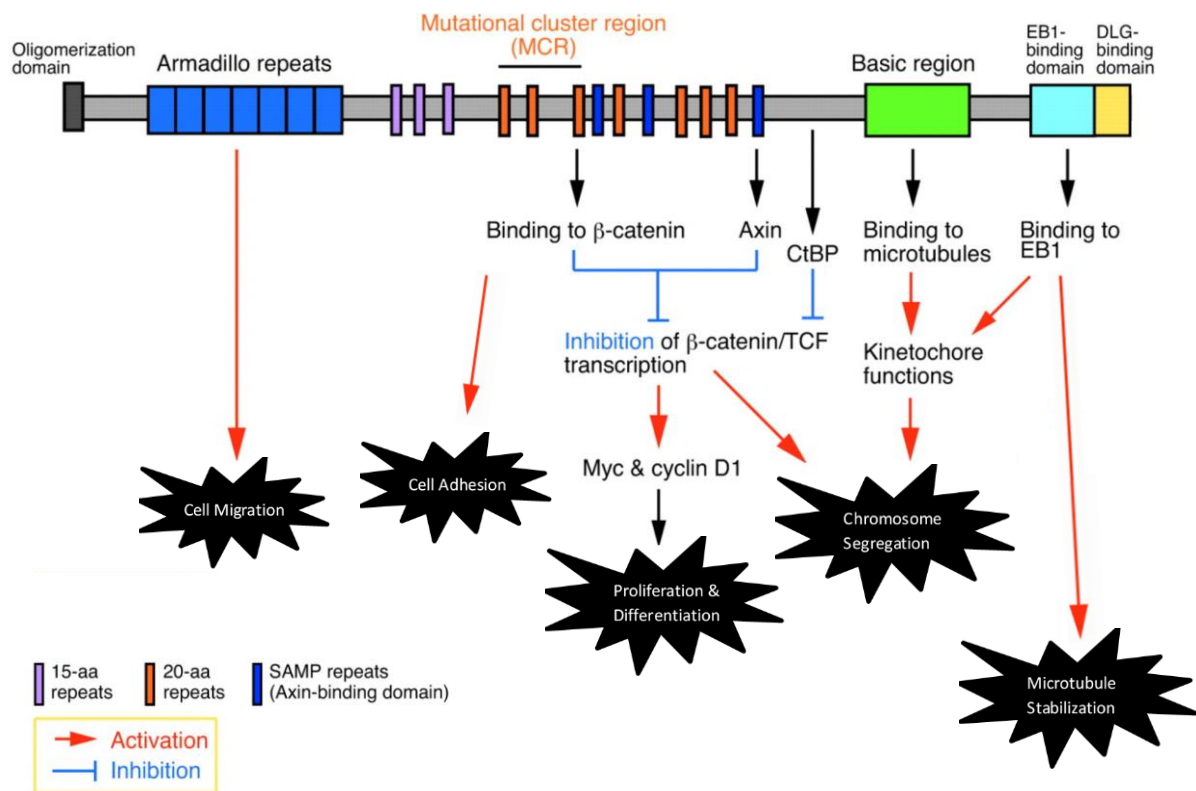


Figure 6: APC protein structure. The pleiotropic nature of APC function is instantly apparent from its structure. Armadillo repeats also exist in β -catenin protein and function in cell adhesion and migration. APC binds β -catenin directly through its 15 and 20 amino acid repeats. Only the 20 amino acid repeats, upon phosphorylation by GSK3, are able to downregulate β -catenin (Fearhead et al., 2001). APC efficiently shuttles β -catenin on to or off the β DC complex (Clevers, 2006), occupying a unique node in the Wnt/ β -catenin pathway. Independently of the β DC complex, APC negatively regulates Wnt signaling by binding β -catenin directly and preventing its nuclear translocation (Ha et al., 2004), or by facilitating CtBP-mediated repression of Wnt target genes (Sierra et al., 2006).

APC also plays a role in microtubule and chromosome segregation. APC binds microtubules to regulate their polymerization and catastrophe. During mitosis, the End Binding protein 1 (EB1) primes APC to bind to the + ends of microtubules, facilitating chromosomal capture to mitotic kinetochore. The same two domains are hypothesized to empower APC to act as a master regulator of apico-basal polarity in epithelial cells (Lesko et al., 2015). Inactivating non-sense mutations of APC tend to strike a short region in APC, called the Mutation Cluster Region (MCR), producing a truncated APC protein devoid of all domains C-terminus of the MCR. [Figure adapted from: (Aoki & Taketo, 2007)].

Although this is the traditionally accepted model of colorectal cancer initiation, research is now demonstrating that, for many tumour-initiating cells, acquiring a selective growth advantage is not sufficient for tumour initiation. This has to be coupled with genetic or genomic instability for tumourigenesis to progress (Fodde et al., 2001). APC mutation achieves this efficiently, as absence of its Basic and EB1-binding domains compromises chromosome capture during mitosis, establishing a route to genomic and chromosomal instability. In a sense, APC is a dual gatekeeper and caretaker

tumour-suppressor gene. No wonder the penetrance of its mutation in tumourigenesis is relatively high: 95% in FAP patients and 80% in sporadic colorectal cancer.

2. AXIN is the rate-limiting factor of Wnt signaling

The AXIN protein family is a rather small one, comprising AXIN1 and AXIN2 (which is also called AXIL or Conductin). Both AXINs contain, in their amino acid sequences, binding sites for LRP5/6, Dishevelled, APC, GSK3, CK1 α and β -catenin (Salahshor & Woodgett, 2005). AXIN binds APC through APC's SAMP repeats (Figure 6). Although AXINs are only 57% similar at the protein level, they perform similar functions. In fact, when AXIN2 is knocked into a mutationally inactivated AXIN1 locus *in vivo*, AXIN2 can rescue AXIN1-deficient phenotypes (van Amerongen & Berns, 2006), but not always.

Like APC, AXIN plays an important role in scaffolding the β DC complex. It is unclear why the β DC complex requires two scaffolding proteins. It is plausible to suggest that they perform different but sometimes overlapping functions. While APC binds to and shuttles β -catenin on and off the β DC complex, AXIN recruits and positions the two kinases, GSK3 and CK1 α , in the correct 3D spatial arrangement in preparation for β -catenin entry and subsequent phosphorylation. Interestingly, cancer cells that lack APC can restore the activity of the β DC complex if AXIN is ectopically overexpressed (Lee *et al.*, 2003). Similarly, AXIN mutants lacking the APC-binding domain can still downregulate β -catenin, but only when overexpressed (Nakamura *et al.*, 1998). These observations imply that AXIN may be the limiting factor of the pathway, in contrast to other more abundant proteins such as GSK-3 β and CK1 α .

Stabilizing AXINs can be an effective way of counteracting elevated Wnt signaling in tumour cells. XAV-939 is a small molecule inhibitor of the poly-ADP-ribosylating enzymes called Tankyrases. Tankyrases destabilize AXIN and encourage their ubiquitination and degradation (Huang *et al.*, 2009). As such, XAV-939 inhibits Tankyrases, which stabilize AXIN to downregulates Wnt signaling. This was shown to be effective in reducing elevated Wnt signaling in cells from multiple cancer types (Tian *et al.*, 2013; Wu *et al.*, 2016; C. Li *et al.*, 2018). In this thesis, I validate XAV-939 as an effective inhibitor of Wnt signaling, and use it to show that renewal of FT stem cells is arrested upon inhibiting Wnt signaling by AXIN stabilization.

It is important to note that AXIN1 and AXIN2, though executing the same functions, are triggered via different stimuli. AXIN1 is thought to be the constitutively expressed AXIN, and is part of the β DC complex that maintains low Wnt signaling in the basal state. Upon Wnt ligand – FZD

receptor pathway activation, AXIN1 is destabilized and degraded. AXIN2 is now upregulated in response, and serves to act in a negative regulatory loop (Salahshor & Woodgett, 2005). This negative feedback loop is presumably a safety net that prohibits cells acquiring pathologically high levels of Wnt signaling that can lead to tumorigenesis. Although activation of Wnt signaling leads to upregulation of a Wnt target gene set, depending on cell types, AXIN2 is a universal marker of Wnt signaling activation in human and mouse cells (Boonekamp et al., 2021). Like many Wnt-target genes, AXIN2 promoter contains eight TCF/LEF elements to which β -catenin/TCF transcriptional complexes bind to activate AXIN2 expression. Targeted mutagenesis or deletion of these elements abolishes AXIN2 expression in response to Wnt activation (Jho *et al.*, 2002). This makes AXIN2 a useful marker of Wnt-active cells *in vitro and in vivo*, as well as a marker of ectopic pathway activation using small molecule inhibitors.

In this thesis, I genetically integrate a fluorescent Wnt reporter into human and mouse FT organoids, and show that endogenous Wnt-active cells (that drive organoid regeneration) have elevated AXIN2 levels. This is also the case for FT organoids treated with Wnt activators. *In vivo*, Axin2 can mark Wnt-active cells, which were shown to be stem cells in several solid tissues (section 1.4).

3. GSK3 and CK1 α are the catalytic core of the β DC complex

GSK3 kinases constitute GSK-3 α and GSK-3 β . Pairwise sequence comparison of their NCBI's RefSeq sequences reveals these kinases share around 72% similarity in amino acid sequence. They are largely redundant in their functions, but not always. GSK3 has a simple structure: a kinase domain and a substrate-binding domain. (Beurel *et al.*, 2015). In general, GSK3 phosphorylates already phosphorylated/primed substrates, or less frequently substrates with an acidic residue in place of the chemically similar phosphorylated residue. This is the case for β -catenin, which is primed for GSK3 phosphorylation by Casein Kinase 1 alpha (CK1 α) kinase.

CK1 α and GSK3 kinases are the catalytic components of the β DC complex. In the absence of a Wnt ligand, GSK3 phosphorylates APC on its 15 and 20 amino acid repeats, increasing APC's affinity to β -catenin (shown earlier, Figure 6). CK1 α and GSK3 also phosphorylate AXIN to increase its binding affinity to β -catenin. This is important to stabilize AXIN, as unphosphorylated AXIN has a much lower affinity for β -catenin (Yamamoto *et al.*, 1999). In fact, a phosphatase exists (PP2A) to reverse and antagonize this GSK3-mediated stabilization of AXIN, dephosphorylating and destabilizing AXIN to positively activate Wnt signaling (Ratcliffe *et al.*, 2000).

GSK3 is not able to bind β -catenin directly *in vitro*. In the presence of AXIN, GSK3's affinity to β -catenin is 20,000-fold higher (Hart *et al.*, 1998). Once the APC-AXIN-kinases destruction complex is in place, CK1 α first phosphorylates β -catenin at Serine 45. GSK3 then phosphorylates β -catenin at Threonine 41, Serine 37 and Serine 33 residues. Phosphorylation of these 4 residues is critical for β -catenin degradation. It creates the phosphodegron, which acts as a docking site for β -TrCP E3 ubiquitin ligase, targeting β -catenin for subsequent proteasomal degradation.

Thus, in the absence of Wnt activation, GSK3 and CK1 α act as negative regulators of Wnt signaling. Upon Wnt activation, interestingly, the pair act in a positive regulatory capacity. They are recruited by Dishevelled to the plasma membrane, where they phosphorylate LRP5/6 co-receptor and create a docking site for AXIN. The result is the sequestering of AXIN away from the cytoplasm, followed by stabilization of β -catenin and activation of the pathway.

Inhibition of GSK3 is a rather effective way of Wnt activation *in vitro*. A number of inhibitors exist for this. The most potent and selective is CHIR99021, exhibiting IC₅₀ values of less than 10 nM for both GSK-3 α and 3 β , and over 500-fold higher IC₅₀ for closely related kinases. CHIR99021 is now an important medium component in promoting stem cell self-renewal of human and mouse embryonic stem cells, as well as in inducing and maintaining pluripotency of iPSC cells (Ye *et al.*, 2012; Ring *et al.*, 2003; W. Li *et al.*, 2009). CHIR99021 is harnessed in this thesis as an activator of Wnt signaling in organoids, to test if functional interference with stem cell pathways, leading to organoid growth inhibition, can be rescued by downstream activation of the pathway.

4. β -catenin is the nuclear effector of Wnt signaling

β -catenin is the ultimate effector of the Wnt signaling pathway. It executes the nuclear functions of the pathway by co-activating the transcription of Wnt target genes containing the WRE elements. It is important to note that there are two pools of β -catenin in a given cell: the structural pool and the signaling pool. These pools of β -catenin are separate and do not intermingle. In fact, these functions are encoded by separate proteins in *C. elegans* (Korswagen *et al.*, 2000). In the structural pool, β -catenin is a component of cell-to-cell adherens junctions. It associates with the cytoplasmic tail of E-cadherin to anchor the exterior of a cell to its internal actin network (Clevers & Nusse, 2012). β -catenin shields a PEST region of E-cadherin which, if exposed, leads to E-cadherin ubiquitination and degradation. E-cadherin shields β -catenin against its destruction complex (Valenta *et al.*, 2012). As such, the pair stabilize each other. Structural adhesion function of β -catenin is embodied in its name; catenin is a Latin word meaning 'chain', which is the historical view of β -catenin as a cell-to-cell anchoring protein.

The catenin family of proteins is composed of α -catenin, β -catenin, γ -catenin (also called plakoglobin) and p120-catenin. With the exception of α -catenin, all members of the catenin family contain the characteristic Armadillo repeats in their amino acid sequence (Figure 7A). Mutations in one Armadillo repeat at a time compromise either β -catenin's structural or signaling function, not both, suggesting each repeat is earmarked for one function (Orsolic & Peifer, 1996).

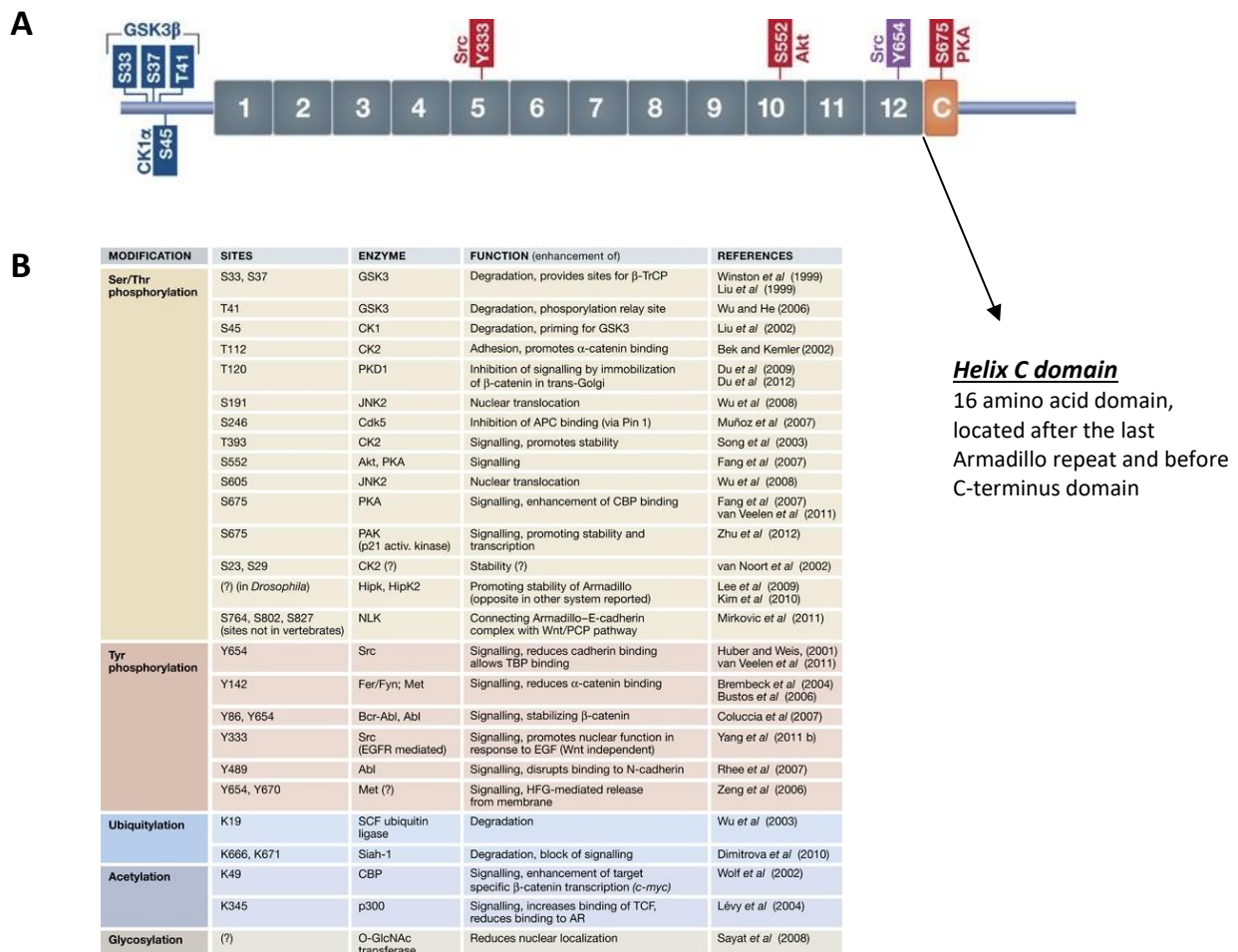


Figure 7: Primary structure of β -catenin protein. (A) β -catenin is composed of three main domains. The N-terminal domain hosts S33, S37, T41 and S45 residues that are phosphorylated by CK1 α and GSK3. This creates the phosphodegrom motif that is eventually recognized by β -TrCP and targets β -catenin for degradation. β -catenin with a deletion in the first 45 amino acids is free from regulation by the destruction complex. This constitutively active β -catenin is employed in this thesis to activate Wnt signaling in organoids (Results, Figure 52-53). The central domain (shown in numbers 1-12) is composed of Armadillo repeats, first identified in *Drosophila armadillo* protein, a homologue of mammalian β -catenin. Armadillo repeats are each composed of 42 amino acids that form triangular helices. Armadillo repeats enable β -catenin to bind AXIN, APC, E-cadherin, WRE elements and many other proteins. Armadillo repeats are subject to extensive post-translational modifications that regulate β -catenin's structural (adherens) and signaling activities, as shown in (B). Once in complex with TCF/LEF and bound WRE elements, the Helix-C domain recruits transcriptional co-activators. The C-terminus tail can fold back and block Helix-C. β -catenin has no DNA-binding domains, so it relies on other transcriptional partners to exert its transcriptional influences [Adapted from: (Valenta *et al.*, 2012)].

The second pool of β -catenin is the cytoplasmic pool that is dedicated for Wnt/ β -catenin signaling. This pool is composed of newly synthesized and unstable β -catenin that is rapidly targeted for degradation by the β DC complex. Upon activation of Wnt signaling, β -catenin is stabilized and translocates into the nucleus. β -catenin contains neither a nuclear localization signal (NLS) nor a nuclear export signal (NES), so the molecular choreography of its shuttling between the nucleus and cytoplasm remain to be determined. It is suggested that β -catenin, as a close relative of the importin / karyopherins family of proteins, is able to bind nuclear pore complexes and facilitate its own import (Clevers, 2006), but direct biochemical and *in vivo* evidence for this is lacking. S191 and S605 phosphorylation on β -catenin is thought to regulate nuclear translocation, and this is mediated by JNK2 (Figure 7B).

β -catenin is subject to several post-translational modifications that promote or inhibit its degradation, adhesion, signaling, stability or nuclear translocation (Figure 7B). An important modification is phosphorylation of Y654, which prevents the C-terminal tail from folding back and blocking Helix-C, the site of recruitment of transcriptional co-activators of the TCF/ β -catenin complex. Mice with a phosphomimetic Y654E mutation show phenotypes similar to hyperactivation of Wnt signaling, including predisposition to intestinal tumourigenesis (Van Veelen *et al.*, 2011). Further phosphorylation by PKA on β -catenin's S675 leads to recruitment of transcriptional co-activators (Piedra *et al.*, 2001) and initiation of transcription of Wnt target genes.

Upon Wnt stimulation, major genome-wide changes in chromatin structure are observed in Wnt-active cells, including rapid acetylation of up to 30kb regions around the site of TCF/ β -catenin binding (Parker *et al.*, 2008). Some of the transcriptional binding partners facilitate chromatin remodelling, including the MLL1/MLL2 SET1-type HMT complex which introduces H3K4 trimethylation (Sierra *et al.*, 2006), nucleosome modulators (such as SWI/SNF and ISWI), histone acetyl transferases (p300, CBP, Tip60, etc) and RNA Pol II (Mosimann *et al.*, 2009). Interestingly, contrary to β -catenin's role as a transcriptional co-activator, β -catenin can also repress transcription of other genes (Delmas *et al.*, 2007).

β -catenin signaling and biology is rather complex. β -catenin is heavily regulated at the protein level by various post-translational modifications. β -catenin is a promiscuous protein with several cytoplasmic and nuclear binding partners. Its nuclear import and export are tightly regulated. The outcome of β -catenin translocation to the nucleus is highly choreographed, and its nuclear activity is the sum of negotiations of various parties with varying influences. Recent advances in protein-DNA interaction technologies, as well as in genome-wide immunoprecipitation and

sequencing approaches have uncovered a growing list of β -catenin interacting partners. Most await functional and *in vivo* validation.

In the next sections, we explore the relevance of Wnt/ β -catenin signaling to two themes relevant to this thesis: stem cell biology (section 1.4) and tumourigenesis (section 1.5).

1.4 Wnt Signaling & Adult Stem Cells

The identification of stem cells in multicellular organisms was met by fascination from scientists and the public alike. Stem cell biology remains one of the hottest areas of medical, biomedical and biological research. Scientists disagree as to the exact definition of a stem cell. However, there is consensus that stem cells maintain themselves (self-renewal) over a long period of time (longevity) and can produce other cell types (multipotency). Two major types of stem cells are realized. The first are embryonic stem cells (ESCs) that exist early in an organism's life. ESCs orchestrate the exquisite symphony of an organism's developmental processes and possess the potency to differentiate to all cell lineages.

The second type are Adult Stem Cells (ASCs), also called tissue-resident stem cells or somatic stem cells. ASCs possess a limited potency, often restricted to only a few cell types of the tissue they reside in. ASCs function to replace dying or damaged cells. Some organs, such as the liver and pancreas, have a limited capacity for regeneration, and ASCs are reactivated only upon injury. Other organs, such as stomach, intestines and colon, have active and rapid stem cell proliferation. It is therefore not strictly correct to view stem cells as being quiescent (Barker & Clevers, 2010), as is the undertone of label-retention experiments. In fact, long-lived differentiated cells may incorporate labels and be mischaracterised as stem cells. Thus, quiescence can be thought of as a characteristic of stem cells in low turnover tissues.

1. Adult Stem cells (ASCs)

ASCs were identified in various organs. Significant progress was made in characterising haematopoietic, neural, cardiac and other stem cells. This is also the case for solid tissues, in which stem cell activity could be high (GI tract) or low (eg. liver, pancreas, etc). It is rather remarkable that the intestine has such vigorous stem cell activity that it replaces its entire epithelium every 5 days. It is thus a hot area of stem cell research. The intestines are also the site of colorectal tumourigenesis,

the second most common cancer. Cancer research into this organ is equally intensive. Once these two but overlapping facets of intestinal research are appreciated, one begins to realise how intestinal biology was always going to be the vanguard of stem cell research in solid tissues. As we shall see in this section, the delineation of stem cell character in the intestines, and the role of Wnt signaling in this process, has had profound implications on our understanding of what a stem cell is; implications that rippled beyond the intestinal biology field, facilitating identification of stem cells in various other tissues.

Various experimental techniques were developed to assay 'stemness' of a cell population. It is fair to say that the gold standard for evidencing 'stemness' is *in vivo* confirmation of multipotency. This can be achieved by either transplantation-based or genetic labelling approaches. In the former, cells of interest are labelled using antibody-based or *in vivo* genetic-based labelling approaches, isolated and transplanted into recipient mice to observe if such cells are able to re-constitute the entirety of the tissue in question *in vivo*. This approach was employed in successfully identifying stem cells in the haematopoietic lineage (Spangrude *et al.*, 1988), mammary glands (Shackleton *et al.*, 2006), skin and hair follicles (Blanpain *et al.*, 2004) and other tissues. The second approach relies on genetic labelling (pulse) and lineage tracing (chase) of labelled cells within live intact animals (Barker *et al.*, 2008). A plethora of examples exist of this, some of which are discussed below.

ASCs generally reside in specified tissue locations known as stem cell niches. Niches provide stem cells with factors that maintain the stem cells' multipotency, as well as factors that protect stem cells against pathogenic or exogenous insults. A paradigmatic example is the intestinal stem cell, housed in crypts under the villi. The niche supplements stem cells with epithelial and mesenchymal-derived Wnt ligands, as well as Noggin and EGF, to maintain stem cell multipotency. The niche also secretes lysozymes, defensins and other anti-bacterial factors to protect stem cells against pathogenic invasions (Sato *et al.*, 2011). In various tissues, physical departure of a stem cell from its niche leads to loss of maintenance signals and differentiation. This argues that stem cells do not possess multipotency due to their intrinsic character *per se*. Multipotency might be a privilege conferred upon stem cells by the niche and microenvironment in which they reside. This notion is corroborated by studies in which stem cells were conditionally deleted *in vivo* (Tetteh *et al.*, 2015). Here, tissue homeostasis is unaffected and new stem cells arise from terminally committed cells that enter the stem cell niche (Murata *et al.*, 2020). Another example is in *C. elegans*, where physical transfer of the stem cell niche induces stemness of cells surrounding the niche's new position, and differentiation of stem cells surrounding the niche's older position (Kimble & White, 1981).

The immediate daughter cell of a stem cell is generally not a terminally differentiated cell, but a committed progenitor. Known as transit-amplifying (TA) cells, these cells have limited proliferation capacity and differentiation potential, and serve to amplify cell numbers to tens of cells (Watt & Hogan, 2000). TA zones were documented in stem cell niches of various tissues (Díaz-Flores *et al.*, 2006). It is not known why such a hierarchical protocol exists. It is reasonable to speculate that this avoids stem cells multiple rounds of unnecessary cell division, guarding their genomic integrity against stochastic replication-associated DNA damage. This is important, as variations in cancer risk between tissues can be partially explained by number of stem cells in those tissues, and their proliferation rate (Tomasetti & Vogelstein, 2015).

2. Signalling Pathways in Adult Stem Cells

A general theme in ASCs from various tissues is the activation of a number of signaling pathways that confer proliferation, self-renewal, specific metabolism and anti-apoptotic programmes. The most prominent of these pathways is Wnt signaling, discussed above (sections 1.1-1.3). Other important pathways in stem cell biology include TGF- β , BMP, Hedgehog and Hippo pathways. Notch signaling is known to regulate proliferation, cell fate specification, differentiation and apoptosis in all metazoans (Figure 8A).

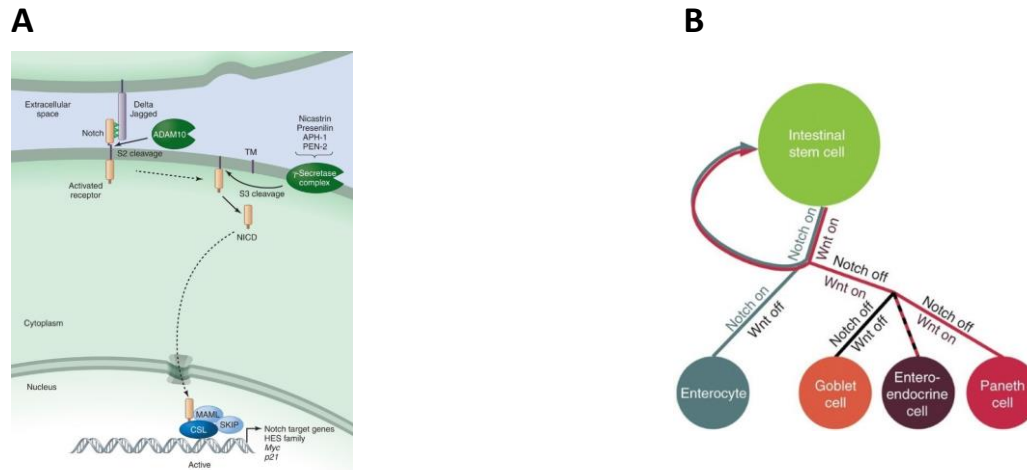


Figure 8: Simplified diagram of the Notch signaling pathway. (A) Notch itself is the pathway's membrane-bound receptor. It is activated by the Delta-like (DLL) and Jagged (JAG) family of ligands, which are also membrane-bound proteins, found on the surface of donor cells. Negative regulatory feedback loops prohibit autocrine signaling within the same cell. Notch is subject to glycosylation and other post-translational modifications, most notably ubiquitination by Numb. Ligand-receptor binding unfolds a region of Notch that allows ADAM10 to cleave away Notch's extracellular domain. This is S2 cleavage. S3 cleavage, mediated by γ -secretase, liberates Notch's intracellular domain (NICD) from the transmembrane domain (TM), facilitating its nuclear translocation. Activated NICD binds to CSL and recruits transcriptional co-activators, which initiate transcription of Notch target genes, including HES family, HEY family, Myc and other. **(B)** An example of the cooperation between Wnt and Notch signaling in dictating stem cell self-renewal and cell fate specification, as seen in intestinal epithelium and organoids. [(A) taken from (Kopan, 2012), (B) taken from (Yin *et al.*, 2013)].

Notch signaling is seen to synergize with Wnt signaling in various contexts. Some investigators believe this to be a novel integrative signaling module, often termed 'Wntch' signaling. The first documented synergy was observed in *Drosophila*, where *vestigial* expression is seen at the interface between dorsal and ventral surfaces of the fly wing. *Vestigial* gene was shown to possess both Wnt and Notch responsive elements, and co-activation of both pathways is required for *vestigial* expression (Klein & Arias, 1999).

For the purposes of this thesis, an important synergy context between these pathways is stem cell maintenance. The nature of this synergy is complex and context-specific. For instance, whereas active Wnt signaling always promotes stemness, Notch activation promotes intestinal stem cell maintenance (Yin *et al.*, 2013; Van Es *et al.*, 2005; see Figure 8B) but its inhibition is required for stem cell maintenance in mammary glands (Zeng & Nusse, 2010; Bouras *et al.*, 2008). Some of the regulatory intersection points between these pathways have been documented. The Notch NICD was shown to directly bind and regulate β -catenin (Sanders *et al.*, 2009). Notch NICD is also able to interact with other Wnt signaling components, including Dishevelled, GSK-3 β and AXIN. The outcome of these interactions could see Notch-mediated inhibition or potentiation of Wnt signaling (Hayward *et al.*, 2008), depending on context. An example of this is *in vivo* deletion of Notch receptor in mouse skin initiates basal carcinomas with elevated Wnt signaling and β -catenin levels (Nicolas *et al.*, 2003). In this thesis, I utilize Wnt-reporter patient-derived organoids to show that Notch signaling potentiates Wnt activation in FT organoids, whereas Notch inhibition compromises organoid regeneration, even upon ectopic Wnt activation.

Overall, among various stem cell pathways, Wnt signaling associates strongly with stemness and maintenance of multipotency. Components of Wnt signaling are established markers of stem cells in various tissues. In this regard, several mouse models were generated, in which stem cells are fluorescently marked, lineage-traced or manipulated in a specific way to understand the role of Wnt signaling and stem cells within those tissues (Aoki & Taketo, 2008). Among these, LGR5/6 and AXIN2-labelled mouse models are the most faithful in reporting Wnt signaling and marking tissue-resident stem cells (Barker & Clevers, 2010). LGR5 and LGR6 are unique and particularly specific to long-lived stem cells, disappearing in the immediate TA daughter cells. Even in low turnover tissues with low spontaneous regeneration, such as the pancreas, kidney and liver, there is no apparent endogenous LGR5/6 expression, but studies have shown activation of these receptors during damage-induced repair or early in development (Barker & Clevers, 2010).

3. Intestinal stem cells

The intestinal epithelium is organized into crypts and villi. Crypts contain Paneth cells that, as mentioned, constitute the niche for stem cells. Villi contain the cell types essential for executing intestinal secretion and absorptive functions, including Enterocytes, Entero-endocrine cells, Goblet and other cells. Between the crypt and villi exists the TA cell zone. As aforementioned, the intestine is one of the most actively proliferating organs, replenishing its entirely epithelium every 5 days.

Using mouse models that express GFP under an endogenous LGR5 promoter, Hans Clevers and colleagues showed that Lgr5+ intestinal stem cells reside at the base of intestinal crypts, are long-lived and replenish the crypts and villi with terminally differentiated progeny (Figure 9). The same finding was reported using lineage tracing of Axin2+ cells (Van Amerongen *et al.*, 2012), which is active in a wider population of cells. Both are surrogates for Wnt-active cells. Troy is another marker that associates with Wnt-active cells, but unlike Lgr5 and Axin2, Troy is an intestine-specific marker. Isolated single Lgr5+ cells regenerate crypts and villi *ex vivo* in organoids (Sato *et al.*, 2009). Upon forced shutdown of Wnt signaling *in vivo*, by loss of Tcf4 (Korinek *et al.*, 1998) or ectopic overexpression of Dkk-1 (Kuhnert *et al.*, 2004), intestinal crypts are lost with no apparent effect on villi. Conversely, activation of Wnt signaling *in vivo* by transgenic expression of the LGR5/6 agonist RSPO1 (K. A. Kim *et al.*, 2005b) or by APC loss (Sansom *et al.*, 2004) leads to pronounced accumulation of nuclear β -catenin, hyperproliferation and ectopic expansion of the crypt compartment. These, and several other *in vivo* and *in vitro* studies, strongly point to the dominant role of Wnt signaling in maintaining intestinal ASCs.

Paneth cells are the Wnt-producing source that supplies Wnt3a to intestinal ASCs (Farin *et al.*, 2016). Both cell types are in direct physical contact at the crypt base. Surprisingly, Paneth cell-derived Wnt3a is dispensable for Wnt signaling activation and ASCs maintenance. Using *in vivo* conditional deletion of WLS (Degirmenci *et al.*, 2018) or PORCN (Shoshkes-Carmel *et al.*, 2018), studies showed that Wnt-producing cells from the mesenchyme provide an extra-epithelial source of Wnt ligands for intestinal ASCs. In the colon, where Paneth cells do not exist, mesenchymal cells are an essential source of Wnt ligands. Moreover, stem cells at the very bottom of a crypt, and therefore closer in range to mesenchymal cells, tend to be the more dominant clones and outcompete other stem cells within the crypt in regenerating the villi (Huels *et al.*, 2018).

Having said that, it is important to note that Axin2+/Lgr5+ cells are stem cells only under homeostatic conditions. Upon genetic ablation of Lgr5+ cells, differentiated cells of the absorptive and secretory lineage are seen to de-differentiate and repopulate the lost Lgr5+ stem cell pool. De-

differentiation here is mediated by Ascl2 transcription factor, and is compromised upon Ascl2 ablation (Murata et al., 2020). Ascl2 expression is generally restricted to Lgr5⁺ stem cells, but under injury conditions it is activated in differentiated cells by mechanisms not completely understood. Differentiated cells in the crypt adopt an open chromatin configuration, which is thought to mediate their rapid response to de-differentiation stimuli (Jadhav et al., 2017).

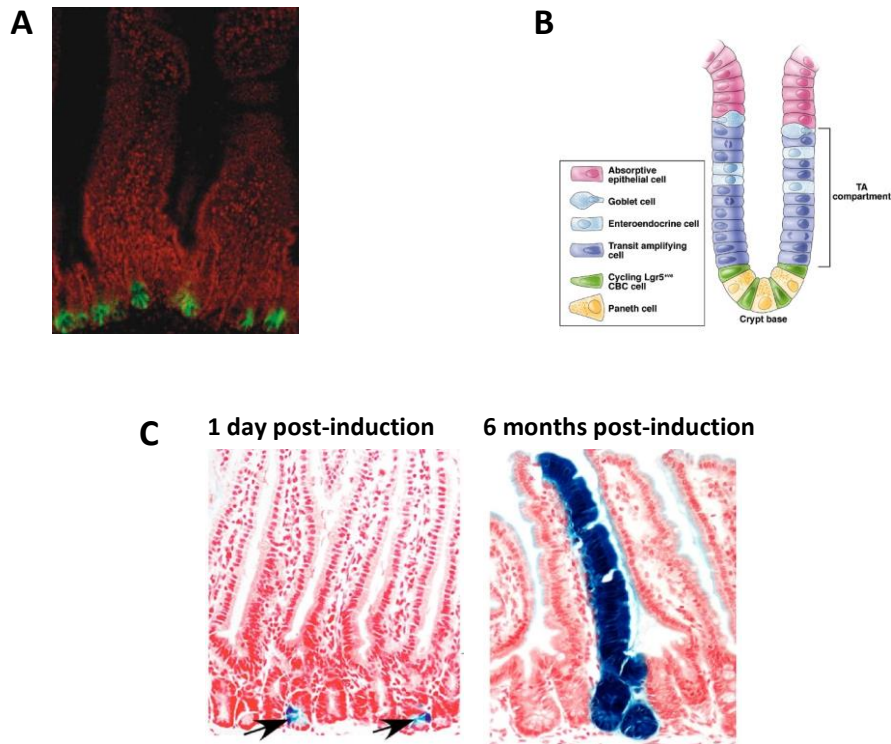


Figure 9: LGR5 is a specific marker of intestinal stem cells. (A) EGFP marking Lgr5⁺ cells. Obtained from intestines of Lgr5-EGFP-ires-Cre^{ERT2} mice. (B) Model of intestinal stem cell location and homeostasis, derived from lineage tracing experiments in mice. (C) Lineage tracing of Lgr5⁺ cells, using Lgr5-EGFP-ires-Cre^{ERT2} harbouring a Cre-activatable R26R-LacZ reporter. Transient tamoxifen treatment activates Cre recombinase, which excises and activates the LacZ reporter specifically in LGR5⁺ cells and their progeny. Limiting doses of tamoxifen were used to stochastically activate Cre in a small number of Lgr5⁺ stem cells [Data from: (Barker & Clevers, 2010)].

Although a useful strategy in examining de-differentiation, specific ablation of stem cells does not accurately reflect physiological damage, which is better modelled by ingested chemicals or pathogenic infections. The latter is particularly relevant, and was shown to induce crypt proliferation despite loss of Lgr5 expression. Instead, inflammation caused by pathogenic infection upregulates interferon- γ (INF- γ) which can drive stemness independently of Wnt signaling (Nusse et al., 2018). We will revisit inflammation and its re-activation of stemness in tumourigenesis (section 1.5.1). Chemical-induced colitis was also shown to drive Lgr5-independent regeneration (Yui et al., 2018). Interestingly, both of these forms of damage upregulate genes seen during fetal development,

suggesting the induction of this fetal transcriptional programme may represent an integral part of damage-induced, Lgr5-independent regeneration of the crypt (de Sousa e Melo & de Sauvage, 2019).

Overall, interrogating differentiation plasticity promises to increase our understanding of non-stem cell regeneration, which can be useful in clinical interventions. For instance, this could be by enhancing plasticity upon tissue damage, using drug-mediated intervention or biasing stem cell differentiation towards a cell type that can assist in resolving the damage. As is discussed in section 1.5.1, cellular plasticity in tumours is a major problem in chemotherapy resistance and targeting of the CSC compartment. If plasticity can be efficiently targeted and inhibited, say by destabilizing Ascl2 or inhibiting its interaction with obligate transcriptional co-activators, or perhaps by reducing plasticity upon targeting tumour-induced inflammation, CSC-based therapies (coupled with plasticity inhibitors) may become an opportune clinical intervention.

4. Gastric (stomach) stem cells

The stomach has a shared embryonic origin with the intestine, and likewise features an actively regenerating epithelium. The stomach has three anatomically different regions: the cardiac, corpus and pyloric antrum. The corpus is the main body of the stomach. It executes digestive functions and secretes hydrochloric acid and zymogens. The pyloric antrum is closest to the intestine and secretes mucus and hormones. While these regions differ slightly in structure and cellular composition, the overall structure of the stomach is composed of repeating structures called gastric units. Each gastric unit is composed of an upper part called the pit and a lower part called the gland (Vries *et al.*, 2010). Gastric units form protruding indentations below the surface lining of the stomach, and depending on the stomach regions, are composed of multiple differentiated cell types, including Mucin-5 (pit) cells, Mucin-6 cells, Entero-endocrine cells, isthmus cells, chief cells, parietal cells and other cell types (Figure 10A).

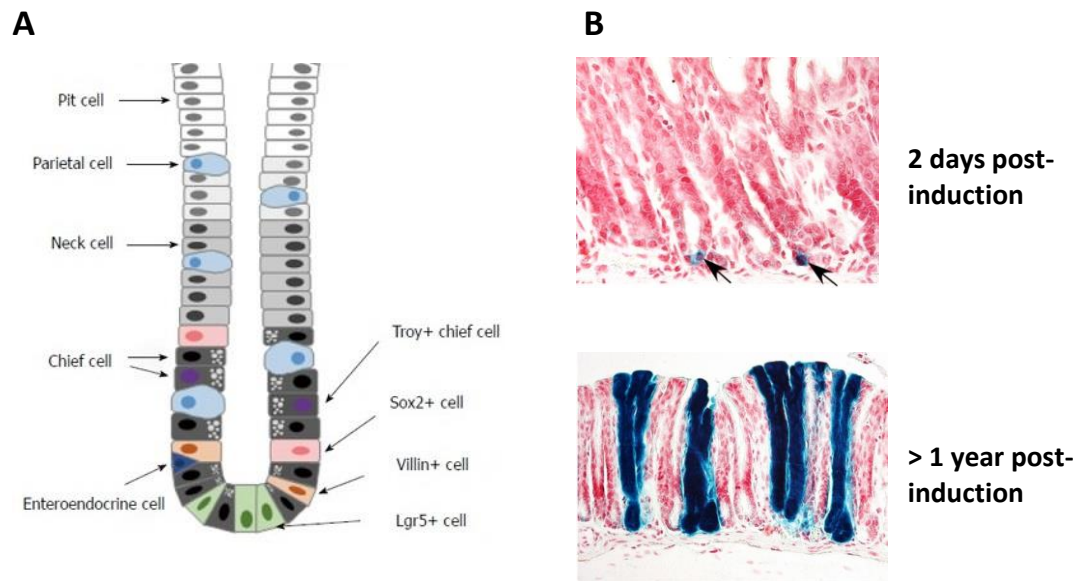


Figure 10: In vivo lineage tracing shows LGR5 is a specific marker of gastric stem cells. (A) Overall structure and cellular composition of stomach gastric units. Cellular composition varies between corpus and pylori regions. (B) Lineage tracing of LGR5+ cells 2 days (upper panel) and > 1 year (lower panel) post-induction, using the mouse model mentioned in Figure 9. [(A) taken from (Zhao *et al.*, 2015); (B) taken from: (Barker *et al.*, 2010)].

Much like intestinal crypts, early studies have shown the base of gastric glands to be a region of active proliferation. *In vivo* clonal mutational studies confirmed the presence of a multipotent stem cell (Bjerknes *et al.*, 2002). Lineage tracing using the Lgr5-EGFP mouse model provided definitive evidence that Lgr5+ Wnt-active cells are the stem cells of the stomach. These cells are long-lived, proliferative and multipotent, regenerating the entire gastric epithelium (Figure 10B). Wnt target genes, most notably Axin2, are enriched in Lgr5+ cells. Single Lgr5+ cells here have the exclusive ability of generating organoids *ex vivo*, containing differentiated cell types (Barker *et al.*, 2010). Upon ablation of Lgr5+ cells, an independent population of Axin2+ Lgr5- cells are activated to repopulate the Lgr5+ pool and repair damage (Sigal *et al.*, 2017). *Helicobacter pylori* infection also triggers activation and expansion of the Axin2+ Lgr5- population during repair. This was shown to be mediated by RSPO3, which is upregulated by mesenchymal cells upon infection (Leung *et al.*, 2018). Interestingly, the Lgr5+ population does not respond to this damage, showing that tissues can possess distinct stem cell pools; one homeostatic for basal regeneration, and another facultative for injury/damage/infection-mediated repair. These two stem cell pools overlap, but can be distinct as is evident in this case.

The above discussion only pertains to pyloric gastric units. In the corpus region of the stomach, however, lineage tracing studies have implicated a small subset of chief cells, expressing

Troy (Stange *et al.*, 2013) and Mist1 (Hayakawa *et al.*, 2015) in long-term regeneration of gastric epithelium. Here, Lgr5+ cells are not involved in homeostatic regeneration, but were seen to contribute to tissue regeneration upon injury. Ablation of Lgr5+ cells has no effect on corpus regeneration, but severely impairs regeneration upon injury (Marc Leushacke *et al.*, 2017). Like Lgr5+ stem cells, the homeostatic Troy+ stem cells express many Wnt target genes (including Axin2) and have the exclusive ability among other cells to form differentiated organoids *ex vivo* (Stange *et al.*, 2013).

Although Troy is a stem cell marker, chief cells are actually terminally differentiated cells that contain zymogen granules and secrete pepsinogen, an enzyme required for protein digestion. Homeostatic stem cell activity performed by differentiated cells is a well-documented phenomenon, as opposed to plasticity which arises in differentiated cells upon tissue injury (Flanagan *et al.*, 2015). This appears to challenge the dogma that stem cells are a distinct population of cells with the sole function of homeostatic tissue regeneration. Along similar lines, I observe in this thesis that putative Fallopian tube stem cells are not a separate population *per se*, but are differentiated Pax8+ secretory cells endowed with active Wnt signaling. As we shall see later, lack of discernible transcriptomic differences between such non-traditional stem cells and some of their progeny poses a technical challenge in identifying them using bulk and even single cell transcriptomic approaches.

5. Hair Follicle stem cells

Hair follicles and epidermal stem cells were identified prior to the discovery of Lgr5/6 as markers of stem cells. Studies have documented expression of CD34 (Blanpain *et al.*, 2004b) and krt15 (Morris *et al.*, 2004) in hair follicle stem cells that showed self-renewal and multipotency *in vivo*. Hair follicle regeneration occurs in cycles of growth and rest. Wnt signaling was known to be important for hair follicle regeneration (Kishimoto *et al.*, 2000), but not epidermal maintenance. This was seen in Lef1-mutant mice. Lef1 is a DNA-binding transcription factor that binds TCF and β -catenin to co-activate Wnt signaling. Lef1-mutant mice have much reduced hair follicles compared to wild type counterparts (Van Genderen *et al.*, 1994). Mice with hyperactivated Wnt signaling, either by Lef1 overexpression or unregulatable β -catenin, exhibit many more hair follicles (Alonso & Fuchs, 2003).

For hair follicles, studies pointed to the hair bulge, above the dermal papilla, as the physical location of the potential stem cell niche (Figure 11A). *In vivo* lineage tracing using Lgr5-EGFP reporter mice showed that Lgr5+ cells reside in the hair follicle stem cell niche, are long-lived, multipotent and replenish the whole hair follicle epithelium (Jaks *et al.*, 2008). Lgr5+ stem cells migrate from the bulge to the hair base, entering a transit-amplifying zone that multiplies and differentiates cells.

Another study attributed the same stem cell characteristics to Axin2⁺ cells residing in the same location as Lgr5⁺ stem cells (Lim *et al.*, 2016). Again, Lgr5 and Axin2 are probably marking the same cells here (Figure 11B).

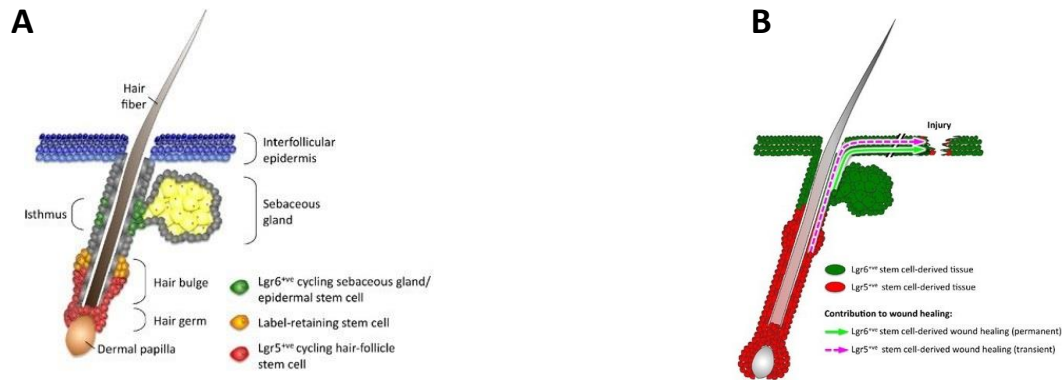


Figure 11: Hair follicle and epidermal stem cells. (A) Overall structure and cellular composition of hair follicles and the epidermis. **(B)** Schematic of lineage tracing results, using *Lgr5-EGFP-ires-CreERT2* or *Lgr6-EGFP-ires-CreERT2*, both harbouring a Cre-activatable R26R-LacZ reporter. LGR5 marks hair follicle stem cells. LGR6 marks epidermal stem cells that contribute to wound healing. [Image taken from (Leushacke *et al.*, 2012)].

Interestingly, the latter study showed that Axin2⁺ cells express their own Wnt ligands, and that ablation of Wnt secretion specifically in Axin2⁺ cells abolishes regeneration. This is rather remarkable, as Wnt-active stem cells are generally maintained by niche-supporting Wnt-expressing cells that supply Wnt ligands to stem cells in a paracrine manner. In hair follicles, we see a rare example of autocrine Wnt signaling, with Wnt synthesized and received by stem cells themselves. Furthermore, Axin2⁺ cells are located in the outer buldge, and express Wnt inhibitors Dkk1 and Sfrp1, seen to localize to Wnt-inactive differentiated progeny in the inner buldge (Lim *et al.*, 2016), presumably to prohibit progeny from benefitting from Wnt ligands. How Lgr5⁺ Axin2⁺ buldge stem cells are able to restrict access of their Wnt ligands to the outer buldge and localize their Wnt inhibitors to the inner buldge remains to be elucidated. It could be speculated that this is down to signaling range and mobility differences between Wnts and Wnt inhibitors Dkk1 and Sfrp1, generating Wnt gradients between the outer and inner buldge, as seen in intestinal epithelium.

6. Epidermal (skin) stem cells

As for epidermal stem cells, these are located above the hair buldge region, just under the sebaceous glands (Figure 11A). Lgr5 expression is undetectable here, but Lgr6⁺ cells were documented to be long-lived, multipotent and replenish the sebaceous gland and all lineages of the skin (Figure 11B).

Prenatally, these Lgr6⁺ stem cells are also the origin of hair follicle cells, including Lgr5⁺ hair buldge stem cells, but with age they become restricted to replenishing the epidermis only (Snippert *et al.*, 2010). Unlike other stem cells discussed, Lgr6⁺ skin stem cells are Wnt-independent (Fuchs & Horsley, 2008). Upon excision of the back skin of mice, Lgr6⁺ skin stem cells were observed to contribute to wound healing. Interestingly, limited and transient contribution is seen from Lgr5⁺ hair follicle stem cells as well. Formation of new hair follicles at the wound site is also facilitated by Lgr6⁺ skin stem cells (Snippert *et al.*, 2010), which might acquire Lgr5⁺ after restoration of homeostasis. Therefore, there is overlap in the biology of Lgr5⁺ hair follicle stem cells and Lgr6⁺ skin stem cells.

Another study arrived at different conclusions using Axin2 reporter mice. Here, Axin2⁺ cells were shown to be multipotent and regenerate the interfollicular epidermis in a Wnt4 or Wnt10a-dependent way, under homeostatic conditions and upon injury. As with Axin2⁺ hair follicle stem cells, Axin2⁺ epidermal stem cells were observed to secrete Wnt ligands in an autocrine manner, as well as secreting Wnt inhibitor DKK1 to restrict the range of Wnt activation (Lim *et al.*, 2013). Overall, although the involvement of Wnt signaling in epidermal stem cells is arguable, marking of epidermal stem cells by Wnt signaling components is established.

7. Mammary (breast) stem cells

This thesis aims to identify Fallopian tube stem cells. Among the tissues discussed thus far, the mammary gland is the closest-resembling tissue to the Fallopian tube, for reasons outlined in section 3.2. Mammary glands are composed of two epithelial layers: a basal KRT5/14-expressing myoepithelial layer and a KRT8/19-expressing luminal layer. The latter differentiates into ductal and milk-producing cells.

Wnt signaling is critical for mammary gland development and homeostasis (Gavin & McMahon, 1992), which is impaired by Loss of Lrp6 (Lindvall *et al.*, 2009), Lef1 (Van Genderen *et al.*, 1994) or transgenic overexpression of DKK1 (Andl *et al.*, 2002). Wnt4 is thought to be the dominant Wnt ligand in relation to mammary biology. Wnt4 is also, interestingly, essential for initiation of female reproductive tract development (section 2.2.1). Wnt4 mediates mammary stem cell expansion in response to ovarian-secreted hormones. Wnt4-mutant mammary tissues are not able to regenerate upon transplantation, and conditional deletion of Wnt4 *in vivo* compromises puberty-induced ductal expansion (Fu *et al.*, 2020). Notably, Wnt4 and Progesterone receptor (PR) co-localize in the luminal compartment, and progesterone was shown to induce Wnt4 secretion by luminal cells (Briskin *et al.*, 2000). Loss of PR or Wnt4 leads to defects in branching morphogenesis, which can be rescued by Wnt1 overexpression. It is thought that luminal-derived Wnt4 drives Wnt-activation in a

subset of Wnt-responsive basal stem cells (Joshi *et al.*, 2010). Finally, inhibition of β -catenin (using a dominant negative mutant) suppresses lobule and alveolar development during pregnancy (Roarty & Rosen, 2010). These and other *in vivo* studies implicate Wnt signaling in mammary gland stem cell biology.

Earlier studies have investigated mammary stem cells and attributed stemness to a fraction of Aldh1+ cells (Ginestier *et al.*, 2007), CD24⁺ CD29^{high} cells (Shackleton *et al.*, 2006), CD24⁺ CD49f^{high} (Stingl *et al.*, 2006) and other cell types. Within the CD24⁺ CD29^{high} cell population, Axin2⁺ marks a minor subpopulation of basal cells that can further enrich for stem cells (Zeng & Nusse, 2010; Van Amerongen *et al.*, 2012). In this interesting study, Axin2⁺ or Axin2⁻ cells were labelled *in vivo* and FACS isolated from within the CD24⁺ CD29^{high} cell population. Upon transplantation into cleared fat pads of recipient mice, Axin2⁺ cells reconstituted the mammary gland at a higher efficiency than Axin2⁻ cells. *In vitro*, Axin2⁺ cells show higher colony-formation efficiency. *In vitro* Wnt ligand-treated Axin2⁺ cells show higher mammary gland reconstitution efficiencies relative to untreated Axin2⁺ cells (Zeng & Nusse, 2010). Based on similar *in vivo* investigations, namely transplantation into mouse mammary fat pads and *in vitro* colony formation, another study quantified a 16-fold enrichment for stem cell frequency in Lrp5^{high} cells within the previously reported basal CD24⁺ CD49f^{high} population (Badders *et al.*, 2009). This goes up to 200-fold enrichment for Lrp5^{high} Lrp6^{high} basal cells (Fu *et al.*, 2020). These studies identify Wnt markers as ‘enrichers’ of stem cell frequencies within reported stem cell population.

Given Lgr5 marks ASCs in various tissues, its role in mammary biology was considered. Specific ablation of Lgr5⁺ cells *in vivo* impairs mammary gland development (Plaks *et al.*, 2013), but not adult mammary gland homeostasis (Trejo *et al.*, 2017). Interestingly, when Lgr5 is coupled with another marker, Tspan8, the pair enrich for mammary stem cell frequency by 20-fold within the CD24⁺ CD29^{high} compartment (Fu *et al.*, 2017). Another study identified Protein C Receptor (Procr) as a Wnt target gene in the mammary gland. Procr⁺ cells are basal CD24⁺ CD29^{high} cells and were evidenced by lineage tracing to differentiate into all lineages of the mammary gland, (Wang *et al.*, 2015).

Overall, a number of cell populations are reported to enrich for mammary stem cells (Figure 12). In contrast to GI tract, hair follicle and skin tissues, it is fair to say that none of these markers specifically and exclusively marks mammary stem cells.

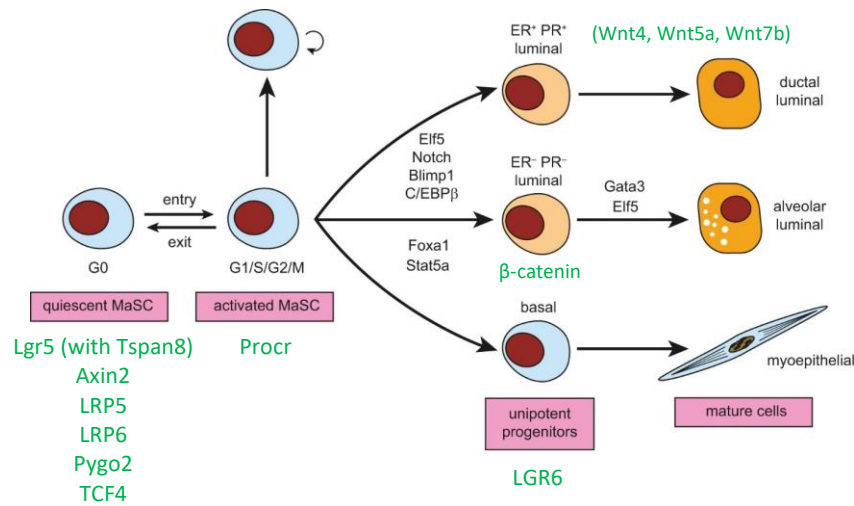


Figure 12: Wnt components enrich for mammary stem cells. Wnt signaling components are shown in green. Enrichment in multipotency was shown by transplantation-based or lineage tracing approaches. Wnt-active mammary stem cells are, by and large, basal cells. Unlike GI tract, hair follicle and epidermis, Wnt signaling components enrich for but do not exclusively mark mammary stem cells. MaSC is Mammary Stem Cell [Modified from: (Fu et al., 2020)].

8. Wnt-active stem cells in low turnover tissues

Unlike the GI tract, which also derives from the endoderm, proliferation and cellular turnover in the liver is rather slow, and is mediated by 1 in 10,000 cells (Sell, 2001). In the adult liver, *Lgr5* expression is undetectable. However, upon injury, liver regeneration is more active, and a population of *Lgr5*⁺ cells appears near the bile ducts. Lineage tracing confirmed that these *Lgr5*⁺ cells are multipotent and can generate hepatocytes and bile ducts *in vivo*, *ex vivo* in organoids and *in vivo* upon transplantation of organoids into mice (Huch, Dorrell, et al., 2013). These *Lgr5*⁺ cells are probably facultative stem cells, regenerating the tissue only upon injury. Another study was able to identify homeostatic stem cells in the uninjured liver, located around the central vein, using *in vivo* lineage tracing for *Axin2*⁺ cells (Wang et al., 2015).

Like the liver, the adult pancreas has a low turnover rate, and Wnt signaling is important for development but not adult tissue homeostasis. Wnt-active pancreatic cells derived from mouse pancreas upon injury were shown to be multipotent *in vivo*, *ex vivo* in organoids as well as *in vivo* upon transplantation of organoids. Both *Lgr5* and *Axin2* marked these Wnt-active facultative stem cells (Huch, Bonfanti, et al., 2013).

In the kidney, turnover is also slow and regeneration happens only upon damage. This is driven by Wnt-active stem cells, shown to be *Lgr5*⁺ by RNA FISH and *in vivo* lineage tracing using the *Lgr5*-EGFP and *Lgr5*-LacZ mice. *Lgr5*⁺ cells establish the epithelium lining the ascending loop of Henle

and distal convoluted tubules (Barker *et al.*, 2012). Other regions, such as the proximal tubule, could be established by Lgr5⁺ cells.

Wnt signaling was shown, using Wnt-reporter mouse lines, to be activated in various regions of the adult lung undergoing regrowth and regeneration. Axin2⁺ cells are involved in repair after bleomycin-induced lung injury *in vivo* (Al Alam *et al.*, 2011; Flozak *et al.*, 2010). More conclusively, Lgr6⁺ cells were identified to contribute to broncho-alveolar lung regeneration using kidney capsule engraftment (Oeztuerk-Winder *et al.*, 2012). Wnt-active progenitors display multipotency and regeneration of differentiated cells in the cochlea, marked by Lgr5 (Chai *et al.*, 2012) or Axin2 (Jan *et al.*, 2013). Axin2⁺ cells were documented in nail and digit regeneration (Takeo *et al.*, 2013). Wnt-active cells in taste buds were shown to replenish all three major subtypes of differentiated cells. The taste bud story is intriguing, as these Wnt-active cells were shown *in vivo* to be marked by both Lgr5 (Yee *et al.*, 2013) and Lgr6 (Ren *et al.*, 2014). This is rare, if not undocumented for any other tissue. More interestingly, KRT5 and KRT14 mark basal cells in this tissue, and Lgr5⁺ Lgr6⁺ cells mark a subset of these KRT5/14 basal cells (Ren *et al.*, 2014; Jiang P., personal communication, 11 Sep 2018).

Finally, Wnt signaling also associates with stemness in non-solid tissues. The best studied stem cell, the haematopoietic stem cell (HSC), is dependent on Wnt signaling for self-renewal. *In vitro* treatment of HSCs with Wnt ligands, or overexpression of β -catenin, promotes HSC self-renewal and inhibits differentiation. Such Wnt-stimulated HSC have an enhanced capacity to reconstitute the entire haematopoietic cell lineage upon injection into irradiated mice (Willert *et al.*, 2003).

9. Perspectives

In summary, the Wnt/ β -catenin signaling pathway has come to define and be defined by stem cells in various organs. Its components, particularly Axin2, Lgr5 and Lgr6 facilitated the marking, isolation and characterisation of stem cells in various tissues, a summary of which is shown in Table 1. Advances in omic and single cell technologies hold promise in deeply characterizing ASCs from multiple organs. Employment of mouse models, organoid culture and scRNA-seq technologies is a particularly powerful trio in interrogating ASCs from multiple organs during multiple points of development (Yu *et al.*, 2020, unpublished data). This will not only serve to characterize ASCs, but also derive a minimal and universal stem cell signature that can be used as a template to define the nature of stem cells and identify them in organs awaiting ASC identification.

The Fallopian tube remains one of these organs, in which regeneration has long been documented to occur, but the stem cells mediating regeneration have not been conclusively

identified. In section 3.3.3 (FT stem cells remain unidentified), we explore the limited attempts made thus far to identify FT stem cells, and how this may relate to ovarian cancer. In the next section, however, we explore the connection between Wnt signaling, stem cells and tumourigenesis. In the Results chapters, the thesis will aim to identify FT stem cells by drawing on some of the lessons learnt from the Wnt signaling epoch of stem cell biology.

Table 1: List of Wnt-active adult stem cells identified by in vivo lineage tracing in mice. In many contexts, these Wnt-active ASCs also establish the tissue epithelia during development. As discussed, some ASCs are actively cycling, others are slowly cycling and some only re-activate upon injury or damage (facultative). Homeostatic ASCs may or may not contribute to injury repair. Markers of facultative-only stem cells are shown in red boxes. This list is not comprehensive, and Wnt-dependent as well as Wnt-independent ASCs in many other tissues are yet to be identified.

Tissue	Location	Marker	Reference
Intestine	Crypt base	Lgr5	Barker et al. (2007)
		Axin2	Van Amerongen et al. (2012)
Stomach	Pyloric (distal)	Lgr5	Barker et al. (2010)
	Corpus (proximal)	Troy	Stange et al. (2013)
		Lgr5	Leushacke et al., (2017)
Hair Follicle	Outer bulge cells	Lgr5	Jaks et al. (2008)
		Axin2	Lim et al. (2016)
Epidermis	Under sebaceous gland	Lgr6	Snippert et al. (2010)
	Basal layer	Axin2	Lim et al. (2013)
Mammary gland	Basal layer	Axin2	Van Amerongen et al. (2012)
		Lgr5	Fu et al. (2017) Plaks et al. (2013) De Visser et al. (2012)
		Procr	Wang et al. (2015)
	Basal and luminal layers, unipotent	Lgr6	Blaas et al. (2016)
Liver	Pericentral hepatocyte	Axin2	Wang et al. (2015)
		Lgr5	Huch, Dorrell, et al. (2013)
Pancreas	Ductal cells	Lgr5, Axin2	Huch, Bonfanti, et al. (2013)
Kidney	Henle ascending loop, distal convoluted tubules	Lgr5	Barker et al. (2012)
	Nephronic	Axin2	Rinkevich et al. (2014)
Lung	Broncho-alveolar (distal lung)	Axin2	Al Alam et al. (2011) Flozak et al. (2010)
		Lgr6	Oeztuerk-Winder et al. (2012)

Ovary	Ovarian Surface Epithelium at Hilum	Lgr5	Ng et al. (2014) Flesken-Nikitin et al. (2013)
Endometrium	Base of glands	Axin2	Syed et al. (2020)
Vagina	Epithelium	Axin2	Ali et al. (2020)
Prostate gland	Luminal cells	Lgr5	Wang et al. (2015)
	Basal and luminal cells	Lgr5	
Eye retina	Amacrine cells	Lgr5	Sukhdeo et al., (2014)
Cochlea	Border cells	Lgr5	Chai et al. (2012) Shi et al. (2012)
		Axin2	Jan et al. (2013)
Taste buds	Circumvallate and foliate taste papillae (posterior)	Lgr5	Yee et al. (2013)
		Lgr6	Ren et al. (2014)
	Tongue (anterior)	Lgr5	Takeda et al., (2013)
Olfactory epithelia	Globose basal cells	Lgr5	Chen et al., (2014)
Central Nervous System	Radial glial cell	Axin2	Bowmana et al. (2013)
Bone	Osteoblasts	Axin2	Tan et al. (2014)
Testis	Spermatogonial stem cell	Axin2	Takase & Nusse (2016)

1.5 Wnt Signaling, Stem Cells & Cancer

We have seen in the last subchapter how Wnt signaling marks stem cells in various tissues. Wnt signaling bestows on stem cells a cellular programme of longevity, multipotency, self-renewal, proliferation and anti-apoptosis. In many contexts, suppression of Wnt signaling is accompanied by loss of stemness (Yin *et al.*, 2013). Tumourigenesis, on the other hand, is the consequence of dysregulated proliferation and anti-apoptosis. In a way, stem cells and tumour cells are two sides of the same coin. An emerging concept in cancer biology is that tumours appropriate the physiological machinery of self-renewal and proliferation already present in stem cells. This theory, often called the cancer stem cell (CSC) theory, postulates that stem cells are the cells-of-origin of tumours, and drive tumour maintenance and progression. Like ASCs, CSCs regenerate the full spectrum of heterogeneity seen in a tumour (Nassar & Blanpain, 2016).

CSC properties are the consequence of ASC longevity and self-renewal, providing a decades-long platform for an iterative process of mutation and clonal selection. Often, the cellular hierarchy within a tumour mirrors that of the tissue of origin. Therefore, consistent with stem cell numbers in normal tissues, CSCs constitute a small fraction of the tumour bulk ranging from 1 in 100,000 to 1 in

1,000 cells (Ishizawa *et al.*, 2010). The presence of a CSC-topped tumour hierarchy was established for a number of malignancies, including breast (Al-Hajj *et al.*, 2003), colorectal (Dalerba *et al.*, 2007), prostate (Collins *et al.*, 2005), lung (Eramo *et al.*, 2008), brain (Singh *et al.*, 2004), blood (Bonnet & Dick, 1997), skin (Driessens *et al.*, 2012) as well as ovarian cancer (Zhang *et al.*, 2008). This was shown by murine xenotransplantation-based approaches in most cases. Confirmation by lineage tracing approaches is pending. Having said that, it should be emphasized that for a subset of other cancers, evidence for CSCs is not solid. In fact, in some tumour types it is established that the CSC model does not apply at all (Ball *et al.*, 2017).

The term Cancer Stem Cell (CSC) is often used to refer to a cell that initiates a tumour, or a cell that possesses the capacity to maintain or regenerate a tumour. The two concepts are not the same. CSCs can be used to refer to cells that display both characteristics. However, if a tumour cell possesses one of these characteristics, it is best referred to as a tumour-initiating cell (TIC) or a tumour-promoting cell (TPCs). In Wnt-dependent tissues, it is often the case that an ASC sustains an initiating mutation, transforming it into a TIC. TICs behave as TPCs in the initial stages of tumourigenesis. Further accumulation of genetic damage may activate stem/tumour cell pathways in differentiated tumour cells, endowing them with stem-like characteristics. At this point, TICs may or may not continue to be the TPC regenerative core of the tumour. It is not an ambition of this subchapter to comprehensively encompass the literature surrounding Wnt signaling and its role in TICs and TPCs. We will, however, explore a few examples that can help us develop an appreciation of the emerging paradigms in this field.

1. Intestinal Cancer

Small intestinal and large intestinal (colorectal) cancers rank second on the list of the most common cancers. Germline and sporadic forms of these cancers arise due to biallelic inactivation of APC, a negative regulator of the Wnt/ β -catenin signaling pathway. Increased β -catenin foci are seen in intestinal tumours from patients and mouse models.

As discussed, Lgr5⁺ intestinal stem cells were shown to be long-lived, multipotent and replenish the entire intestinal epithelium *in vivo* (Sato *et al.*, 2009). In an elegant study, Barker *et al.* (2009) induced the driving mutation of intestinal cancers in different intestinal cell types. Neoplastic transformation arose only when the initiating mutation was induced under an Lgr5 promoter, targeting Lgr5⁺ stem cells. This decisively indicated that the Lgr5⁺ Axin2⁺ Wnt-active stem cell is one of the cells-of-origin of colorectal cancer.

Lgr5⁺ stem cells also act as TPCs. This was shown by lineage tracing using a multicolour Cre reporter, where Lgr5⁺ cells were shown to fuel the growth and maintenance of adenomas (Schepers *et al.*, 2012). Direct lineage tracing of human tumours is not feasible, but human-derived organoids provide a platform for circumventing this. CRISPR-Cas9 genome editing was used by one study to integrate a fluorescent reporter into the LGR5 locus of human colorectal tumour organoids. These organoids were transplanted into mice, and LGR5⁺ cells were shown to fuel tumour growth and progression. Interestingly, upon xenotransplantation of organoids with an LGR5 inducible Caspase 9 knock-in allele (Shimokawa *et al.*, 2017) or an LGR5 inducible Diphtheria toxin (De Sousa E Melo *et al.*, 2017), selective ablation of LGR5⁺ cells lead to tumour regression, but only briefly. Tumour growth was restored after the inducer was removed, by re-emerging LGR5⁺ TPCs (Shimokawa *et al.*, 2017), suggesting cell fate plasticity within tumour cells, as documented for normal crypts (section 1.4.3). However, colorectal cancer metastasis to the liver did not happen upon LGR5 ablation, implicating LGR5⁺ tumour cells in metastasis (Melo *et al.*, 2017). Endogenously, Lgr5⁺ CSCs can emerge *de novo* without ablation of existing Lgr5⁺ CSCs. This is driven by the inflammatory environment, that triggers stemness in differentiated tumour cells by re-activating Wnt signaling in an NF- κ B dependent mechanism (Schwitalla *et al.*, 2013). Non-epithelial derived growth factors, such as HGF, can signal through c-met to stabilize β -catenin and hyperactivate Wnt signaling, independent of Lgr5⁺ CSCs (de Sousa e Melo & de Sauvage, 2019).

Although upregulation of Wnt signaling was known to initiate intestinal tumourigenesis since the late 1980's, the cell-of-origin of colorectal cancer remained elusive. This was the case until advances were made in intestinal homeostasis and stem cell biology. We now not only appreciate that LGR5⁺ stem cells are TICs, but that they can also behave as TPCs. This underscores the importance of understanding normal tissue homeostasis and biology, and how advances in this can help us understand tumour biology. Tumourigenesis, therefore, is best conceptualized as a form of defective tissue homeostasis.

2. Gastric (stomach) Cancer

Stomach cancer, also called gastric cancer, is the fourth most common cancer worldwide. Gastric cancer classifies into two major types: intestinal and diffuse cancer. The former arises from the pyloric antrum region of the stomach, and is characterised by an intestinal-like hyperplasia (Vries *et al.*, 2010). As discussed (section 1.4.4), Lgr5 marks long-lived multipotent stem cells at the base of gastric units in this region of the stomach.

In gastric cancer patients, tumour regions show accumulation of nuclear β -catenin (Clements *et al.*, 2002). Using *in vivo* targeted mutagenesis, it was confirmed that Apc loss specifically in Lgr5+ gastric stem cells leads to tumour formation, suggesting that the Lgr5+ stem cell is the cell-of-origin of gastric cancer (Barker *et al.*, 2010). No tumours were observed in the gastric corpus region, so the cell-of-origin of corpus tumours remains to be elucidated.

3. Hair Follicle & Epidermal tumours

Wnt signaling may be a cause of tumourigenesis in the hair follicle. In humans, pilomatricomas, a tumour emerging from hair follicles, features activating mutations in β -catenin (Chan *et al.*, 1999). Likewise in mice, a constitutive activation of β -catenin leads to pilomatricoma tumours in the hair follicle (Gat *et al.*, 1998). It is likely these oncogenic hyperactivating mutations in Wnt signaling components target Lgr5+ stem cells of the hair follicle, but this has not been experimentally evidenced. However, it was shown that targeting p53 loss and Kras^{G12D} activation to these Wnt-active stem cells leads to epidermal squamous cell carcinomas (White *et al.*, 2011).

4. Breast Cancer

Tumourigenesis in the mammary gland was observed to closely associate with mammary stem cells. As mentioned at the outset of this chapter, Wnt1 ligand was initially identified as the preferred locus of integration of the Mouse Mammary Tumour Virus (MMTV), causing breast cancer. Transgenic overexpression of Wnt1 or Wnt3 expands the mammary progenitor compartment leading to tumourigenesis (Shackleton *et al.*, 2006; Teissedre *et al.*, 2009). As we saw, the mammary progenitor compartment can be enriched by Wnt markers. (Figure 12). This is supported by lack of Wnt1-driven tumourigenesis in Lrp5^{-/-} mice (Lindvall *et al.*, 2006).

Notably, targeting Lgr6+ cells with loss of p53 and Brca1, as well as activation of Kras, gives rise to luminal tumours only (Blaas *et al.*, 2016). Although Lgr6 is an Rspo receptor, it is not generally seen to mark Wnt-active cells (Clevers H., personal communication, 23rd Oct 2020). Overall, breast cancer can be conceptualized as a multistep process that ensues upon perturbation in the precise control of the switch between mammary stem cell self-renewal and differentiation. Wnt signaling plays an important role in this switch.

5. Perspectives

Advances in CSC biology have inspired therapies aimed at striking a tumour at its regenerative core. However, as mentioned, differentiated tumour cells may de-differentiate upon compromising TPCs

within a tumour. How this happens is not well-understood, but it's likely to be mediated, at least in part, by plasticity mechanisms already present in normal tissues. For instance, given what we know about the intestinal niche, it is hypothesized that ablation of LGR5⁺ stem cells creates physical vacancies within the intestinal crypt. Cells that fill this vacancy are endowed with EGF, Notch as well as Wnt ligands from Paneth cells (Sato *et al.*, 2011) and the underlying mesenchyme (Degirmenci *et al.*, 2018). This, coupled with protection offered by the niche against differentiation-inducing signals such as BMP and TGF- β (Batlle & Clevers, 2017) may enable triggering of stemness into otherwise differentiated cells. Similar examples have been observed in other tissues, where *in vivo* ablation of Wnt-active stem cells leads to rapid de-differentiation of some committed cells, replacement of stem cells and tissue regeneration (Tata *et al.*, 2013; Kusaba *et al.*, 2014). This was observed in some tumours upon CSC ablation (Shimokawa *et al.*, 2017). There are exceptions, however. For instance, in glioblastoma, specific ablation of TPCs halts tumour growth and prolongs survival without replenishment of the TPC pool by other tumour cells (J. Chen *et al.*, 2012). Therefore, the emerging paradigm is that stem cells are unique by virtue of their location and niche environment, as opposed to being unique *per se*.

Therefore, cellular plasticity makes therapeutic targeting of CSCs impractical. However, this can have short-term benefits in receding tumour progression or inhibiting metastasis (De Sousa E Melo *et al.*, 2017). Practically, since CSCs survive therapy, it might be more efficacious to employ combinatorial approaches, to target the tumour by bulk/standard chemotherapy, as well as CSC-targeted therapy. Two studies successfully showed the feasibility of targeting Lgr5⁺ CSC in xenograft models, using cytotoxic drugs conjugated to anti-LGR5 antibodies (Gong *et al.*, 2016; Junttila *et al.*, 2015). Other efforts have focused on targeting the microenvironment that facilitates de-differentiation of committed tumour cells (Batlle & Clevers, 2017). Although an attractive concept, this might not be an ideal approach for two reasons. Firstly, normal stem cells also rely on the microenvironment for ASC-mediated regeneration of healthy tissue, so targeting this microenvironment will come at a cost to healthy tissue. Secondly, upon tumour progression and increasing genomic instability, cancer cells become gradually independent from niche signals. This is seen clearly in colorectal cancer, in which APC loss, KRAS activation and SMAD4 loss give tumour cells independence from niche-supplied Wnt, EGFP and BMP/TGF- β inhibitors, respectively (Batlle & Clevers, 2017). Nonetheless, although independent from niche signals, tumour cells remain dependent, and in some cases addicted, to these pathways. Therefore, inhibition of these pathways at a very downstream level might be more opportune. An example of this is PRI-724, which is a small molecule that interferes with β -catenin's binding with its transcriptional partner CBP to activate Wnt signaling. Employing such a drug at doses sufficiently low to inhibit the elevated Wnt signaling levels in tumours, without compromising the basal Wnt signaling levels in healthy ASCs, represents a

reasonable approach. PRI-724 is under clinical trials for colorectal, pancreatic and leukemia (Batlle & Clevers, 2017), and is employed in this thesis as a downstream inhibitor of Wnt signaling in FT organoids.

1.6 Chapter Summary

Wnt signaling has associated with development and tumourigenesis ever since its discovery. Canonical Wnt signaling culminates in β -catenin stabilization, nuclear translocation, binding to WRE elements and expression of Wnt-target genes. This gene set is customizable dependent on context, tissue type and developmental stage. However, AXIN2 is a universal marker of Wnt activation. Non-canonical Wnt signaling includes Wnt/PCP and Wnt/ Ca^{2+} pathways, both independent of β -catenin and LRP5/6 co-receptor. The perception that canonical and non-canonical pathways are linear signaling nodes is gradually fading. It is increasingly evident that these pathways crosstalk and compete for shared components, but molecular detail of this crosstalk is lacking. Both pathways are the subject of active research and continual discovery.

Wnts are the primary activators of Wnt signaling. They undergo post-translational glycosylation and lipid modifications, which are important for their secretion and activity. PORCN and WLS are essential for Wnt secretion. Cysteine residues are crucial for correct 3D folding of Wnts. FZDs are the natural receptors of Wnt ligands, capturing Wnts through CRD domains. RSPOs bind LGR4-6 to potentiate Wnt signaling, by degrading RNF43/ZNRF3, an E3 ubiquitin ligase that targets FZD receptors for degradation.

Today, Wnt signaling is the one of the most important pathways in ASC biology. ASCs reside in niches that cater for their Wnt and other requirements, but few ASCs express their own Wnts in an autocrine loop. TA zones are common in ASC niches, and may exist as a protective mechanism against stochastic replication-associated DNA damage. Other pathways collaborate with Wnt signaling in maintaining ASCs, notably Notch signaling. Findings in intestinal ASC research were instrumental in delineating the Wnt pathway and extracting near universal markers for ASCs, including Axin2, Lgr5 and Lgr6. In some tissues, such as GI-tract and hair follicle/epidermis, Wnt-active cells are the exclusive stem cells. In other tissues, such as the mammary gland, Wnt-active cells enrich for stem cells but do not exclusively mark them. In low turn-over tissues such as the liver, pancreas and kidney, Wnt-active cells are more relevant to facultative damage/injury-induced

regeneration. The list of tissues that feature Wnt-dependent homeostatic or facultative regeneration is steadily increasing. Unfortunately, FT stem cells have not been identified to date.

An interface between stem cell and cancer biology is becoming increasingly evident. Wnt-active ASCs were shown to act as TICs, TPCs, both (CSCs) or none, depending on the tumour type. The intestinal story of ASC, LGR5 and LGR5+ TICs shows that understanding normal tissue homeostasis is important to advance our knowledge of tumour initiation. Hierarchical plasticity in tumours, driven by ASC niches, complicates the targeting of CSCs. As such, targeting the Wnt pathway at downstream levels might be more opportune. A number of Wnt signaling modulators are currently in clinical trials. Overall, Wnt signaling, stem cells and cancer have emerged as an important triangular axis in tissue biology, that has improved our understanding of homeostasis, regeneration, cell fate specification, cancer initiation and tumour progression, and opened promising future avenues to pursue in cancer treatment and regenerative medicine.

CHAPTER 2

WNT SIGNALING & FALLOPIAN TUBE
DEVELOPMENT

WNT SIGNALING & FALLOPIAN TUBE DEVELOPMENT

The developmental process of the female reproductive tract (FRT) is rather remarkable. A plethora of literature exists on the subject, some looking at whole tissue transcriptomes while others investigating the contribution of a single gene or gene family to every stage of development of the FRT. The literature on the subject is therefore too vast to be comprehensively covered here. Given that this thesis aims to highlight the critical role of Wnt signaling in FT regeneration and stem cell maintenance, the discussion in this section shall be confined to Wnt signaling and the role of Wnt ligands in the phased development and patterning of the FRT. The intention is to understand the embryonic origins of Wnt-expressing cells, and how this transcends beyond development into adult tissue maintenance.

For obvious moral and ethical reasons, little is known about *in situ* development of the human FRT. Much of our knowledge on the subject has been extrapolated from seminal studies on mouse FRT development. The earliest primordial anlage of the mouse FRT is the Mullerian duct, also referred to as the paramesonephric duct. It emerges from the mesoderm germ layer. The Mullerian duct runs down the two lateral sides of the urogenital ridge, very close to the Wolffian ducts, the embryonic origin of the male reproductive system. The Mullerian and Wolffian ducts co-exist early in development. In response to sex-specific signaling cues, one of the ducts is promoted and the other regresses. These signaling cues can come from genetic sexual determination, as seen in humans and mice, or from environmental factors, as is observed in reptiles in which sex is determined depending on egg's incubation temperature (Waters *et al.*, 2007).

2.1 The Mullerian Duct

The Mullerian duct is the embryonic origin of the oviduct (anterior most), uterus, cervix and upper vagina (posterior most). The oviduct is the mouse equivalent of the human FT. The ovaries have a different embryonic origin, arising from the urogenital ridge of primordial gonads, and are related to the peritoneal mesothelium (C. Miller & Sassoon, 1998). This difference in embryonic origins has implications for treatment and understanding of the natural history of Ovarian cancer (see section 3.4.2). The neonatal Mullerian duct is relatively plastic and undifferentiated. At birth, it is negative for oviduct, uterine and vaginal epithelial markers (Kurita *et al.*, 2001). An exquisite process of development and patterning ensues in the following 7-9 days. Wnt signaling is intimately involved in this patterning process.

The oviduct, uterine and the upper part of the cervix epithelium differentiate into simple columnar epithelium. A columnar-squamous epithelium transitional zone exists in the cervix, marking the end of the columnar epithelium and the beginning of the stratified squamous epithelium, which lines the remaining part of the FRT, namely the lower cervix and vagina (Heikkilä *et al.*, 1999). Cervical and vaginal squamous epithelium express the basal cell marker KRT14, while the simple columnar epithelium lining the oviduct, uterus and upper cervix is KRT14-negative (Kurita *et al.*, 2001). I confirm in this thesis that the human FT and mouse oviducts do not express KRT14. However, I do note the presence of rare KRT5+ cells in tissue and tissue-derived organoids.

1. The mesenchyme directs neonatal Mullerian duct differentiation

The neonatal Mullerian duct epithelium relies on the underlying mesenchyme for tissue-specific cytodifferentiation and patterning. This was evidenced by an elegant set of tissue heterografting experiments, conducted using an established protocol (Cunha, 1976). When neonatal uterine epithelium was isolated and heterografted on vaginal mesenchyme, the neonatal uterine epithelium differentiated into vaginal-like epithelium, with stratified squamous morphology and KRT14 expression, both features of vaginal epithelium. Similarly, neonatal vaginal epithelium displayed uterine features when recombined with uterine mesenchyme, morphing into a simple columnar epithelium and lacking KRT14 expression (Cunha, 1976; Kurita *et al.*, 2001). Importantly, this plasticity is only seen in neonatal epithelium 5-7 days old. After this temporal window, differentiated epithelium is resistant to this mesenchyme-induced heterotypic differentiation.

2. Mullerian duct regression in male mice

Further development of the Mullerian duct requires the absence of regression signals. In male mice, gonad specification is triggered by the action of the Y-chromosome gene SRY, a master regulator of male differentiation. SRY directs XY fetal gonad towards testis differentiation. The testis thereafter secretes hormones that virilise and masculinize the rest of the body. Testosterone promotes differentiation of the Wolffian duct to the epididymis, vas deferens and seminal vesicles (McClelland *et al.*, 2012). Cells within the testes, called Sertoli cells, produce Mullerian Inhibiting Substance (MIS), also known as Anti-Mullerian Hormone (AMH). Mullerian duct mesenchyme expresses MIS receptor, which binds to MIS to cause Mullerian duct regression in males (McClelland *et al.*, 2012). Mutations inactivating MIS or its receptor render the Mullerian duct mesenchyme incapable of responding to MIS signaling. This leads to FRT persistence in male mice (Behringer *et al.*, 1990). It is therefore widely held that female sexual development is the default sexual differentiation pathway. It ensues in the absence of active male, anti-female sexual differentiation signaling, mostly driven by SOX9,

FGF9 and SRY (McClelland *et al.*, 2012). This concept is helpful as an overall paradigm, but in terms of molecular details this is too simplified as we shall see later.

2.2 Wnts in FRT Development

A number of Wnt ligands have been reported to be expressed in the adult mouse oviduct. Four ligands are epithelium-specific, including Wnt7a, Wnt7b, Wnt11 and Wnt16. On the other hand, Wnt2b, Wnt5a and Wnt5b are expressed in both the epithelium and mesenchyme, while the remaining ligands of the 19 member family are not expressed (Ghosh *et al.*, 2017). This is for the adult oviduct. However, only Wnt4, Wnt5a and Wnt7a have been implicated in oviductal and Mullerian duct embryonic development (Miller *et al.*, 1998a). At birth, Wnt7a expression is seen throughout the epithelium of the Mullerian duct, while Wnt5a expression is in the mesenchyme. Wnt4 expression is restricted to the mesenchyme in the anterior part of the Mullerian duct. During postnatal development and FRT patterning, Wnt7a becomes restricted to the anterior parts of the FRT epithelium (oviduct and uterus). Wnt5a localizes to the mesenchyme of distal uterus, cervix and vagina while Wnt4 expression is activated in the posterior parts of the FRT (Miller *et al.*, 1998a).

1. Wnt4 is required for initiation of FRT development

Wnt4 KO mice lack the Mullerian duct. Wnt4 is therefore critical for Mullerian duct initiation. The primordial anlage of the male reproductive system, the Wolffian duct, persists in Wnt4 KO female mice. This indicates that Wnt4 is required for Wolffian duct regression. Under wild type conditions, ovaries produce female sex hormones to drive the differentiation of the Mullerian duct. In female Wnt4 KO mice, testosterone biosynthesis is detectable in the ovary. Testosterone promotes Wolffian duct development, so this might explain the persistence of the Wolffian duct in Wnt4 KO female mice (Vainio *et al.*, 1999). Targeted loss of RSPO1 (section 1.2.5) phenocopies loss of Wnt4, suggesting that Wnt4 acts through the Wnt/ β -catenin signaling pathway (Chassot *et al.*, 2008). Male sexual development in Wnt4 KO male mice is unaffected by Wnt4 mutation. Taken together, there is evidence to suggest that Wnt4 is essential for the initiation of female sex determination and FRT development.

Wnt4 loss is neonatally lethal, and thus it has not been possible to look at the role of Wnt4 in adult oviduct or FRT homeostasis. Most studies were conducted decades ago. With current advances in inducible mouse mutagenesis, it will be interesting to investigate the effect of Wnt4 KO on the

adult oviduct and FRT. One study came close to achieving this, although Wnt4 was deleted not in an inducible but in a tissue-specific manner, under the MIS receptor promoter. This targets the Mullerian duct mesenchyme. Authors noted that Mullerian duct initiation was normal, and that Wnt4 deletion in Mullerian duct mesenchyme had no effect on FRT development (Kobayashi *et al.*, 2011), further substantiating the notion that Wnt4 is important for initiation but not maintenance of the FRT. More studies are needed to understand the role of Wnt4, if any, in the epithelial compartment of the FRT.

2. Mesenchymal Wnt5a is not required for oviduct development

In wild type mice, Wnt5a is expressed in the Mullerian duct mesenchyme at birth but restricts to the posterior uterus, cervix and vagina during postnatal development (Cary Miller *et al.*, 1998a). Wnt5a KO mice die shortly after birth due to global defects in antero-posterior axis development (Yamaguchi *et al.*, 1999). Examination of their Mullerian ducts reveals that anterior structures, namely oviduct and uterus, develop normally. Posterior parts, the cervix and vagina, were lacking. This suggests Wnt5a is important for posterior FRT development (Mericskay *et al.*, 2004). Importantly, the normally developed uterus lacks endometrial glands. Expression of Wnt7a and Wnt4 appear unaffected, but deletion of Wnt7a disrupts Wnt5a levels (Dunlap *et al.*, 2011).

To circumvent neonatal lethality of Wnt5a KO mice, tissue heterograft techniques into ovariectomized mice were employed. When wild-type mesenchyme was recombined with uterine epithelium (wild-type or Wnt5a KO), glands formed normally. However, when Wnt5a KO mesenchyme was used, no endometrial glands developed in wild type or Wnt5a KO epithelium (Mericskay *et al.*, 2004). This suggests that mesenchymal Wnt5a, much like epithelial Wnt7a (see below), is involved in the genesis of endometrial glands. Ectopic Wnt5a overexpression rescues the KO phenotype. Wnt4 overexpression does not, indicating absence of molecular redundancy (Mericskay *et al.*, 2004). As a general phenomenon, Wnt5a is expressed in the mesenchymes of other developing organs (Lickert *et al.*, 2001). It is an open question as to why this is the case, but consistent with its association with Wnt/PCP signaling, it is plausible that Wnt5a is a mesenchymal-to-epithelial signal that instructs dividing epithelial cells to extend epithelial sheets in a polarized and unidirectional orientation. Tight and strict organization is a feature of epithelial cells, that is lacking in the loosely organized stromal or mesenchymal cells underneath.

3. Wnt7a specifies the oviduct and uterus during development

Unlike Wnt4 and Wnt5a, Wnt7a is expressed exclusively in epithelial cells in the prenatal Mullerian duct. Postnatally, Wnt7a expression becomes restricted to the oviduct epithelium and uterus luminal epithelium. In contrast to Wnt4 and Wnt5a KO mice, Wnt7a KO mice are viable but are reproductively sterile, both males and females (Parr & Mc Mahon, 1995). Upon Wnt7a loss, the Mullerian duct is able to initiate development and persist, but its correct patterning is disrupted. Oviducts in Wnt7a KO mice are almost always absent. When present, oviducts are under-developed and display significantly reduced internal coiling, reminiscent of uterine epithelium. This implies that Wnt7a is necessary for oviduct specification during development.

On the other hand, Wnt7a KO uterus epithelium changes morphology to resemble vaginal epithelium, with a morphological change from simple columnar to stratified epithelium, as well as acquisition of vaginal epithelium markers. Collectively, these observations are referred to as posteriorization of the FRT (Miller & Sassoon, 1998). The uterus in Wnt7a KO mice is smaller and thinner compared to wildtype, and distinctly lacks endometrial glands, which are plenty in wild type mice and differentiate from uterine luminal epithelium. Ablation of Lgr5+ cells in the luminal compartment also leads to loss of endometrial glands (Chai *et al.*, 2012), suggesting that Wnt-active Lgr5+ cells are the benefactors of the Wnt7a signal. Endometrial glands are critical for conception but not fertility. Loss of endometrial glands, mediated by LIF mutation, renders female mice incapable of conception (Stewart *et al.*, 1992; Bhatt *et al.*, 1991). Therefore, Wnt7a KO mice could be sterile due to lack of functional oviducts and endometrial glands.

The above studies looked at FRT phenotypes of Wnt7a KO mice, generated by targeted insertion of a neomycin resistance cassette into exon 2 of Wnt7a (Parr & Mc Mahon, 1995). Another study deleted Wnt7a conditionally, using floxed Wnt7a and Cre recombinase driven under Progesterone Receptor promoter. Consequences to the uterus were as previously reported, but less severe phenotypes were seen in the oviduct (Dunlap *et al.*, 2011). This discrepancy between the two studies could be attributable to Progesterone Receptor expression, which may be restricted only to a subset of oviduct cells. Recent *in vivo* studies on the oviduct are perhaps more appreciative of this, and employ Ovgp-1 (Sherman-Baust *et al.*, 2014) or Pax8 (Shuang Zhang *et al.*, 2019) to drive oviductal-specific expression.

Wnt7a loss also carries implications for non-epithelial cells as well, which in wild type mice do not express Wnt7a. Upon Wnt7a loss, the uterine smooth muscle layer becomes thickened, disorganized and irregular. The sharp boundary between the smooth muscles and stroma disappears.

The size of the stroma reduces and glands, which generally heavily populate the uterine stroma, are absent (C. Miller & Sassoon, 1998). These observations suggest that epithelial-derived Wnt7a not only affects the epithelium, but also signals to the mesenchyme and contributes to its correct patterning. As aforementioned, the mesenchyme is important for tissue-specific differentiation of the FRT in the first 7-9 days after birth. Heterozygous mice, harbouring a wild type and a mutant Wnt7a allele, resemble wild type mice in oviduct and uterus morphology (C. Miller & Sassoon, 1998). This suggests that Wnt7a is haplosufficient in nature, and that no dosage compensation mechanisms operate between Wnt7a and other patterning ligands.

4. Wnt7a primes the neonatal Mullerian duct to respond to regression signals

Studies document another interesting phenotype in Wnt7a mutants, this time in male mice. Recall that the early Mullerian duct expresses MIS receptor in its mesenchyme. This binds to secreted MIS in males, leading to Mullerian duct regression. In Wnt7a KO mice, MIS receptor is not expressed in the Mullerian duct mesenchyme, leading to Mullerian duct persistence in male mice (Parr & McMahon, 1998). Persistence of the Mullerian duct blocks sperm passage, and could be the reason for sterility of Wnt7a KO male mice. Therefore, epithelial-derived Wnt7a is crucial in epithelial to mesenchymal signaling, and in priming the mesenchyme and rendering it competent to respond to Mullerian duct regression signals.

This is reminiscent of the role of Wnt7a in limb-bud patterning. Here, Wnt7a signals from the dorsal ectoderm to the underlying mesenchyme, where it promotes expression of *Lmx-1b*, leading to dorsal limb patterning. In fact, loss of Wnt7a results in the dorsal ectoderm acquiring ventral characteristics in the dorsal half of the limb (Riddle *et al.*, 1995; Cygan *et al.*, 1997), much like loss of Wnt7a results in oviducts and uterus acquiring a uterine and vaginal morphology, respectively (C. Miller & Sassoon, 1998). Both are manifestations of posteriorization in patterning. Therefore, Wnt7a is intimately involved in the patterning of the FRT and other organs.

5. Wnt7a promotes Mullerian duct differentiation

Male mice mutant for Wnt7a contain no oviducts, or if present, undifferentiated and under-developed oviducts with little or no coiling or invaginations (C. Miller & Sassoon, 1998). This indicates that Wnt7a is required for further differentiation of the Mullerian duct. This is consistent with Wnt7a's role in 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). Notably, male mice mutant for MIS or its receptor contain a persisting Mullerian duct (Behringer *et al.*, 1990), in which

oviducts are well developed and differentiated (Mishina *et al.*, 1996). Therefore, Wnt7a's promotion of FRT differentiation is independent of MIS receptor.

Studies have thus far investigated the role of Wnt7a (as well as Wnt4 and Wnt5a) in FRT development. These Wnt ligands, particularly epithelial-derived Wnt7a, are clearly of significant importance. To understand the role of Wnt7a in tissue homeostasis and regeneration, inducible and conditional disruption of the Wnt7a gene in adult FRT is required. Such studies do not exist hitherto. Furthermore, given Wnt7a's involvement in oviduct differentiation, a number of questions arise from developmental studies: do early epithelial Wnt7a-expressing cells represent the cellular origins of the adult oviduct? Are adult stem cells in the oviduct relics and remnants of early multipotent progenitors that are Wnt7a-expressing or Wnt7a-receiving? Do these cells remain multipotent during adult life? If so, do these putative stem cells respond to hormonal and environmental cues to regenerate the oviduct? These important questions remain unanswered; some are the subject of investigation in this thesis.

2.3 Wnts and Ovarian Hormones in FRT development

1. Estrogen and Progesterone

Estrogen and Progesterone are known to interact *inter se*, and the outcome of their interactions regulates Wnt signaling. Estrogen acts through its nuclear receptor Estrogen Receptor ER α (also called ESR1) and ER β (also called ESR2). Liganded ER α translocates to the nucleus, dimerizes and binds to Estrogen responsive elements to recruit transcriptional co-activators, which relax chromatin and initiate transcription of target genes. Liganded ER α may also exert its functions independently of genomic DNA, by interacting with various nuclear proteins and transcription factors (Björnström & Sjöberg, 2005). On the other hand, ER β is thought to antagonize ER α , although its exact role is not known. Progesterone also acts through a dedicated nuclear receptor (PR) using similar mechanisms, with a different but sometimes overlapping set of estrogenic interactions and target genes.

Progesterone is known to antagonize Estrogen signaling in the FRT (Dougherty & Sanders, 2005), by downregulating expression and activity of ER (Hsueh *et al.*, 1976), or by directly influencing the availability of Estrogen itself (Bulun *et al.*, 2010). Estrogen, however, promotes PR receptor expression, constituting a negative feedback loop where Estrogen downregulates itself via Progesterone. Estrogen biology is quite complex, given ER α has at least 3 isoforms, ER β comes with 5

different isoforms and all of these may cross-regulate each other. Expression of ER isoforms and their binding partners is stage and tissue-specific. For instance, while Tamoxifen is an ER agonist in the uterus, it acts as an ER antagonist in the breast, used in endocrine therapy for breast cancer (Deroo & Korach, 2006). An essential action of Estrogen is dimerization of liganded ERs, which may allow any of these 8 isoforms to homo- or hetero-dimerize, adding to functional complexity (Björnström & Sjöberg, 2005).

2. Estrogen activates Wnt-target gene expression and promotes proliferation

Estrogen regulates Wnt signaling. Most studies document this to be a positive regulation. A report showed that nuclear β -catenin is enhanced during the proliferative phase of the menstrual cycle. This phase is characterized by elevated Estrogen signaling (Figure 13A). Upon progression to the secretory phase, dominated by Progesterone signaling, β -catenin nuclear localization disappears (Hideyuki *et al.*, 1999). In colon cancer cell line SW480, in which APC mutation promotes high basal Wnt signaling levels, Estrogen treatment upon expression of ER α enhances expression of Wnt target genes in a Wnt-reporter (TOPFlash) assay (Kouzmenko *et al.*, 2004). On the other hand, Wnt signaling promotes proliferation. In the endometrium, Wnt signaling induces rapid proliferation of cells. In two studies, female mice were administered lithium chloride, a potent inhibitor of GSK-3 β , through drinking water. These mice displayed enhanced endometrial proliferation and hyperplasia (Gunin *et al.*, 2004; Polotsky *et al.*, 2009), mimicking the effects of Estrogen-dominant proliferative phase of the menstrual cycle. Overall, Estrogen, Wnt signaling and proliferation are concordant processes.

The molecular details of Estrogen-mediated activation of Wnt signaling are not completely understood. However, Estrogen treatment of patient-derived endometrial primary culture induces stabilization of β -catenin and hyperproliferation. This Estrogen-induced proliferation is abolished upon treatment with Wnt signaling inhibitor Sfrp2 (Hou *et al.*, 2004), suggesting that Estrogen-induced proliferation is mediated upstream in the Wnt pathway, by the action of a Wnt ligand. On the contrary, in rat models, ER α was shown to interact with Akt and p85, the regulatory subunit of PI3K, and together the complex inhibits GSK-3 β . This stabilizes β -catenin and activates Wnt signaling. Liganded ER α may also bind β -catenin directly (Kouzmenko *et al.*, 2004) and stabilize it (Figure 13B), suggesting that Estrogen's activation of Wnt signaling can also be downstream in the Wnt pathway, independent of a Wnt ligand. Overall, the molecular details of Estrogen's activation of Wnt signaling are not completely understood. Different mechanisms operating in different tissues cannot be ruled out.

Progesterone is known to inhibit Estrogen, Wnt signaling and proliferation. This was shown in endometrial biopsies from postmenopausal patients who underwent control, estradiol (a potent

form of Estrogen) or estradiol + MPA (medroxyprogesterone acetate, a synthetic form of Progesterone) treatments for 3 weeks. The study showed enhanced expression of Wnt signaling genes in samples of Estrogen-treated patients, which is counteracted upon simultaneous administration of Progesterone (Hanifi-Moghaddam *et al.*, 2007; Wang *et al.*, 2010). Progesterone was shown to be effective in inhibiting TOPFlash reporter activity in endometrial Ishikawa cell line. Progesterone-mediated inhibition of Wnt signaling is abolished upon siRNA knockdown of either Dickkopf-1 or FOXO1, suggesting the pair mediate progesterone's inhibition of Wnt signaling (Figure 13C). It is also possible that Progesterone inhibits Wnt signaling by directly antagonizing Estrogen or its ER receptors.

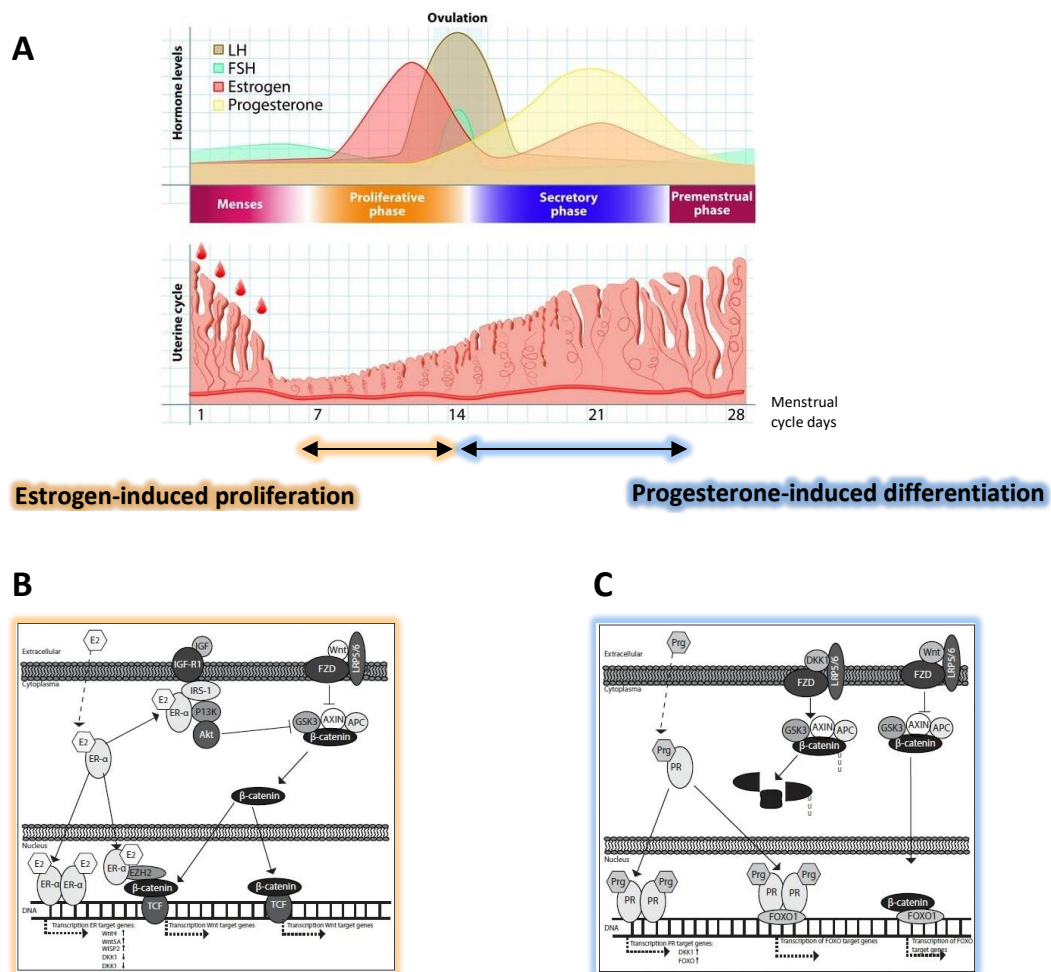


Figure 13: Hormonal levels and Wnt signaling regulation during the menstrual cycle. (A) Upper panel shows the levels of ovarian-secreted Estrogen and Progesterone hormones, as well as pituitary-secreted Luteinising Hormone (LH) and Follicle Stimulating Hormone (FSH). Whilst LH and FSH peak briefly around ovulation, the cycle is defined by an Estrogen-dominated proliferative phase and a Progesterone-dominated secretory phase. Lower panel shows effect of ovarian hormones on the endometrium, an organ containing similar cell types and of common embryonic origin to the oviduct. **(B)** Estrogen and its nuclear receptor ERα promote Wnt signaling directly, by inhibiting GSK-3β or acting as a transcriptional co-activator to β-catenin, or indirectly by downregulating Dickkopf (DKK1), an inhibitor of the Wnt co-receptors LRP5/6. **(C)** Progesterone and its nuclear receptor inhibit Wnt signaling indirectly, by antagonizing Estrogen signaling, and by upregulating Dickkopf-1 and FOXO1. FOXO1 binds and sequesters β-catenin away from TCF, disabling transcription of Wnt target genes. [(A) adapted from (Walker S., 2019); (B-C) taken from (Wang *et al.*, 2010)].

These studies, and others, focused on the relationship between ovarian hormones and Wnt signaling in the endometrium. The endometrium has a similar embryonic origin and development to the oviduct, as well as harbouring the same cell types, excluding glands. Therefore, there is basis for extrapolating conclusions of these studies to the oviduct. In fact, oviducts from chicks subcutaneously injected with DES (an Estrogen analogue, see below) displayed massive cellular proliferation, with oviducts gaining 8-fold and 150-fold weight increase after 3-day and 2-week treatment, respectively (Munro & Kosin, 1943). Having said that, conclusive evidence is yet to be derived from studies on murine oviduct. The emerging picture, however, is that the Estrogen / Progesterone signaling axis exerts functions, partially through Wnt signaling, that regulate the balance between proliferation and differentiation. Perturbations of this fine balance can lead to reproductive tract pathologies, including cancer.

3. Estrogen inhibits Wnt7a

Wnt7a KO mice display the phenotypes above-mentioned. These phenotypes are strikingly similar to those of mice prenatally exposed to Diethylstilboestrol (DES), an Estrogen analogue. After birth, these mice contained remnants of the Wolffian duct, and oviducts were under-developed and lacked coiling. Neonates born to DES-exposed mice also displayed posteriorization of the FRT, lack of endometrial glands and other Wnt7a KO phenotypes (McLachlan *et al.*, 1980). This hinted at the possibility that Estrogen negatively regulates Wnt7a expression. This was confirmed by the observation that Wnt7a RNA levels are significantly reduced in the FRTs of neonates of DES-exposed mice relative to unexposed mice (Miller *et al.*, 1998).

DES was first manufactured in 1938 and mimics the action of naturally occurring estrogens, such as Estrone and Estradiol-17 β (Dodds *et al.*, 1938). It was prescribed to pregnant women in the US between 1940 and 1971, to counteract a drop in Estrogen levels, which precedes and was thought to be responsible for premature delivery and *in utero* fetal death. DES was prescribed to 5-10 million women, before its long-term harms and predisposition to cervical and vaginal cancers were realized (Giusti, 1995). Women who were exposed to DES *in utero* showed similar congenital phenotypes to Wnt7a KO mice, including FT abnormalities, lack of tubal coiling, withered Fimbria and infertility (DeCherney *et al.*, 1981).

As mentioned, mice neonatally exposed to DES have significantly reduced Wnt7a levels. This DES-induced downregulation of Wnt7a does not take place in ER α KO mice (Couse *et al.*, 2001), indicating this is an estrogenic effect. Another report documented that Estrogen administration to endometrial carcinoma cell line Ishikawa decreases endogenous Wnt7a levels (Wagner & Lehmann,

2006). During the human menstrual cycle, Wnt7a expression peaks at the secretory phase, during which progesterone levels are high (Bui *et al.*, 1997). Similarly, in mice, Wnt7a levels peak at diestrous and metestrus, mirroring Progesterone levels (Cary Miller *et al.*, 1998b). It can be speculated that Progesterone achieves this by inhibiting Estrogen signaling, liberating Wnt7a from inhibition by Estrogen. Progesterone treatment of DES-treated ER α knockout mice can help clarify this in an *in vivo* setting. In this thesis, I show that Estrogen signaling downregulates WNT7A in hFT organoids, both at the RNA and protein levels.

Therefore, evidences from studies supports the idea that Estrogen negatively regulates Wnt7a during development. It is interesting that DES phenotypes are only seen in neonates, and not in adult female mice (McLachlan *et al.*, 1980). This seems to suggest, as was hinted by tissue heterograft experiments (Cunha, 1976; Kurita *et al.*, 2001), that there is a short temporal window during which Wnt7a imposes its patterning effects on the FRT. Therefore, it can be hypothesized that restoration of Wnt7a expression after this temporal window will not reverse the Wnt7a KO / DES phenotype.

Knowledge of the effect of Estrogen on the developing FRT led to the optimization of a new heterograft protocol for studying FRT development. For example, to study uterine development, neonatal uterine heterografts used to be injected under the kidney capsules of wild type female mice. In this setting, neonatal uterine heterografts take 5 weeks to develop, with very few endometrial glands. This is contrary to the development of intact neonatal uterine *in situ*, which normally takes 1-2 weeks to develop many glands populating the endometrium. It was hypothesized that exposure to adult sex hormones (in particular Estrogen) could be delaying the rapid development of heterografted uterus. Performing the same heterografting procedure under the kidney capsules of ovariectomized female mice (lacking Estrogen) leads to rapid development of many uterine glands in heterografted neonatal uterus (C. Miller *et al.*, 1998). This further substantiates the negative regulation imposed by Estrogen on Wnt7a-driven patterning.

On a side point, ER expression in any *in vitro* model is crucial to interrogate Estrogen regulation of Wnt signaling in hormone-responsive tissues. Work in our lab confirmed ER expression in fresh human FT tissue (Hu *et al.*, 2020) and FT organoids (this thesis). Crucially, ER expression is completely absent in patient-derived FT cells cultured in standard 2D culture. This highlights the importance of employing physiologically relevant models of tissue biology to address important research questions, and underscores the superiority of organoids in modelling tissue biology relative to traditional 2D cultures. This further highlights the importance of appreciating the crosstalk between Estrogen and Wnt signaling, and that withdrawal of the confounding effects of Estrogen

might be important to precipitate the role of Wnt signaling in organoid regeneration and FT stem cells. Finally, from our discussion above, it is notable that Estrogen activates Wnt signaling but inhibits Wnt7a expression. There is an apparent contradiction, which is the subject of discussion in sections 8.3.4 and 9.3.

4. Estrogen inhibition of Wnt7a may be mediated by mesenchymal Wnt5a

As mentioned, mice prenatally exposed to DES (an Estrogen analogue) show reduced Wnt7a expression and Wnt7a KO phenotype (McLachlan *et al.*, 1980). Interestingly, DES is unable to repress Wnt7a in Wnt5a-mutant heterografts. This hints at the possibility that Estrogen's inhibition of Wnt7a is mediated by Wnt5a. In fact, an endogenous example of this is seen in the endometrium. Normally, Wnt7a is expressed in the luminal epithelium, while Wnt5a is expressed in the mesenchyme. Wnt7a downregulation is seen in luminal epithelium which invaginates into the mesenchyme, generating endometrial glands (Mericskay *et al.*, 2004). Given evidence that glands are monoclonal in origin (Lipschutz *et al.*, 1999), this mesenchymal Wnt5a-driven downregulation of Wnt7a in invaginating luminal epithelium may possibly happen in one cell, that goes on to expand and form a monoclonal gland. For the purpose of our discussion, this corroborates the notion that Wnt5a mediates suppression of Wnt7a expression.

Uterine grafts planted in wild type female hosts showed that, at diestrus when Estrogen levels are low and Progesterone is high, Wnt7a expression is normal. However, in late proestrus when Estrogen levels are high and Progesterone levels are low, wild type grafts show an Estrogen-mediated suppression of Wnt7a. This Estrogen-mediated suppression of Wnt7a is absent in Wnt5a mutant grafts, again confirming that Wnt7a suppression is Wnt5a-mediated (Mericskay *et al.*, 2004). In section 1.1.3, we discussed examples of how non-canonical Wnt signaling can inhibit canonical Wnt/ β -catenin signaling. We saw how Wnt5a can bind Ror2 (He *et al.*, 2008) to inhibit Wnt signaling, how Wnt5a siRNA depletion activates Wnt signaling in mouse mammary cell lines (Olson & Gibo, 1998) and how Wnt5a overexpression suppresses Wnt signaling in colon cancer cell lines (Topol *et al.*, 2003). Therefore, non-canonical Wnt signaling, driven by Wnt5a, could be the mediator of Estrogen-induced inhibition of Wnt7a. However, the mechanisms of this regulation remain to be elucidated.

2.4 Wnts and Hox genes in FRT development

1. Hox genes

We have so far discussed the role of Wnt7a and other Wnts in development and patterning of the FRT. Generally speaking, when exploring embryonic patterning of organs, we will not have done the topic justice if we overlooked the role of Hox genes. However, this is not the only reason we ought to consider Hox genes, for this fascinating family of genes cooperates intimately with Wnt7a and other Wnts during FRT development. The literature on Hox gene patterning of the FRT, its molecular developmental biology as well as hormonal regulation is rather vast. Discussion here will focus on crosstalk between Wnts and Hox genes.

Hox genes are a family of conserved genes that encode transcription factors, with a distinctive homeobox domain encoding 61 amino acids. The homeodomain motif encodes DNA-binding helices, and recognizes DNA with core sequences TAAT or TTAT. Four families of Hox genes exist in humans and mice: Hoxa, Hoxb, Hoxc and Hoxd. Each family is located on a separate chromosome, and encodes a set of 9-13 genes clustered in an organized tandem arrangement along chromosomes. This tandem arrangement remarkably mirrors the spatial expression of Hox genes along the anterior-posterior axis of an organ or set of organs (Du & Taylor, 2004), specifying positional identity. One such axis is the Mullerian duct, the primordial FRT. Hoxa9, Hoxa10, Hoxa11 and Hoxa13 specify oviduct, uterus, cervix and upper vagina, respectively (Figure 14A).

Hox gene expression is generally such that the most posterior Hox gene is phenotypically dominant and suppresses the more anterior Hox genes; a property known as posterior prevalence (Manzanares *et al.*, 1997). Hox gene expression is considered a switch that transiently activates during development to direct lineage specification, before deactivating. In this respect, the FRT is unique in that it maintains Hox gene expression beyond embryonic development and into adult tissue homeostasis (Du & Taylor, 2004). Why this is the case is an open question. It is conceivable that the FRT is a dynamic set of organs that require rapid and global changes in response to female sex hormones during the menstrual cycle and pregnancy. It is observed that, with aging, female mice lose Hoxa11 expression in the uterus, leading to changes in uterine morphology (C. Miller & Sassoon, 1998) and probably age-related infertility. Therefore, as in development, Hox genes can provide a tool for delivering these rapid and global changes, and so the FRT might be programmed to remain in a constant state of plasticity and molecular receptivity.

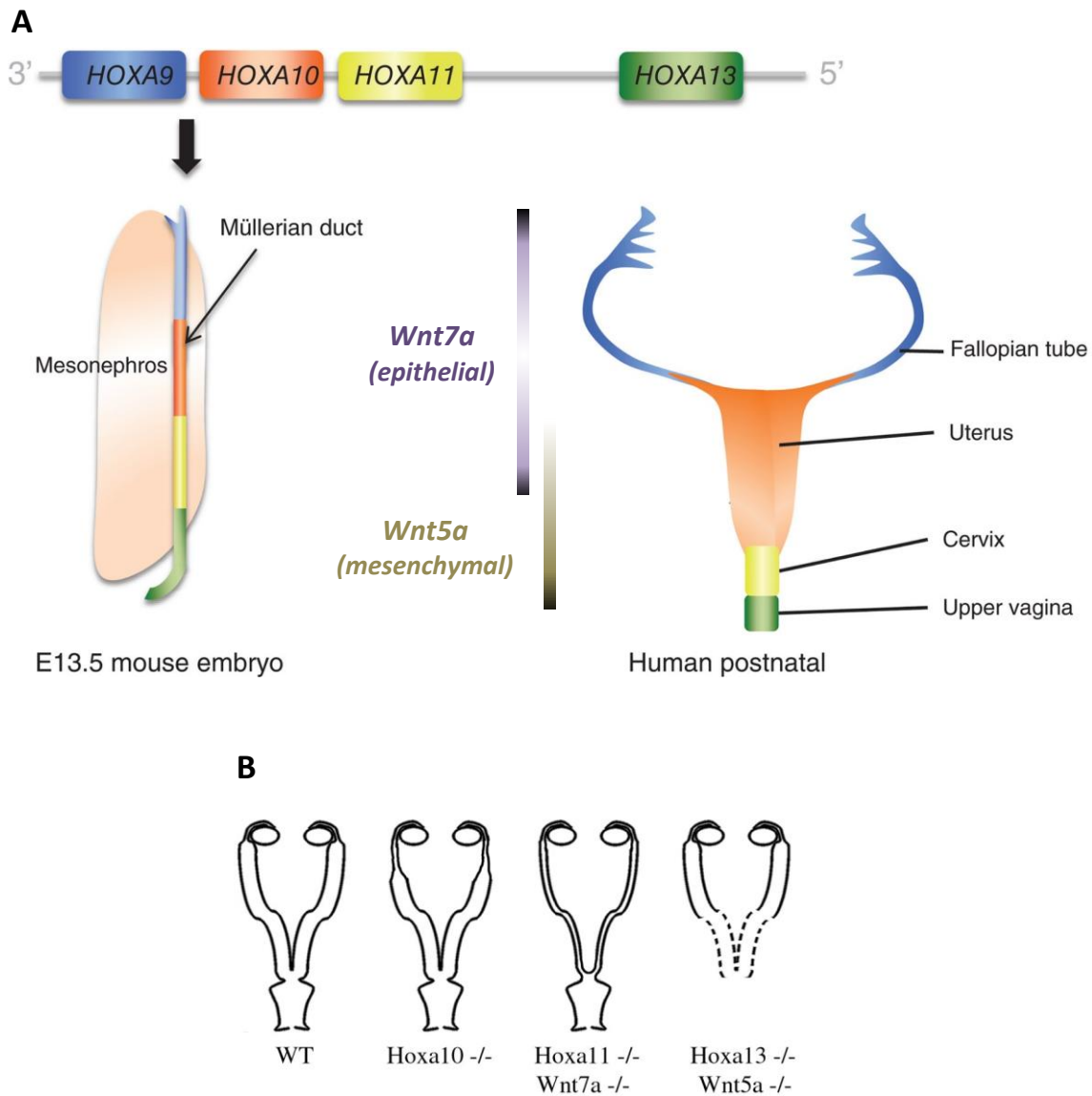


Figure 14: Wnt and Hox genes in FRT development. (A) Colour-coded Hox gene expression in the early mouse Mullerian duct and postnatal human FRT. Hoxa9 is expressed in what is destined to develop into the oviduct (or FT in humans). Hoxa10 is expressed in the developing uterus, Hoxa11 in the posterior uterus and cervix while Hoxa13 is expressed in the posterior cervix and upper vagina (Cheng et al., 2005). The lower vagina originates from a different embryonic origin separate from the Mullerian duct. Wnt7a is expressed in epithelial cells in the anterior FRT. Wnt5a expression is mesenchymal and posterior, overlapping with Wnt7a in the mid uterus. **(B)** Summary of the knockout phenotypes of Wnt and Hox genes on FRT development. Hoxa10 knockout transforms the anterior uterus to oviduct-like structures. Loss of Wnt7a abolishes or severely compromises oviduct development, and leads to FRT posteriorization. Loss of Wnt5a or Hoxa13 abolishes posterior FRT development. [(A) adapted from (Roly et al., 2018), (B) taken from (Mericskay et al., 2004)].

2. Wnts and Hox genes cooperate in patterning the Mullerian duct

One notable observation in Wnt7a KO mice is that expression of Hoxa10 and Hoxa11 is lost postnatally (Miller & Sassoon, 1998). Therefore, FRT posteriorization phenotype in Wnt7a KO mice

could be the consequence of Hox gene posterior prevalence. Indeed, Hoxa10 and Hoxa11 KO mice show similar defects in the anterior FRT as reported for Wnt7a KO mice (Branford *et al.*, 2000). In Wnt7a KO mice, Hoxa9 expression is also significantly reduced (Block *et al.*, 2000), explaining the absence of a functional and properly coiled oviduct. Since anterior Hox gene expression initiates but is lost only postnatally, around the time Wnt7a expression is activated, this suggests that Wnt7a does not initiate but takes over control of maintaining Hoxa10 and Hoxa11 expression from an undefined factor. Recall that *in utero* DES treatment leads to loss of Wnt7a expression and Wnt7a KO phenotypes in mice (McLachlan *et al.*, 1980). DES treatment also represses Hoxa10 and Hoxa11 expression. It is helpful to think of Wnt7a expression and Hoxa10/11 expression as concomitant. It is clear that regulation of this concomitant yet uncharacterized mechanism is dependent on sex hormones.

Hox genes may also explain the Wnt5a KO mouse phenotype, which is characterized by normal development of the oviduct and uterus, but loss of uterine glands, cervix, vagina (Mericskay *et al.*, 2004). In fact, the Wnt5a KO phenotype resembles the Hoxa13 KO phenotype (Warot *et al.*, 1997). Both are clearly concomitant and together specify and pattern the posterior FRT. As previously mentioned, Wnt7a expression is repressed physiologically during proestrus in mice, when Estrogen levels are high, and also upon exogenous supplementation of the Estrogen analogue DES. In a Wnt5a KO background, this repression does not happen. As expected, repression of Hoxa10 and Hoxa11 happens physiologically at proestrus and upon DES treatment, but not in a Wnt5a KO background (Mericskay *et al.*, 2004). It is unclear which gene family, Wnt or Hox gene, acts upstream of the other, but recent single cell data of wild type and Hox9,10,11-mutant uterus suggests that both gene families cross-activate and maintain each other's expression (Mucenski *et al.*, 2018).

Overall, it is evident that Wnt/Hox genes collaborate in patterning the FRT. The anterior-specifying genes are Wnt7a, Hoxa10 and Hoxa11, and are concomitantly regulated. The posterior-specifying genes are Wnt5a and Hoxa13. Genes within each group and between groups collaborate, and are interdependent in the initiation or maintenance of each other. This very same collection of Wnt and Hox genes is involved in the patterning of vertebrate limb, and can serve as a model from which general concepts of this patterning axis can be extrapolated to FRT development (Du & Taylor, 2004). The exception might be that in FRT development, this patterning axis is subject to hormonal oversight.

2.5 Chapter Summary

The Mullerian duct is the primordial anlage of the adult FRT. At birth, the neonatal Mullerian duct is relatively plastic and undifferentiated. In the first week after birth, the Mullerian duct undergoes global changes in response to hormone signaling, as well as spatial expression of Wnt and Hox genes. Oviduct, uterus, cervix and upper vagina develop along the anterior-posterior axis. The oviduct, uterus and cervix epithelium differentiate into simple columnar epithelium. A columnar-squamous transitional zone exists in the cervix, marking the end of the columnar epithelium and the beginning of the stratified squamous epithelium, which lines the lower cervix and vagina. The Mullerian duct mesenchyme plays a central role in directing epithelial patterning of the early FRT. The mesenchyme is also responsible for regressing the duct in the presence of male-specific MIS hormone.

Wnt4, Wnt5a and Wnt7a have been implicated in oviductal and Mullerian duct embryonic development (Miller *et al.*, 1998a). Wnt4 is required for initiation of FRT development. Wnt7a expression is strictly anterior and epithelial, while Wnt5a is posterior and mesenchymal. Wnt5a does not participate in oviduct development, but is important for endometrial adenogenesis and specification of cervix and upper vagina. Wnt7a specifies oviduct lineage. Wnt7a KO mice have no or severely compromised oviducts, are reproductively sterile and show FRT posteriorization. Wnt7a KO uterus lacks glands and acquires vaginal-like epithelium. Wnt7a loss also affects non-epithelial tissues, with marked changes in uterine smooth muscles and lack of expression of mesenchymal MIS receptor, required for regressing the Mullerian duct in the presence of male-specific MIS hormone.

In general, Estrogen promotes Wnt/ β -catenin signaling and proliferation. Progesterone antagonizes these. Exposure of neonatal Mullerian duct to Estrogen (or its derivative DES) phenocopies Wnt7a KO in mice. This suggests Estrogen inhibits Wnt7a expression, and is corroborated by low Wnt7a levels in the Estrogen-dominated proliferative phase of the menstrual cycle. Wnt7a inhibition by Estrogen might be mediated by Wnt5a, which acts as a mesenchymal-to-epithelial signal. Hox genes interplay with Wnts in patterning the neonatal FRT. Hoxa10 and Hoxa11 loss phenocopies Wnt7a KO in mice. Wnt7a KO mice lose expression of Hoxa9, 10 and 11. Posteriorly, Wnt5a and Hoxa13 have a similar relationship, so Wnts and Hox genes cross-maintain each other's expression. Anterior-specifying and posterior-specifying groups of Wnt/Hox genes interact to pattern neonatal FRT. Their expression is maintained beyond FRT development and into adult homeostasis, under hormonal instruction. Given Wnt expression is regulated by hormones, and that Wnt is

involved in FT/oviduct regeneration (section 3.3.3), it is plausible that FT stem cell self-renewal and differentiation is regulated by ovarian hormones.

CHAPTER 3

OVARIAN CANCER & FALLOPIAN TUBE BIOLOGY

OVARIAN CANCER & FALLOPIAN TUBE BIOLOGY

3.1 Ovarian Cancer

Ovarian Cancer is the fifth most common cause of cancer-related death in women. On average, a woman is diagnosed with ovarian cancer every 2 minutes, and up to 150,000 women die from it annually (CRUK, 2017). The seriousness of the disease precipitates upon looking at survival statistics, which remained persistently low with little improvement over the last few decades (Figure 15A). Comparison with breast cancer is often made, as this is also a predominantly female cancer with hormonal involvement and BRCA predisposition. The mortality-to-incidence ratio for ovarian cancer is three times worse than that for breast cancer (Kroeger & Drapkin, 2017). Survival rate from ovarian cancer can be high, up to 90%, if the disease is diagnosed at stage I, when the tumour is limited to the ovary. Upon tumour metastasis to the pelvic region (stage 2), abdomen (stage 3) or beyond the peritoneal cavity (stage 4), survival rate decreases dramatically (Figure 15B). Therefore, there is an urgent and hitherto unmet clinical need for early detection methods using sensitive and specific screening methods.

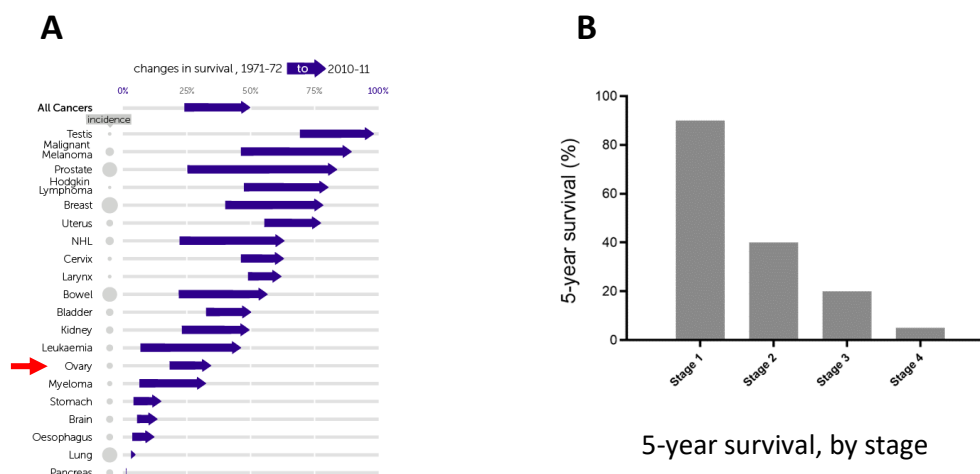


Figure 15: Ovarian cancer survival. (A) Trend of 10-year net survival for various cancers over a four-decade period. Note that survival from ovarian cancer (red arrow) ranks in the bottom third of the list. Improvement in survival has been relatively modest. **(B)** Ovarian cancer net survival, by stage. [(A) taken from CRUK (2014); (B) based on statistical figures obtained from CRUK (2020)].

1. Histological subtypes

Ovarian cancer is an umbrella term encompassing a number of tumour types that grow on and within ovaries, including epithelial, germ cell, sex cord-stromal and other tumour types. Epithelial Ovarian Cancer (EOC) accounts for over 90% of ovarian cancer. EOC itself is subdivided into a number of histological types (or histotypes), which were thought to be molecular subtypes of the same disease, but are now increasingly appreciated to be separate diseases. These are broadly categorized into two groups: type I and type II tumours (Figure 16A). With the exception of clear cell carcinoma, which is ungraded, all type I tumours are low grade.

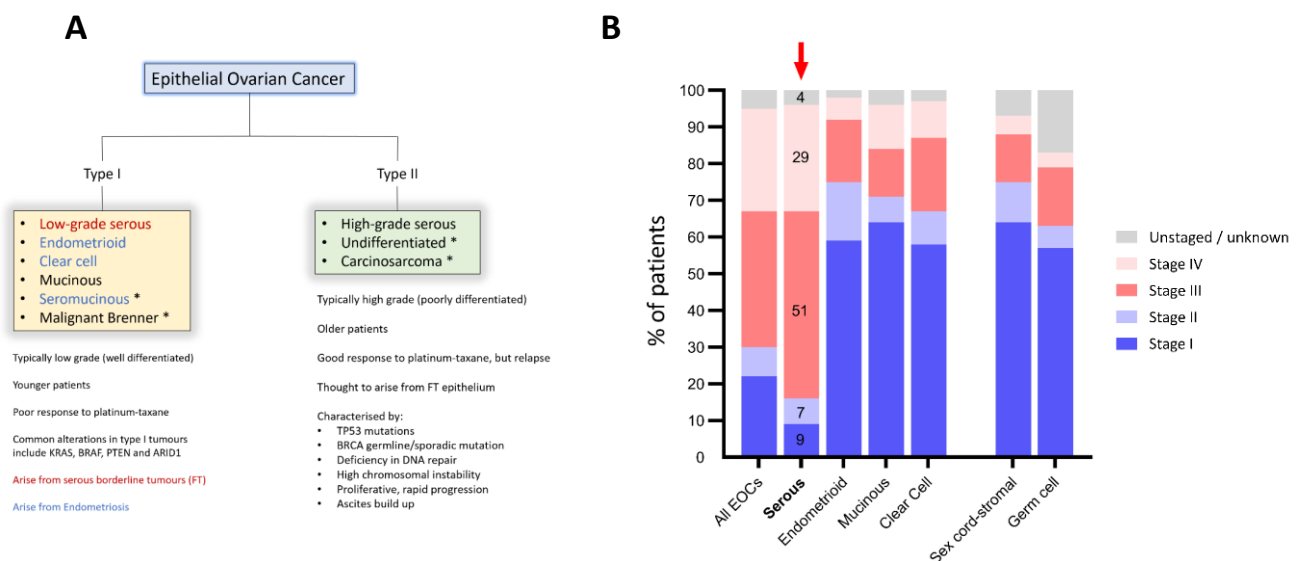


Figure 16: Ovarian cancer histotypes. (A) Histological subtypes of epithelial and non-epithelial ovarian cancer, showing recurrent genetic aberrations. High grade serous ovarian cancer (HGSOC) accounts for over 70% of epithelial ovarian cancer. It is also the most aggressive and exhibits lowest survival. **(B)** Ovarian cancer stage at diagnosis, shown by breakdown of histotypes. For serous histotype, % of patients is shown in numbers within the histogram. The trend of late diagnosis and low survival in EOC is driven by HGSOC. EOC is all epithelial ovarian cancers. Sex cord-stromal and germ cell are non-epithelial ovarian cancers and account for less than 10% of all ovarian cancers. Asterisk * indicates rare malignancies. [(B) based on statistical figures obtained from (Torre et al., 2018)].

Although EOC histotypes grow on ovarian surface, different subtypes morphologically resemble normal cells from the Fallopian tube (serous), endometrium (endometrioid), lower endometrium / cervix (mucinous) or vaginal epithelia (clear cell) (Cheng *et al.*, 2005). Histotypes have separate tissues-of-origin, and each is characterised by its own pathophysiology and a distinct set of genetic and genomic alterations. The majority of HGSOC patients are diagnosed at advanced stages when survival is low. In fact, it is often unhelpful to view ovarian cancer statistics with pooled subtypes, as this averages figures and masks the truly deadly nature of HGSOC, which is responsible for overall late detection (Figure 16B) and low survival (Figure 15B) in ovarian cancer.

It is unclear how EOCs can be so diverse in histotypes, resembling Mullerian-derived tissues in the FRT. Different histotypes are thought to develop from retrograde menstruation, during which cells in menses, normally extruded posteriorly from the uterus to the outside of the body, find their way to anterior FRT and eventually the ovary. Such cells undergo malignant transformation and, depending on their anatomical location in the FRT, a closely associated EOC histotype emerges (Kurman & Shih, 2011). This retrograde menstruation hypothesis argues that the only true 'ovarian' tumours are the non-epithelial ones, namely sex chord-stromal and germ cell tumours, as well as ovarian surface epithelium (OSE)-derived neoplasms as we shall see later.

A second hypothesis argues that all EOC histotypes are OSE-derived, and that resemblance of EOC histotypes to neighbouring tissues is aberrantly acquired during oncogenesis. Different Hox genes are known to be expressed during embryonic development and patterning of the Mullerian duct, as well as maintained in adult FRT (section 2.4 and Figure 14). These FRT-specific Hox genes are not expressed in the OSE, but are seen in the corresponding EOC histotypes. A study showed that aberrant activation of FT, endometrium, endocervix and upper vagina-specific Hox genes produces serous, endometrioid, mucinous and clear cell carcinomas, respectively. This was shown *in vitro* and *in vivo* (Cheng *et al.*, 2005). Although conceptually intriguing, there is little evidence that such ectopic Hox gene activation happens spontaneously during the natural progression of EOC. Overall, the presence of precursor FT lesions in serous EOCs (section 3.3.1), as well as endometriosis lesions adjacent to endometrioid and clear cell EOCs, suggests these malignancies have extraovarian origins. Ectopic HOX gene activation may be more relevant to mucinous carcinoma, for which no precursor lesions are known, and vaginal epithelia are spatially too distant to support a vaginal origin.

3.2 High Grade Serous Ovarian Cancer (HGSOC)

As abovementioned, HGSOC is the EOC histotype with the greatest incidence, and is responsible for 90% of ovarian cancer deaths. Fortunately, HGSOC tumours are highly sensitive to platinum/taxane-based chemotherapy, and many patients initially respond well to treatment. It is not known why this is the case, but it is likely due to the widespread deficiency of DNA homologous recombination (or HR) repair in HGSOC. Although response is initially promising, HGOSC almost always recurs within 18 months (Jayson *et al.*, 2014). Recurrent tumours are amenable to further rounds of chemotherapy. However, chemoresistance eventually arises in 80-90% of patients (Bowtell *et al.*, 2015), leading to bowel obstruction and mortality.

1. Genetic and genomic landscape

Type I tumours display actionable mutations in ERBB2, KRAS and BRAF, which may be targeted with MAPK kinase inhibitors. Unlike type I and other cancers which come with a somatic mutational load sufficiently high to produce actionable mutations or immunotherapy-targetable neoantigens, the somatic mutational landscape of HGOSC is strikingly simple (Figure 17A). TP53 mutations are detected in almost all HGSOCs (Ahmed *et al.*, 2010). The next most frequently mutated genes occur in less than 5% of patients. This includes BRCA1 and BRCA2, which are somatically mutated in only 3.2% and 2.9% of patients, respectively. However, when epigenetic loss of BRCA expression, as well as BRCA germline HGSOC cases are factored in, BRCA1/2 alterations are seen in a third of HGSOCs. HGSOC shows extensive inter-patient genetic heterogeneity. This complicates research efforts to build a consensus model for tumour initiation and progression. The somatic mutational load of HGSOC is actually one of the lowest in comparison to other cancers (Figure 17B). Instead, HGSOC is one of the most genomically unstable tumours (Figure 17C), displaying a remarkable degree of aneuploidy, chromosomal instability (CIN) and somatic copy number alterations (CNAs).

Like all cancers, HGSOCs must have an Achilles' heel, an addiction or vulnerability that can be therapeutically exploited. The Cancer Genome Atlas (TCGA) has extensively analysed 489 HGSOC tumours, using data from exome sequencing, copy number changes, mRNA expression, epigenetic methylome mapping and microRNA expression. Integrated analyses precipitated a set of pathways that are hyperactivated or silenced in a large fraction of HGSOCs, including HR repair, PI3K/Ras, Retinoblastoma (Rb) and Notch pathways, altered in 50%, 45%, 67% and 22% of HGSOC cases, respectively (Figure 17 D-F). Current therapeutic strategies are focused on counteracting HGSOC's dependency on these pathways.

However, HGSOC operates active mechanisms promoting CNAs. CNAs may recurrently affect some genes, including copy number gains in MYC, CCNE1 and PI3K kinase, or copy number losses in PTEN, Rb1 and NF1. Gene duplications (gains) are seen in a fifth of tumours, while the gene losses by CNAs occur in at least 2% of tumours (TCGA, 2011). These frequencies are rather small, so the majority of genes affected by CNAs are, by and large, random and different intra- and inter-patient. These CNA mechanisms provide a platform for clonal selection and chemoresistance, posing a challenge against successful drugging of the pathways that HGSOCs may rely on.

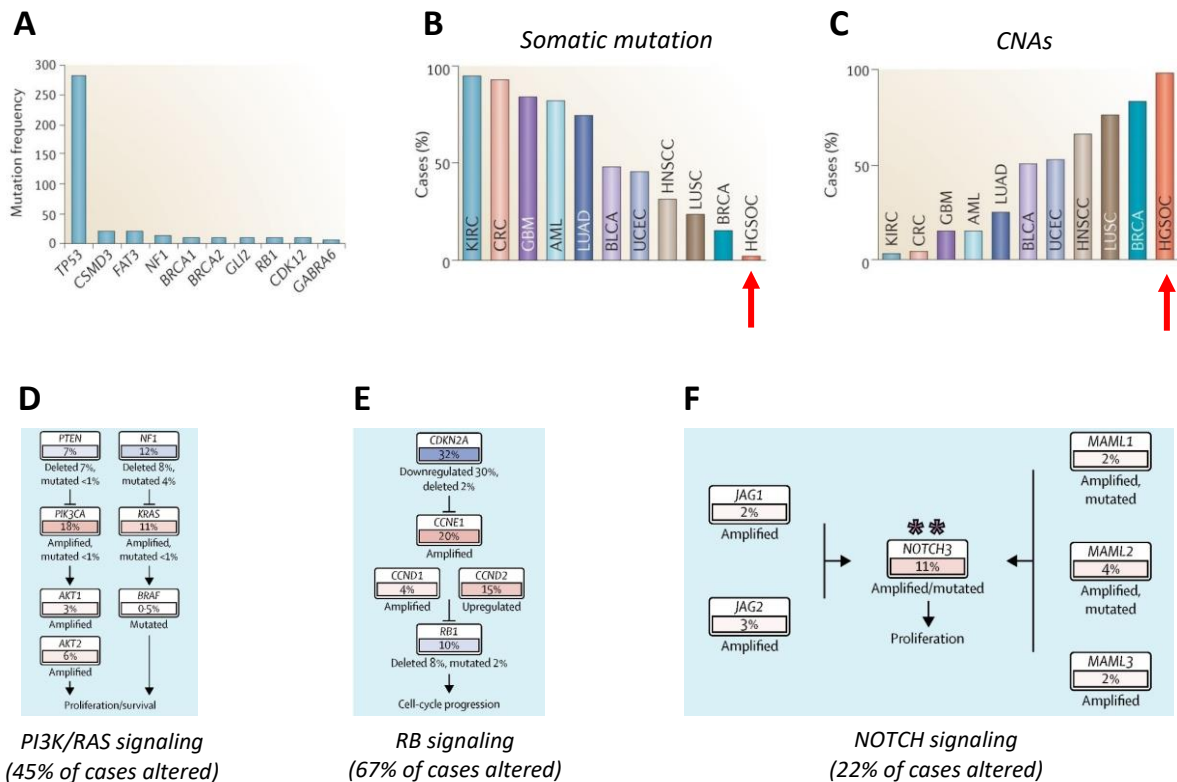


Figure 17: HGSOC genetic and genomic landscape. (A) Genes with the highest frequency of somatic mutations in HGSOC (out of 300 patients). Apart from TP53 mutation, which is near universal, all recurrent somatic mutations are seen in less than 5% of cases. (B-C) Comparison of HGSOC to other cancers in frequencies of somatic mutation (B) and copy number alterations (C). AML (acute myeloid leukaemia), BLCA (bladder urothelial carcinoma), BRCA (breast invasive carcinoma), CRC (colorectal carcinoma), GBM (glioblastoma), HNSCC (head and neck squamous cell carcinoma), KIRC (kidney clear-cell carcinoma), LUAD (lung adenocarcinoma), LUSC (lung squamous cell carcinoma) and UCEC (uterine carcinoma). HGSOC, along with breast cancer, feature a low mutation burden but a remarkably high frequency of CNAs. (D-F) Pathway alterations in HGSOC: (D) activation of PI3K/RAS signaling, (E) upregulation of proliferation through Rb signaling and (F) activation of Notch signaling. Current stratification-based therapeutic strategies focus on counteracting HGSOC's dependency on these pathways. [(A-C) taken from (Bowtell et al., 2015); (D-F) is an adaptation of data from (TCGA, 2011), taken from (Jayson et al., 2014)].

2. Genetic predisposition & BRCA alterations in HGSOC

BRCA stands for Breast Cancer gene. Germline mutations in BRCA predispose patients to breast and ovarian cancer. BRCA1 mutation carriers have an 80% lifetime risk of developing breast cancer by the age of 70 (O'Donovan & Livingston, 2010) and 30-60% risk of developing HGSOC (Bast et al., 2009). For BRCA2 germline mutation carriers, breast and ovarian cancer risk is 50% and 15-30%, respectively. BRCA2 mutations also predispose to male breast, pancreatic, melanoma and prostate cancer. Germline BRCA mutation carriers (who we shall refer to as BRCA carriers) account for 15% of all HGSOC cases, 13% of all basal-like breast cancers and 4% of all luminal-like breast cancer (TCGA et al., 2012). The figures increase if BRCA somatic mutation and epigenetic loss are appended to this.

For HGSOC, this increases from 15% to 33%, and up to 50% when all known components of the HR repair pathway are considered (Figure 18B). Alterations in some genes, such as EMSY and FANCD2, compromise HR repair in a BRCA-dependent way while others, such as PTEN, do so independently of BRCA (Gupta *et al.*, 2009)

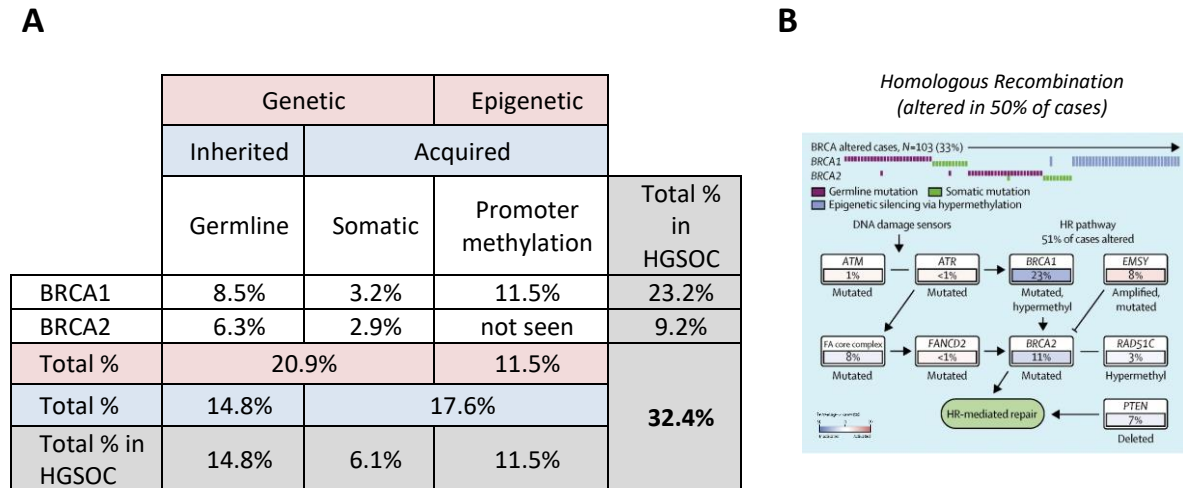


Figure 18: Frequency of BRCA1/2 gene and pathway alterations in HGSOC patients. (A) Breakdown of BRCA alteration frequencies in HGSOC. BRCA1 and BRCA2 loss are largely mutually exclusive. BRCA1 promoter hypermethylation and BRCA1/2 somatic mutations are mutually exclusive. Epigenetic loss of BRCA2 has not been reported. Overall, BRCA alterations are observed in a third of HGSOC cases. (B) HRR deficiency may arise due to alterations in non-BRCA genes, as shown. When rare mutations in these genes are accrued to BRCA mutations, deficiency in homologous recombination as a whole is observed in half of HGSOC patients. For each gene, % indicates alteration frequency, red or blue denote activation or deactivation, respectively, as shown in the key at the bottom left of the figure. Diagrammatic representation of the table in (A) is shown in the upper panel of B. [(A) generated based on numerical figures from (TCGA, 2011), (B) taken from (Jayson *et al.*, 2014)].

BRCA alterations associate strongly with HGSOC. In a study looking at BRCA alterations in EOCs, all samples containing BRCA alterations were HGSOC tumours, and none were observed in endometrioid, mucinous or clear cell carcinomas (Press *et al.*, 2008). It is intriguing that BRCA loss in breast cancer produces pathologically distinct diseases, with BRCA1 associated with basal-like breast cancers while BRCA2 associates with hormone receptor-positive luminal-like breast cancer, respectively (Bowtell, 2010). Not much is known about whether such differences, if any, exist between BRCA1-altered versus BRCA2-altered HGSOCs.

BRCA genes are classic caretaker tumour-suppressor genes, playing a role in maintaining genome integrity. Given similarities in nomenclature, BRCA1 and BRCA2 are often assumed to be members of a protein family, which is not strictly true. In fact, the pair are only 40% similar at the mRNA level and 26% similar at the protein level (based on pairwise sequence alignment). BRCA1s do

not share protein domains, but function as partners in the repair of DNA double-stranded breaks, via the HR repair pathway (Figure 19).

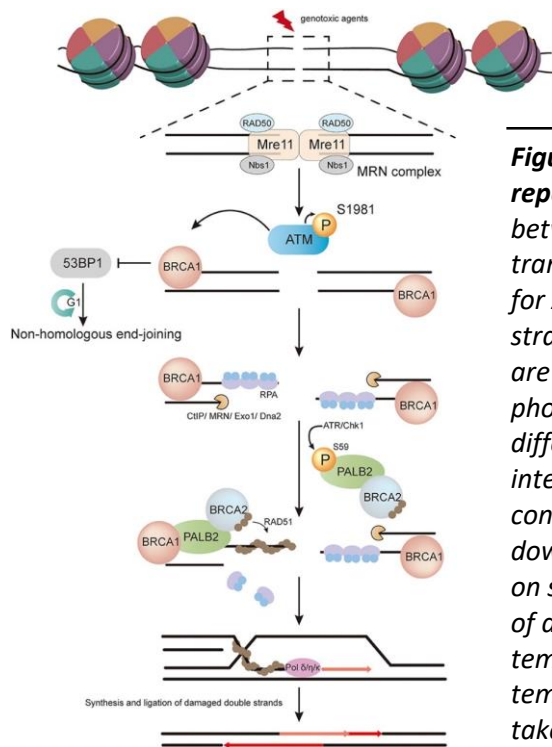


Figure 19: Simplified diagram of BRCA-mediated HR repair pathway. BRCA1 functions as a molecular link between damage sensing and initiation of repair. BRCA1 translocates early to the damage site, and is a substrate for ATR, ATM and CHK2. Different insults yield double-stranded breaks (DSBs) with distinct biochemistries. These are detected by different sensors that activate and phosphorylate BRCA1 on different motifs, eliciting different responses (Venkitaraman, 2002). BRCA1 interacts with the MRN complex to recruit BRCA2-containing complexes. BRCA2 functions more downstream, and acts as a 'loader' of RAD51 recombinase on single-stranded DNA. RAD51 promotes the formation of a nucleoprotein filament that invades into the repair template. This enables cross-strand priming and template-directed error-free repair. HR-based DNA repair takes place largely during the S/G2 mitotic phase, when sister chromatids are in close proximity to avail a repair template to this process. [Taken from Wu et al., 2020)].

3. BRCA and Copy Number Alterations (CNAs)

Double-stranded DNA breaks are repaired preferentially via the HR repair pathway, which is error-free. In the absence of BRCA or essential components of the pathway, resolution of double-stranded breaks is tilted towards another pathway: non-homologous end-joining (NHEJ). As the name suggests, this pathway re-joins broken pieces of DNA. Unlike HR repair, NHEJ is error-prone and leads to loss of intervening bases, or the joining of DNA breaks to other regions of the genome, causing chromosomal rearrangements and CNAs (Gudmundsdottir & Ashworth, 2006). This was shown in early studies, evidencing that loss of BRCA1 or BRCA2 in cell lines drives widespread CNAs and CIN (Patel *et al.*, 1998).

Thence, it is intuitive to assume that the high prevalence of CNAs and CIN seen in HGSOC is due to deficiency in HR repair. However, HR deficiency is absent in 50% of HGSOCs, which also display extensive CNAs. Furthermore, the TCGA (2011) study investigated the level of CNAs in BRCA-wild type versus BRCA-altered cases in HGSOC. Authors found BRCA-altered cases did not exhibit higher levels of CNAs. Other large-scale studies that employed whole genome sequencing, mRNA

expression and copy number analyses of many HGSOC patients did not report increased CNAs in BRCA-deficient tumours, although increased somatic mutations are sometimes observed (Patch *et al.*, 2015). Taken together, these and other studies suggest that BRCA mutation and HR deficiency not drive CNAs in HGSOC, although may promote early disease onset or specific CNA signatures (Macintyre *et al.*, 2018).

4. TP53, BRCA and HGSOC initiation

As we shall see later, the FTs of BRCA carriers are enriched for HGSOC precursor lesions containing TP53 mutation. This, coupled with the near ubiquitous TP53 mutation in BRCA-deficient HGSOCs, suggests that BRCA mutation alone is insufficient to drive tumourigenesis. Theoretically speaking, unresolved DNA damage in BRCA-deficient cells will elicit p53-dependent cell cycle arrest and apoptosis. This will, parenthetically speaking, continue to be happen until BRCA deficiency meets TP53 dysfunction, or dysregulation in cell cycle checkpoint. This concept was validated in mouse models, where Cre-mediated loss of BRCA1 in mouse models causes transformation of the mammary gland, but only when accompanied by sporadic TP53 alteration. Biallelic inactivation of BRCA is embryonically lethal, but rescued upon TP53 mutagenesis (Xu *et al.*, 2001). For BRCA2, the TP53 requirement is more stringent. BRCA2 conditional mutagenesis does not lead to mammary tumourigenesis or sporadic TP53 mutation, but can cause breast tumours when driven in a TP53-mutant background (Jonkers *et al.*, 2001). Although the addiction of BRCA-deficiency to TP53 mutation in tumour initiation is clear in HGSOC and BLBC, it is not universal. A good example of this is luminal-like breast cancer in BRCA carriers, where frequency of TP53 mutation is low (TCGA, 2012).

5. HGSOC, BRCA status and treatment

Although BRCA mutations may not contribute to HGSOC initiation and CNA, BRCA-altered samples show distinct features which are enriched but not unique to BRCA-altered samples (Table 2). BRCA hypermethylation may affect HGSOC genomic landscape in a way different to BRCA mutation. This is not without precedent, as colon cancers from patients with inherited defects in DNA mismatch repair genes have distinct features compared to colon cancers with epigenetic silencing of the same genes (McGivern *et al.*, 2004).

	BRCA status				Reference
	Wild type	Germline	Somatic	Epigenetic	
PTEN / PI3K		decreased PTEN		amplified PI3K	Press <i>et al.</i> (2008)
Overall survival	41.9 months	66.5 months		41.5 months	TCGA (2012)
Myc amplified	24%	49%			TCGA (2012)
CCNE1 amplified	26%	8.2%			Bowtell <i>et al.</i> (2015)

Table 2: BRCA-associated features of HGSOCs. Myc amplification and reduced CCNE1 amplification are enriched in BRCA-altered tumours. Overall survival tends to be better for BRCA-altered than for BRCA wild type patients, although BRCA-hypermethylated patients have lower survival equivalent to BRCA wild type patients. BRCA mutation reversion has been reported in a subset of HGSOC cases and is associated with poor survival (Milanesio *et al.*, 2020). In sporadic HGSOC, epigenetic silencing and somatic mutation of BRCA occur in 11.5% and 6.1% of cases, respectively (TCGA, 2012), pointing to promoter hypermethylation as a more efficient route to BRCA inactivation. Patients with BRCA hypermethylation are younger in age.

Recently, BRCA-mutant tumours were shown to rely on PARP1, which is involved in repair of single-stranded DNA breaks (Amé *et al.*, 2004). Inhibition of PARP in BRCA-deficient cell lines and mouse models causes selective lethality of tumour cells (Farmer *et al.*, 2005), showing up to a thousand-fold sensitivity to BRCA-deficient compared to BRCA-proficient cells (TCGA, 2011). In this respect, HGSOC organoids were shown to be better predictors of PARP sensitivity in patients compared to DNA sequencing. Employing both platforms provided stronger predictive power than either (Hill *et al.*, 2018). Organoids may serve as a useful platform for PARP sensitivity testing, as well as more generally as good predictors of response to treatment in patients of cancer and non-cancer diseases (see section 3.4.2).

Although PARP inhibitors hold promise for treating HGSOC, they are only effective against HR-deficient HGSOCs. Furthermore, chemoresistance mechanisms may arise in similar ways to platinum resistance; for instance, by reversion of germline BRCA mutation (Edwards *et al.*, 2008; Sakai *et al.*, 2008) or even reversion of BRCA hypermethylation (Patch *et al.*, 2015). There is a consensus among ovarian cancer investigators that the single most effective route towards improving HGSOC survival remains the detection of patients at early stages of the disease, by means of specific and sensitive screening tools. Research into early detection can take many forms; a greater understanding of the mysterious steps surrounding HGSOC initiation is undoubtedly a big part of this.

6. Molecular Subtypes of HGSOC

Ovarian cancer can manifest in one of many histotypes, epithelial and non-epithelial, serous or otherwise. Within HGSOC, we saw how heterogeneity can be driven due to BRCA, homologous

recombination status and other factors shown in Table 2 and Figures 17 and 18. Furthermore, in our discussion in the next section, we will see that, although HGSOC is thought to arise from the distal FT, some HGSOCs may arise from the ovarian surface epithelium and are distinct in their features (Löhmußaar *et al.*, 2020).

To add to this complexity, studies have shown that HGSOC categorizes into a number of molecular subtypes (Table 3). The two largest studies, conducted by the Australian Ovarian Cancer Study (AOCS, Tothill *et al.*, 2008) and The Cancer Genome Atlas (TCGA, 2011), discovered that HGSOCs can be molecularly subtyped into a mesenchymal, proliferative, immunoreactive or differentiated type. These molecular subtypes are largely based on expression studies and transcriptomic analyses. To solidify observations, these and other studies integrated transcriptomic subtyping with exome sequencing subtyping, copy number subtyping, epigenetic methylome subtyping and microRNA subtyping. Each transcriptional subtype was shown to associate with one or two subtypes from other platforms, corroborating the robustness of the transcriptional subtyping.

The consensus is that the mesenchymal subtype exhibits the poorest prognosis and survival. Interestingly, none of the subtypes associates significantly with BRCA alterations or HR deficiency (TCGA, 2011; Bowtell, 2010; Hu & Ahmed, unpublished data), rationalizing our hypothesis that BRCA and HR repair alterations may catalyse tumour initiation and endow tumours with specific alterations (Table 2), but BRCA and HR alterations play a limited role in the overall genetic and phenotypic landscape of HGSOCs, the bulk of which is probably driven by TP53-mediated CNAs. A number of challenges arise from tumour subtyping studies and it might be years before HGSOC molecular subtyping can be used to guide treatment in the clinic, but it is a step in the right direction.

Table 3: HGSOC molecular subtypes identified by studies.

Study	Molecular Subtypes	Genes, Pathways, & Features
Australian Ovarian Cancer Study (AOCS) Tothill <i>et al.</i> (2008)	C1 Stromal	↑ High EMC production and remodelling ↑ High ACTA2, FAP, vascular endothelial cells ↑ PECAM1 & PDGFRB ↑ Tissue desmoplasia, myofibroblast infiltration ↓ Low immune infiltration Early relapse, poor prognosis, short survival
	C2 Immunoreactive	↑ Extensive intratumour T-cell infiltration ↓ Low expression of stromal genes Better prognosis and survival
	C4 Differentiated	More mature and differentiated Shares features with serous borderline tumours Intermediate prognosis and survival
	C4 Mesenchymal / Proliferative	↑ high N-cadherin, P-cadherin, Wnt signaling, Hox genes ↑ high in proliferation markers ↓ low E-cadherin, MUCA1, CA125 ↓ low immune infiltration ↓ low MHC class I and II Dedifferentiation Poor prognosis & survival. But better than C1 Stromal subtype (Helland <i>et al.</i> , 2011): Stem-cell associated factors, MYCN, LIN28B, HMGA2, LET-7 family of microRNAs
The Cancer Genome Atlas (TCGA, 2011)	Mesenchymal	↑ Mesenchymal genes ↑ Hox genes ↑ Stromal components (FAP) ↑ Microvascular pericytes (ANGPTL1/2) ↓ Epithelial genes
	Proliferative	↑ Transcription factors HMGA2 & SOX11 ↑ Proliferation markers (eg. MCM2 & PCNA) ↓ Low tumour markers (MUC1 & CA125)
	Immunoreactive	↑ T-cell chemokine ligands (CXCL11, CXCL10, CXCR3) ↑ Myc amplification
	Differentiated	↑ Tumour markers (MUC1 & CA125) ↑ Fallopian tube secretory cell markers (eg SLP1) ↑ Myc amplification
Dana-Farber / Harvard Cancer Center Bentink <i>et al.</i> (2012)	Angiogenic (S1g1)	↑ High angiogenesis genes ↑ High ECM proteins ↑ High cell adhesion 71% of C1 Stromal subtype (AOCS) categorized here 58% of Mesenchymal subtype (TCGA) categorized here 20% of Immunoreactive subtype (TCGA) categorized here

	Non-angiogenic (S1g0)	↑ Single-stranded DNA binding
Konecny <i>et al.</i> (2014)	Mesenchymal	Largely confirmed TCGA molecular subtyping
	Proliferative	Adapted TCGA subtyping & created <i>de novo</i> subtyping
	Immunoreactive	Best prognosis found for Immunoreactive subtype
	Differentiated	↑ B7-H1 & IRF7 genes characterise Immunoreactive subtype Poor prognosis for Mesenchymal & Proliferative subtypes
Yang <i>et al.</i> (2013)	Integrated Epithelial (iE)	↑ Epithelial genes Resembles Fallopian tube epithelium Probably corresponds to Differentiated subtype in other studies
	Integrated Mesenchymal (iM)	Mesenchymal-like Elongated tumour cells Weaker connection between cells Lacks well-organized epithelia All TCGA mesenchymal subtype samples cluster here But other TCGA clusters appear here as well Poor overall survival Suggest mrR-506, a potent EMT inhibitor, as a therapeutic tool
Zhang <i>et al.</i> (2016)	C1 subtype	↑ Immune-activation; ↑ Hormone metabolism
	C2 subtype	↓ Hormone metabolism
	C3 subtype	↓ MAPK pathway
	C4 subtype	↓ Immuno-activation
	C5 subtype	↓ Mesenchymal development; ↓ MAPK pathway
	C6 subtype	↑ MUC family; ↓ Mesenchymal development; ↑ MUC family
	C7 subtype	↑ Mesenchymal development
	C8 subtype	↑ Immuno-activation
	C9 subtype	↑ MAPK pathway
Oxford Classifier (52-gene signature) Derived from FTE cells; applied on HGSOC tumours from external datasets Hu <i>et al.</i> (2020)	Mesenchymal	↑ Enriched in ECM pathways (eg. SPARC & TIMP3) ↑ High in PI3K-Akt signaling ↑ Mesenchymal markers TWIST1, TWIST2 & SNAI2 ↓ Repressed miRNA-200 family (that suppress EMT) Correlates well with poor survival Captures many of the TCGA and AOCS mesenchymal tumours
	Cell Cycle	↑ Enriched in cell cycle genes (MCM2-7, Ki-67, TK1, STMN1) ↑ Elevated DNA repair (FANCD2, FANCI, MSH2) ↑ High in chromatin remodelling (eg. HMGB2 & SMC1A) Rare subtype (1.6%)
	KRT17 subtype	↑ MHC class II genes (e.g. HLA-DQA1, HLA-DPA1, and HLA-DPB1) ↑ Cytokeratins (KRT17 and KRT23) ↑ Aldehyde dehydrogenases (e.g. ALDH1A1 & ALDH3B2) ↑ Upregulation of CDKN1A (also called p21) Seems to be the equivalent of the Immunoreactive subtype in TCGA and AOCS studies.
	Differentiated	↑ RNA synthesis & transport (e.g. PTBP1, ZNF259 & PRPF38A) Shares several markers with Cell Cycle subtype May represent a transient differentiating cell population
	Ciliated	Correlates with low-grade tumours

3.3 The Fallopian Tube

The Fallopian tube (FT) is the anterior-most part of the FRT and is of Mullerian origin. It stretches from the uterus to the ovaries, forming an anatomical channel through which ovulated eggs travel to the womb (Figure 20A). As discussed in Chapter 2: WNT7A and FT development, the FTs are lined with simple columnar epithelium composed of secretory and ciliated cells (Figure 20B). It is in the FT that fertilization occurs, and the zygote travels to the uterus for implantation. Strictly speaking, on humans and great apes have FTs; other animals such as mice do not, and the oviduct here is the equivalent of the FT (Crow *et al.*, 1994).

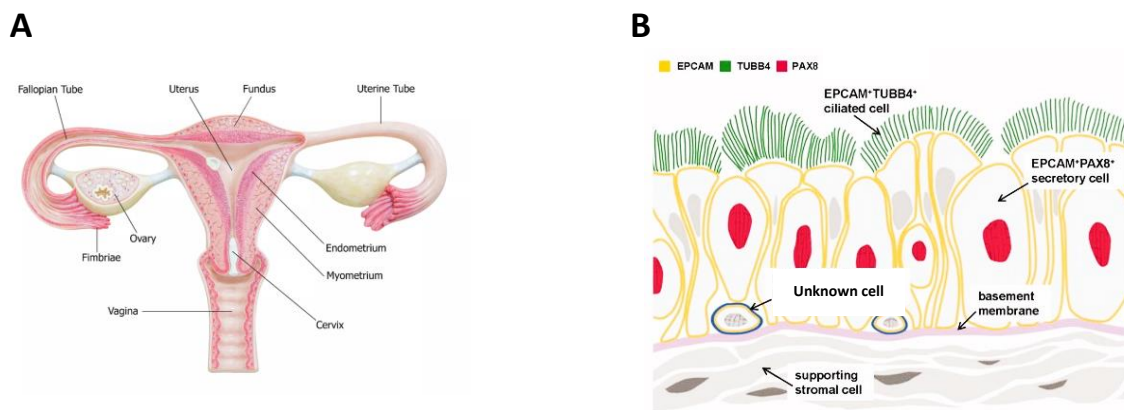


Figure 20: Fallopian tube anatomy and cellular composition. (A) The FTs are part of the FRT. From the distal end near the ovary to the proximal end near the uterus, the FT is divided into 4 parts, namely the infundibulum (with fimbriae at the distal-most end), the ampulla, the isthmus and the intramural part. The fimbria possesses finger-like projections that are in very close proximity to the ovaries. The fimbria is thought to be the site of origin of HGSOC. **(B)** Established cell types in the FT. Secretory cells secrete factors that nourish ovulated eggs or the zygote. Ciliated cells use cilia to beat rhythmically and facilitate transport of the ovulated egg or zygote to the uterus. PAX8 and TUBB4 mark secretory and ciliated cells, respectively. [(A) taken from (Boskey, 2020); (B) adapted from (Paik *et al.*, 2012)].

A limited number of markers exist for identifying the two FT cell types, such as Pax8 and Myc for secretory cells, and β -tubulin 4 (TUBB4) and tyrosinated tubulin that stain the cilia of ciliated cells. FOXJ1 is a master regulator of ciliogenesis in different tissues (You *et al.*, 2004; Yu *et al.*, 2008) and marks FT ciliated cells (shown later, Figure 33A). Unfortunately, none of these markers are cell surface markers, precluding isolation and characterization of these cells. Recently, this thesis has contributed to a study that characterized, at the single cell level, patient-derived non-diseased FT cells (Hu *et al.*, 2020). This has improved our understanding of the cellular biology of the FT, and

uncovered a previously unappreciated heterogeneity particularly within FT secretory cells. However, the study fell short of identifying FT stem cells, which have thus far evaded identification.

1. The Fallopian tube as the tissue-of-origin of HGSOC

HGSOC generally presents with tumour deposits in the ovary, FT, peritoneum, omentum and other anatomical locations. The observation that early stage tumours were confined to the ovary, while metastatic spread increased with progressive disease intuitively pointed to the ovary as the site of HGSOC initiation. BRCA carriers at risk of developing HGSOC undergo risk-reducing prophylactic removal of their ovaries and FTs. A Dutch study documented an interesting observation from these prophylactically-removed FTs. While having no HGSOC or any of its symptoms on the ovary, half of these BRCA carriers harboured pre-neoplastic regions in their FTs (Piek *et al.*, 2001). Later and larger studies documented this in up to 12% of asymptomatic BRCA carriers (J. Kim *et al.*, 2018).

Collectively, these studies identified the precursor lesion of HGSOC, which almost always concentrates at the fimbria: the distal end of the FT near the ovary. The precursor lesion was called STIC, an abbreviation for Serous Tubal Intraepithelial Carcinoma (Figure 21).

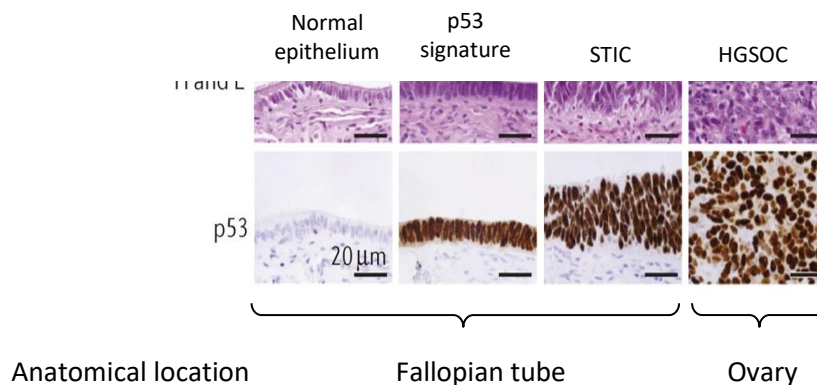


Figure 21: STIC is the precursor lesion for HGSOC. STICs are non-invasive pre-malignant lesions with enlarged nuclei, dark nuclear staining (hyperchromasia), coarse chromatin aggregation, prominent nucleoli, cellular multi-layering and a proliferative index, all prominent morphological features of HGSOCs (Kim *et al.*, 2018). STICs harbour TP53 mutations identical to patient-matched HGSOC (Kuhn *et al.*, 2012). STICs display DNA damage (stain positively for γ -H2AX), expression of p16, FAS, Rsf-1, cyclin E and many features seen in HGSOC (Sehdev *et al.*, 2010), most notably PAX8, the defining marker of FT secretory cells. TP53 mutation and DNA damage precede other morphological features, and cells exhibiting only these two features are referred to as p53 signatures, falling short of the criteria for STIC categorization. TP53 signatures are seen in half of patients containing STIC lesions (Lee *et al.*, 2007). Two-thirds of TP53 signatures are positive for γ H2AX (Lee *et al.*, 2007), suggesting that TP53 mutation precedes DNA damage. Typically, only one TP53 signature or/and one STIC lesion is observed in each patient. STIC = Serous Tubal Intraepithelial Carcinoma. [Image taken from (Bowtell *et al.*, 2015)].

In the normal FT, secretory and ciliated cells are spread out evenly, and intermingle in small alternating groups. Secretory Cell Outgrowths (SCOUTs) are benign but unusual expansions of secretory cells, that stretch for 30 or more cells without interluding ciliated cells. Although SCOUTs are benign, they are enriched in the FTs of HGSOC patients and asymptomatic BRCA carriers, and are thought to precede p53 signatures and STICs (Figure 21). However, SCOUTs do not display DNA damage and only a quarter of them harbour TP53 mutations, sharing the same TP53 mutations as STICs and HGSOC (Chen *et al.*, 2010).

Integrated characterizations of HGSOCs, particularly by the TCGA (2011) and the AOCs (Tothill *et al.*, 2008) studies were useful in profiling HGSOC's genetic and genomic landscape. However, the majority of tumour samples in these studies came from patients with advanced stage HGSOCs. For instance, only 0.05% (TCGA) and 6.3% (AOCs) of tumour samples came from stage II HGSOC patients. For stage I samples, this was 0% (TCGA) and 8% (AOCs) in total. Therefore, excluding one study (Labidi-Galy *et al.*, 2017) which looked at early precursor lesions but only in 5 patients, no detailed and large-scale studies have thus far managed to capture and characterize the early changes in HGSOC, limiting our understanding of HGSOC initiation.

Several lines of evidence indicate that STICs are indeed the precursor lesions of HGSOCs. Some of these evidences are summarized in Table 4. Although the association of p53 signature, STIC and HGSOC is compelling, there are cases of STICs that are not accompanied by HGSOCs, particularly amongst BRCA carriers. Although this could suggest some STICs do not progress to HGSOC, it can also mean that these STICs were intercepted *en route* to forming HGSOC. In fact, studies have estimated a 7-year window of progression between the pair (Labidi-Galy *et al.* 2017), suggesting an opportune window of intervention had there been an effective screening tool.

Evidence	Study
STICs are found in the FTs of sporadic HGSOC patients (up to two-thirds of patients)	Howitt <i>et al.</i> (2015) Chen <i>et al.</i> (2017)
STICs are found in the FTs of BRCA carriers with HGSOC (up to one third)	Howitt <i>et al.</i> (2015)
STICs are found in prophylactically removed FTs from asymptomatic BRCA carriers (up to 11.5%)	Finch <i>et al.</i> (2006) Shaw <i>et al.</i> (2009)
Salpingectomy (surgical removal of the FT) significantly reduces the risk of developing HGSOC in BRCA carriers	Falconer <i>et al.</i> (2015) McAlpine <i>et al.</i> (2014)
STICs contain many of the alterations that appear early in HGSOC	Labidi-Galy <i>et al.</i> (2017) Yamamoto <i>et al.</i> , (2016)
STICs and HGSOCs show intra-patient similarity in copy number alterations	Salvador <i>et al.</i> (2008) Labidi-Galy <i>et al.</i> (2017)
HGSOC is histologically and morphologically more similar to normal FT than to OSE	Li <i>et al.</i> (2012)
Transcriptomic profile of HGSOC resembles that of normal FT, not the normal ovary (most notably Pax8, expressed in normal FT and HGSOC, but not OSE)	Ducie <i>et al.</i> (2017) Xiang <i>et al.</i> (2018)

Non-genetic heterogeneity of normal FT cells reflect molecular subtypes of HGSOC	Hu <i>et al.</i> (2020)
Identical TP53 mutation is always seen in STICs and patient-matched HGSOC	Kindelberger <i>et al.</i> (2007) Kuhn <i>et al.</i> (2012)
Fallopian Tube cancer has similar genomic features and pathogenesis to HGSOC	Tone <i>et al.</i> (2008) Marquez <i>et al.</i> (2005)
Engineering HGSOC mutations specifically in the oviduct leads to formation of STIC and HGSOC in mouse models	Perets <i>et al.</i> (2013) Kim <i>et al.</i> (2012)
Engineering these mutations in oviduct organoids and orthotopic transplantation into mouse bursa leads to HGSOC	Zhang <i>et al.</i> (2019); Löhmusaar <i>et al.</i> (2020)

Table 4: Summary of selected studies identifying STICs as the precursor lesion of HGSOCs.

On the other hand, there are cases of HGSOCs that are not accompanied by STICs. This may be explained by scrutinizing STIC detection protocols, which may be missing a fraction of STIC lesions. Indeed, initial reports were shown to have missed a large proportion of STICs in sporadic and familial HGSOCs (Kurman & Shih, 2016). This was the case until the introduction of the SEE-FIM protocol for STIC detection. SEE-FIM stands for Sectioning and Extensively Examining the Fimbria and entails sectioning the fimbria into multiple longitudinal regions, with each region sectioned vertically at every 1mm interval. This has greatly improved the chances of STIC detection (Medeiros *et al.*, 2006), but is not exhaustive enough to capture such a localized region of 10-50 cells.

Another reason that can lead to under-reporting of STICs is staining protocols. STICs always harbour TP53 mutations, which manifest as an overexpression of TP53 (Figure 21). Many studies identify STICs by TP53 immunohistochemistry (IHC). However, although two thirds of patients harbour TP53 missense mutations that produce mutant overexpressed protein detectable by IHC, one-third of patients harbour null TP53 mutations that produce no protein expression (TCGA, 2011). Thus, up to a third of STIC lesions are missed when employing TP53 IHC for STIC detection. Stathmin and p16 (Novak *et al.*, 2015), as well as laminin γ 1 (Kuhn *et al.*, 2012) show promise in IHC detection of TP53-null STIC lesions, but these are not routinely employed in the clinic yet. Finally, although the vast majority of STICs localize at the distal end of the FT, a minor fraction of STICs (estimated at around 5-10%) occur at more proximal FT regions, which are not accounted for by most studies. Overall, these limitations suggest that STICs are under-reported, and their association with HGSOC may be under-appreciated.

Lack of STICs in the FTs of all HGSOC patients could also be explained by ovarian endosalpingiosis (Bristol-Gould *et al.*, 2005), a rare process in which FT-like cells are observed in the ovary, probably as a consequence of cell seeding or shedding. This shedding process may be promoted by ovulation, which bathes the fimbria with Follicular Fluid (FF) rich in pro-inflammatory

and pro-mitotic factors, including EGF, Estrogen, FSH hormone, LH hormone and TGF (Revelli et al., 2009). Hyaluronic Acid, another factor in FF, promotes cell-matrix detachment and activates expression of ECM degrading enzymes (Yu & Stamenkovic, 1999; Zhu et al., 2006). Its receptor, CD44, is widely expressed in the distal FT (Hu et al., 2020). Therefore, Ovarian endosalpingiosis of a STIC lesion will see its complete transfer to the ovarian surface, leading to HGSOC initiation and leaving no trace of the STIC in the FT (Bowtell *et al.*, 2015). A more common prelude to this outcome is STIC seeding into the ovary upon ovulation. This creates cortical inclusion cysts in an inflammatory environment, leading to export of the oncogenically-induced STICs to the OSE. This is consistent with statistical evidence showing that decreased ovulation, either through natural (pregnancy and lactation) or induced (using oral contraceptive pill) methods significantly decreases the risk of HGSOC (Kurman & Shih, 2011).

Finally, STIC-negative HGSOCs can be explained by assuming a non-FT origin for this fraction of HGSOC. The most likely candidate in this respect is the OSE (Cheng *et al.*, 2005), which was shown to possess the potential of transformation in mouse models (Flesken-Nikitin *et al.*, 2003). However, unlike FT-derived STICs, early precocious HGSOC lesions were not reported for the ovary. Furthermore, a multi-centre study employed DNA sequencing, copy number analysis, miRNA data and gene expression platforms to conduct a comparative analysis between HGSOC tumours in patients with versus without STICs. The study found no substantial differences between STIC+ and STIC- HGSOC tumours (Ducie *et al.*, 2017). The study also found that the molecular profiles of STIC- HGSOC, like those of STIC+ HGSOC, are much more similar to normal FT epithelium than to normal OSE. Crucially, the demographic and clinical outcomes of both tumours are also very similar. Overall, these studies, supported by culminating evidences (Table 4), STIC under-reporting and the precocious nature of STICs relative to advanced HGSOC (Labidi-Galy *et al.*, 2017), suggest that on balance, the evidence lends significant credence in support of a tubal origin for HGSOC.

2. Cell-of-origin of HGSOC remains unidentified

Although the tissue-of-origin of HGSOC is now known, the cell-of-origin of HGSOC remains obscure. STICs stain positively for Pax8 and MUC1 (Bowtell, 2010), both markers of FT secretory cells. Mouse models support the notion that STICs and HGSOCs are the consequence of neoplastic transformation of FT secretory cells (Perets *et al.*, 2013; Kim *et al.* 2012). At the gene expression level, HGSOCs represent the transcriptomes of normal FT secretory cells, not ciliated cells (Karst *et al.*, 2014; Zhang *et al.*, 2015). These and other studies point to the FT secretory cells as the cell-of-origin of HGSOCs. However, there are multiple cell types of secretory cells (Hu *et al.*, 2020), and a minority of these were shown, by *in vivo* lineage tracing, to possess multipotent potential, giving rise to terminally

differentiated secretory and ciliated cells (Ghosh *et al.*, 2017). No studies, research efforts or reports have yet pinpointed which FT secretory cell type is the TIC of HGSOC. Among other reasons, this is due to the lack of knowledge about the cell types residents in the FT.

However, STIC localization might provide a clue. We mentioned that STICs, by and large, concentrate at the fimbria, the distal end of the FT. A number of studies have identified the fimbria as a FT region enriched in stem-like cells, possessing longevity and multipotency (Erickson *et al.*, 2013; Ghosh *et al.*, 2017; Paik *et al.*, 2012; Xie *et al.*, 2018; Wang *et al.*, 2012; Zhu *et al.*, 2020). This is consistent with the fimbria as the distal-most region of the FT, susceptible to ovulation-mediated inflammatory damage, which requires regeneration at periodic intervals.

In section 3.2.3, we discussed how TP53 mutation is responsible for driving CNAs and HGSOC initiation, although BRCA or HR deficiency may play a role in facilitating this process. In essence, loss of TP53 or BRCA here represents dysfunction in cellular caretaker functions. Tumourigenesis is a process that ensues upon dysregulation of a cell's caretaker and gatekeeper functions. In other words, tumourigenesis is a process where genetic instability meets hyperproliferation, and the pair provide a platform for an iterative process of mutagenesis and clonal selection. Constant proliferation is a feature of cancer cells. As a matter of fact, four of the six hallmarks of cancer initially proposed by Hanahan & Weinberg (2000) pointed to this feature, including self-sufficiency in growth signals, insensitivity to anti-growth signals, limitless replicative potential and evading apoptosis. Genetic and genomic instability were included as hallmarks of cancer in a revised version of these hallmarks a decade later (Hanahan & Weinberg, 2011).

For HGSOC, TP53 mutation and less frequently BRCA alterations, provide a molecular path to loss of caretaker function. However, it is not yet clear how the gatekeeper function is lost, to promote constant cell division in an otherwise senescent / differentiated cell. Our exploration of Wnt signaling, ASCs and cancer in section 1.5 points to the possibility that gatekeeper function need not require to be lost, if genetic instability can target an already actively and constantly cycling cell. In many tissues, slow and long-term cycling under homeostatic conditions is a characteristic unique to tissue-resident stem cells. Coupled with the unusually specific localization of STICs to stem cell-enriched regions of the FT, this provides a rationale for interrogating whether stem cells provide an abode for TP53/BRCA mutations, and nest their decades-long nurture towards HGSOC.

In the hypothesized progression model from SCOUTs, through to p53 signature and STIC and ending in HGSOC, SCOUTs are the earliest precursor lesion and the only entity not significantly associated with TP53 mutation and CNAs, but shows expansion and proliferative capacity. If true, this

model proposes that loss of gatekeeper function already exists and precedes loss of caretaker function induced by TP53 mutation. SCOUTs are therefore cells capable of proliferation and expansion under benign conditions, and are thought to represent aberrant stem cell outgrowths (Yamamoto *et al.*, 2016). This further strengthens the rationale for interrogating the role of FT stem cells in HGSOc initiation.

The Helene Harris Memorial Trust (HHMT) forum is an international 4-yearly forum that convenes the world's leading ovarian cancer experts. In its 2015 meeting, focused on HGSOc, investigators and scientists highlighted the pressing need for understanding the origins of HGSOc, stating:

“A better understanding of the normal cellular biology of FTSECs as a whole, including growth factor requirements and determinants of self-renewal, is warranted to help interpret how specific mutations and copy number changes affect the behaviour of HGSOc.”

Unfortunately, however, it has thus far remained a challenge to investigate the role of FT stem cells in HGSOc initiation. This is because FT stem cells have not been identified or characterized yet.

3. Fallopian Tube Stem Cells remain unidentified

A number of studies have indicated the presence of FT stem cells. A report noted that mouse oviducts contain a population of label-retaining cells (LRCs) at the distal/fimbrial end of the oviduct (Snegovskikh *et al.*, 2014). Another study corroborated this finding using Tet-inducible histone 2B-GFP mice. In this study, LRCs persisted for 12 weeks (progenitor LRCs) or long-term for 47 weeks (stem-like LRCs), and were only seen in the distal oviduct. None were observed in the proximal oviduct. Progenitor LRCs possessed 71-fold higher spheroid-formation efficiency (Wang *et al.*, 2012). Stem-like LRCs are ER- PR- CD44- while progenitor LRCs are ER+ PR- CD44+. The study did not employ transcriptomic profiling of LRCs, nor investigated LRCs multipotency *in vitro* or *in vivo*. Another report also associated stemness with CD44+ cells in human FT (Paik *et al.*, 2012). However, these epithelial CD44+ cells showed a modest 5-fold increase in spheroid-forming capacity, albeit multipotency here was confirmed. The basal FT cell type claimed to be stem cells (Figure 20B) were shown, by this thesis and others, to be infiltrating lymphocytes (see Figure 36).

Conclusive evidence for the presence of FT stem cells came from a Pax8^{rtTA} TetO^{Cre} YFP^{fl/fl} mouse model. This allows doxycycline-inducible labelling of oviduct secretory cells. Authors confirmed that ciliated cells emerge from Pax8+ secretory cells. Using another inducible mouse model, Pax8^{rtTA} TetO^{Cre} Ctnnb1^{fl/fl}, authors showed that oviduct regeneration is partially compromised when β -catenin loss is induced specifically in secretory cells (Ghosh *et al.*, 2017). However, this compromise is minor, by a factor of 1.5-2 compared to controls, unlike the major abnormalities seen

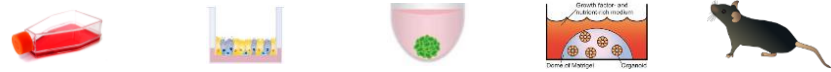
in Wnt-dependent organs. Authors did not account for Estrogen signaling, which might activate Wnt target gene expression independently of β -catenin (section 2.3 and Figure 69 D-G in Results chapters).

Given these observation, the dominant role of Wnt signaling in ASC biology in various organs (section 1.4) and the role of Wnt signaling in Mullerian duct development (chapter 2), it may be conceivable that oviduct regeneration is driven by Wnt-active stem cells. On this basis, Ng *et al.* (2014) performed Lgr5 lineage tracing in mice, showing that during Mullerian duct development, Lgr5+ cells appear in the anterior-most part of the Mullerian duct. Lgr5+ cells contribute to development. However, Lgr5 expression disappears by day 7 after birth (Seishima *et al.*, 2019). In adult mice, Lgr5+ cells were detected at the ovary-ligament-fimbria junction, but these Lgr5+ cells did not contribute to oviduct homeostasis (Ng *et al.*, 2014). As we saw earlier, organs with low turn-over such as the liver, pancreas and kidney, express Lgr5 only upon injury-mediated repair. This could be the same for the oviduct, which is a slowly cycling organ. Furthermore, we saw that Axin2 may be a better marker than Lgr5 in labelling homeostatic Wnt-active stem cells in some tissues, such as the uninjured liver (Wang *et al.*, 2015). Therefore, alternative strategies for marking Wnt-active cells should be investigated in the mouse oviduct, before the presence of Wnt-active stem cells can be definitively ruled out.

Interestingly, whereas Lgr5 lineage tracing did not detect Lgr5+ cells in the adult vagina (Ng *et al.*, 2014), Axin2 lineage tracing identified a self-renewing subpopulation of Axin2+ basal vaginal cells. Vaginal Axin2+ cells reconstitute the entire vaginal epithelium and possess enhanced organoid formation efficiency (Ali *et al.*, 2020). Again, this underscores the importance of considering alternative marking strategies of Wnt-active cells. In the endometrium, Axin2+ Wnt-active cells were also shown, by *in vivo* lineage tracing, to be long-lived bipotent stem cells, giving rise to endometrial ciliated and secretory cells in the gland epithelium, as well as in luminal epithelium in the long run (Syed *et al.*, 2020). Given what we know about the primordial origin, embryonic continuum and co-development of the oviduct and uterus (see Chapter 2), as well as the above-mentioned effect of inducible β -catenin loss on oviducts *in vivo*, it is conceivable that Axin2 marks FT/oviduct stem cells, but this is yet to be evidenced. However, these and other studies helped narrow down the search for FT stem cell, which are now thought to be a subset of secretory cells with potential Wnt-activation. Deeper characterisation of these cells is urgently required to understand their contribution to normal tissue homeostasis, and their role in STIC formation and HGSOc initiation.

3.4 Organoids: the next generation of *in vitro* modelling

The lack of knowledge about FT stem cells is perhaps a direct consequence of the lack of reliable models of FT biology. In its 4-yearly report, the Helene Harris Memorial Trust ranked the development of better experimental and three dimensional models of HGSOC near the top of its list of priorities for reducing incidence and improving outcomes in HGSOC (Bowtell *et al.*, 2015). The FT remains understudied and under-researched in relation to other organs. In the last decade, interest in FT biology has re-kindled following a plethora of reports implicating the FT in HGSOC pathogenesis (Table 4). Subsequently, various studies pioneered FT models that can be harnessed in understanding HGSOC initiation. It is beyond the scope of this thesis to comprehensively elaborate on these. However, broadly speaking, FT models can be categorized into one of five categories. As shown in Table 5, these models have different advantages and drawbacks.



	2D culture	Air-Liquid Interface culture	Spheroid culture (suspension)	Organoid culture	<i>In vivo</i> models
Longevity	short-term (1-2 months)	short-term	medium	long-term	long-term
Ease of handling	easy	medium ease	medium ease	medium ease	highly technical
Incubation times	short	long	medium	medium	very long
Cost	cheap	affordable	cheap	affordable	Expensive
Human-derivation	yes	yes	yes	yes	mouse-specific
Dimensionality	2D	2D	3D	3D	3D
Mature FT features (e.g. invaginations)	no	no	no	yes	yes
Cell-matrix interactions	no	limited	no	yes	yes
Epithelial cellular heterogeneity	no	yes	yes	yes	yes
Non-epithelial microenvironment	no	may be possible	no	possible	present
Model complexity					
Example studies	Karst & Drapkin (2012) Shan <i>et al.</i> (2012) Jazaeri <i>et al.</i> , (2011)	Zhu <i>et al.</i> (2020) Levanon <i>et al.</i> (2010) Fotheringham <i>et al.</i> (2011) (Y. Yamamoto <i>et al.</i> , 2016)	Paik <i>et al.</i> (2012) Lawrenson <i>et al.</i> (2013) Chang <i>et al.</i> (2020)	Kessler <i>et al.</i> (2015) Xie <i>et al.</i> (2018) Hoffmann <i>et al.</i> , (2020)	Perets <i>et al.</i> (2013) Kim <i>et al.</i> (2012) Wang <i>et al.</i> (2012) Huang <i>et al.</i> (2015)

Table 5: Approaches for modelling FT biology *in vitro*. Each culture method has its advantages and drawbacks, as shown. Advantages are shown in green boxes. Traditional 2D culture homogenizes cellular heterogeneity and exhausts the stem cell compartment, including in the FT (see Figure 25). 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, casting doubts on its physiological relevance. Air-liquid interface approaches produce well-differentiated cultures that can be effective in modelling stem cell self-renewal and differentiation. However, they are single-use cultures that cannot be expanded, 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. *In vivo* lineage tracing methods are the gold standard for evidencing stem cell activity, but are expensive, highly technical and require protracted incubation times. Organoids are a miniaturized form of organs, capturing some of their *in vivo* complexities while maintaining the convenience of *in vitro* models. As such, organoids bridge the gap between simplistic and inadequate *in vitro* models and expensive highly-technical *in vivo* models.

Generally speaking, effective research on stem cell biology necessitates the utility of a model that is capable of reliably re-constructing the stem cell niche *in vitro*. Current preclinical models suffer from deficiencies in dimensionality, tractability and longevity. *In vivo* lineage tracing is the gold standard for evidencing endogenous stem cell activity of a population. However, mouse transgenics

is highly technical, expensive and not amenable to the high throughput preliminary investigations often required before embarking on mouse experiments. Further research on FT stem cells requires *in vivo* strategies targeting oviduct-specific stem cells; these are largely lacking. Amongst these models with variable technical limitations, organoids emerge as a powerful tool that bridges the gap between inadequate and simplistic *in vitro* models and expensive highly-technical mouse models.

1. What are Organoids?

Organoids are primary tissue-derived, three-dimensional cultures that are self-organizing and self-renewing in nature. Organoids are stem cell-driven cultures that can propagate without genetic immortalization, keeping them in a native state. Organoids strikingly resemble the tissues from which they derive, in overall architecture, cellular heterogeneity and tissue biology (Figure 22). Organotypic cultures have been optimised for various organs, from several species, including human and mouse tissues (Nusse & Clevers, 2017).

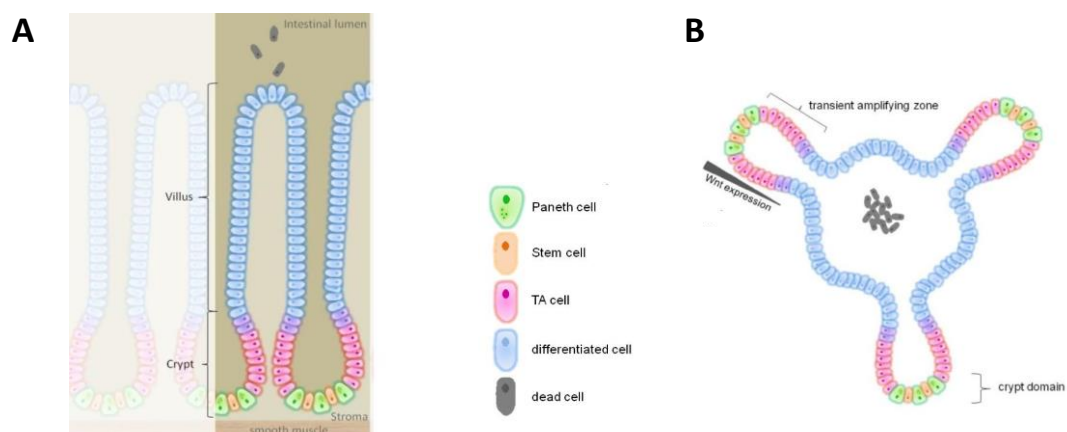


Figure 22: Prototype of organoids, first shown for intestinal tissue. Organoids are *in vitro* mini organ-like structures with long-term and stable growth, sustained by stem cell self-renewal and differentiation in a 3D gel matrix. Organoids mimic structural and functional features of tissues they derive from. Shown here is a schematic intestinal organoid, which was the first 3D organotypic culture to be established a decade ago. *In vivo* cellular diversity and organization in the native tissue (A) is reflected in the tissue-derived organoid (B). [Taken from: de la Roche (2014)].

Organoid culture techniques have attracted a multitude of applications. Tissue-specific organoids can now be derived from embryonic stem cells, induced pluripotent stem cells or adult stem cells (ASCs). ASCs-based organoids are established from matched healthy and diseased tissue (such as cancer), and have been heavily characterized at genomic, transcriptomic, proteomic and epigenomic levels, to further our understanding of disease mechanisms. Broadly speaking, organoid

cultures are in essence stem cell-driven cultures, and thus an early application of organoids was to further our understanding of stem cell biology in multiple organs. Other applications include modelling tissue biology, drug screening, modelling host-pathogen interactions *in vitro*, biobanking, as well as for personalized and regenerative medicine.

Organoids were initially established as epithelia-only cultures. Although they advanced our understanding in various fields, they were devoid of microenvironmental components. Recent advances have attempted to address this limitation. Recently, it was shown that induced pluripotent stem cells could be employed to generate tissue-specific organoids containing all three germ layers. These complex organoid cultures were composed of mesenchymal and beating neuronal innervations, that self-organized as seen in the *in vivo* setting. This was shown for gut (Well, 2020, unpublished data) and skin (Koehler, 2020, unpublished data) tissues; the latter is the first report of hair-generation in skin organoids.

At the clinical level, several studies have shown that patient-derived tumour organoids are good predictors of patient response to clinical treatment (Ooft *et al.*, 2019; Vlachogiannis *et al.*, 2018; Yao *et al.*, 2020), paving the way for organoid-based personalized cancer medicine. We've alluded earlier to how HGSOC organoids predict patient response to PARP inhibitors (Hill *et al.*, 2018). A remarkable success story for organoids was seen in cystic fibrosis research. Patients with this disease inherit mutations in the CFTR gene, causing life-long respiratory and digestive complications. Several drugs are in clinical use, targeting the most common mutations, but effective patient stratification for drug treatment was lacking. Normal intestinal organoids were shown to swell upon treatment with Forskolin, but those of CFTR patients do not. Treatment with drugs not only restore swelling in CFTR patient-derived intestinal organoids, but this so called 'swelling assay' is now a reliable organoid-based assay for predicting individual patient response to a number of cystic fibrosis drugs (Dekkers *et al.*, 2013; Dekkers *et al.*, 2016), showcasing the power of organoids in personalized medicine. Finally, gut organoids were successfully harnessed to repair damaged gut tissue in mice, due to colitis, inflammatory bowel disease and other gut pathologies (Sato, 2020, unpublished data). Separately, the Tokyo Medical and Dental University in Japan is conducting the first organoid-based clinical trial in regenerative medicine, whereby GMP- and clinical-grade organoids will be generated from patients, expanded in suspension using industrial-scale production and re-injected into patients with gut pathologies (Watanabe, 2020, unpublished data).

In comparison to more established culture methods such as traditional 2D culture, spheroids and *in vivo* models, organoid culture technology is a relatively recent advent in tissue modelling. A major limitation of organoid technology is the lack of expertise in the technique, as well as the lack of

confidence in the fidelity of organoid modelling of human tissue. To address these issues, a major programme was launched as part of the Human Cancer Models Initiative (HCMI), to generate a pan-cancer organoid biobank annotated with clinical data, characterized in tandem with the parent tumours and detailing streamlined protocols for organoid generation (Ledford, 2016). This is a partnership between ATCC, Hubrecht Organoid Technology (HUB), Sanger Institute, Broad Institute, Spring Cold Harbours and others, and pertains only to tumour models. To address the question on fidelity of organoids to model normal human tissue, the Organoid Cell Atlas was launched in December 2020 as a 'biological network' within the Human Cell Atlas project, to deeply characterize normal non-diseased human tissue and derived organoids at the single cell level (Bock *et al.*, 2020). Another aim of the Organoid Cell Atlas is to improve organoid protocols by, for instance, increasing differentiated cell types, including microenvironmental components, etc. relative to human tissue. Both of these initiatives promise to create highly-curated, single cell resolution maps of organoids from multiple tissues, and better improve our understanding of how closely organoids model primary tissue.

2. Organoids for modelling FT and HGSOc biology

Organoid research is a relatively recent player in FT and HGSOc research. The first study to report establishment of organoid culture technique for the FT was published 5 years (Kessler *et al.*, 2015). Although a significant step forward, the reported protocol is poorly optimized, includes undefined differentiation-inducing factors and in its current format is not amenable to isolation of specific populations of cells within organoids, or to single cell organoid approaches (Table 13 in Results). These aspects are critical for isolation and characterisation of putative stem cells, as well as interrogating the role of these cells in HGSOc initiation. In this thesis, I build on this protocol and optimise it further. I use the optimised protocol to interrogate requirements of stem cell self-renewal *in vitro*. Upon identification of stem cell character, I employ genetic labelling of stem cells within live organoids, for further isolation and characterisation using RNA FISH, single cell technologies and functional approaches. In contrast to the human protocol, the published mouse oviduct organoids is robust and tractable (Xie *et al.*, 2018). This is perhaps unsurprising, given the reported ease of handling mouse organoids for various tissues relative to the more technically challenging human counterparts. Apart from the limited efforts to shed light on FT stem cells in these two publications, no work has hitherto employed organoid technology for FT stem cell research.

For the purpose of cancer research, organoids were employed to model HGSOc initiation, by genomic engineering of HGSOc-specific mutations into FT- and OSE-derived organoids (Zhang *et al.*, 2019; Löhmußaar *et al.*, 2020). Upon orthotopic transplantation of mutant organoids into mice, both

studies concluded that TP53 mutations alone are insufficient to drive tumourigenesis.

Tumourigenesis ensues upon addition of further mutations in Rb (Zhang *et al.*, 2019) or Brca and Pten (Löhmußaar *et al.*, 2020).. Importantly, both studies show that HGSOC can derive from the OSE. However, OSE-derived tumours were less aggressive, had a longer latency and were more chemoresistant, all characteristics of non-HGSOC type I EOC tumours. Furthermore, as mentioned, deep characterisation of STIC+ vs STIC- HGSOC tumours from patients concluded that these tumours were indifferent (Ducie *et al.*, 2017). Finally, organoids were also established directly from HGSOC tumour samples. HGSOC organoids have been heavily characterised and shown to effectively predict patient response to clinical treatment (de Witte *et al.*, 2020), as well as capture tumour heterogeneity seen in parent tumours from patients (Kopper *et al.*, 2019). However, establishment of HGSOC organoids has a low success rate (Hoffmann *et al.*, 2020; Kopper *et al.* 2019). A summary of the major organoid-based studies of FT and HGSOC biology is shown in Table 6.

Table 6: Summary of organoid-based research into FT and HGSOC biology.

Study	Species	Starting material	Summary
Kessler <i>et al.</i> (2015)	Human	Normal Fallopian tube	• First study to optimize organoid culture for FT • Confirmed presence of secretory and ciliated cells • Confirmed organoids are monoclonal • Proposes Wnt and Notch signaling as important in maintenance of stem cells within organoids • Showed organoids are responsive to Estrogen • Organoid protocol was poorly optimised (see Table 13 in Results) • Did not investigate the character of organoid-forming cells
Xie <i>et al.</i> (2018)	Mouse	Normal oviduct	• Conducted analysis of growth factor requirements, showing mouse organoids have minimal culture requirements compared to human organoids • Showed organoids are hormone-responsive • Showed distal/fimbrial oviduct is enriched in organoid-forming cells
Zhang <i>et al.</i> (2019)	Mouse	Oviducts and ovarian surface epithelium (OSE) From control and transgenic mice with alterations in TP53 or TP53 + Rb	• Dox-induced Cre in mice showed that TP53 and Rb mutations in oviduct secretory cells cause STIC and HGSOC in mice • Established organoids from oviducts of transgenic mice • Induced Cre recombination <i>in vitro</i>, generating mutant organoids • Orthotopic Injection of mutant organoids into mice recapitulates disease features • Same genetic lesions were introduced, specifically in the ovary, causing less aggressive HGSOC with longer latency • FTE-derived and OSE-derived HGSOC had distinct transcriptomes and chemosensitivities

Löhmusaar <i>et al.</i> (2020)	Mouse	Normal oviduct and OSE From Cas9-P2A-EGFP mice	• Mouse oviduct organoids established • Small guide RNAs transfected to generate organoid lines with mutations in TP53, TP53+BRCA1 or TP53+BRCA1+PTEN • Show that oviduct-derived organoids are more sensitive to paclitaxel and PARP inhibitor treatment compared to OSE-derived mutant organoids • Both oviduct-derived and OSE-derived tumour organoids are resistant to cisplatin treatment • Orthotopic injection of mutant organoids into mice showed that TP53 or TP53+BRCA1 mutant organoids did not have tumorigenic potential • Only upon adding PTEN mutation to this does tumorigenic potential transpire • HGSOC in mice showed differentiated or immunoreactive molecular subtypes, but limited evidence of this is presented • Authors also show evidence of mesenchymal-like tumours, but only upon subcutaneous injection in mice, not orthotopic • Choice of genes to mutate is less relevant to sporadic HGSOC
Kopper <i>et al.</i> (2019)	Human	All EOC histotypes, including HGSOC tumours	• Developed a protocol for HGSOC organoid culture • Conducted detailed comparative analyses of tumour and tumour-derived organoids at the genomic, transcriptomic and copy number levels • Overall, organoids correlate very well with matched parent tumours • This correlation does not change significantly upon long-term culture of organoids • Shows evidence at the single cell level that organoids capture intra-patient heterogeneity present in the parent tumour • Developed miniaturized high throughput drug screening platform for organoids • Showed proof-of-principle that tumour organoids can be xenografted into mice for <i>in vivo</i> drug sensitivity assays • Low success rate for establishing HGSOC organoids (~55%)
Hill <i>et al.</i> (2018)	Human	HGSOC tumours	• Shows that HR-deficient organoids correlate with sensitivity to PARP inhibitors • Shows that functional defect in replication fork protection correlates with sensitivity to carboplatin and CHK1 and ATR inhibitors • Observes examples where BRCA mutations do not confer HR deficiency or PARP inhibitor sensitivity • Suggests organoids can be used in tandem with DNA sequencing to predict response and identify patients that may benefit from treatment with PARP inhibitors
de Witte <i>et al.</i> , (2020)	Human	All EOC histotypes, including HGSOC tumours	• Generated organoids from tumour samples obtained at interval debulking surgeries • Subjected organoids to the same treatments administered to patients • Shows that treatment response in organoids correlates well with clinical response in patients • Small sample size (36 organoids from 23 patients), but the study is ongoing

3. Mouse models

Although organoids are powerful models to investigate many features of tissue and disease biology, mouse models remain the gold standard, particularly in evidencing stem cell character and its association, if any, with tumour initiation. Most HGSOc mouse models do not fully recapitulate disease features, due to low efficiency of STIC formation, lack of progression from STIC to HGSOc or absence of peritoneal metastasis and ascites (Kim *et al.*, 2018). Most importantly, unlike in well-defined cancers such as colorectal cancer, in which induction of the driver mutation (APC loss) alone in mouse colon can initiate adenomas, insertion of driver mutation (TP53 loss) alone is insufficient to trigger formation of STIC or HGSOc.

Efficient colorectal tumourigenesis in mice ensues when the disease's driver mutation is induced in the cell-of-origin of this cancer, namely the tissue-resident stem cell. A study has shown that inducing driver mutations in other cell types does not lead to tumourigenesis (Barker *et al.*, 2009). It can be speculated that in HGSOc mouse models, the most reliable of which is the doxycycline-inducible Pax8^{rtTA} TetO^{Cre} TP53^{-/-} (Perets *et al.*, 2013), induction of TP53 driver mutation targets the pan secretory compartment of the FT. Depending on the duration of induction, this may miss HGSOc's cell-of-origin, which could be rare within the secretory compartment. Thus, refinement of the targeting strategy is required, but this is not possible without clues about the cell-of-origin and its unique active promoters. Adding PTEN (Perets *et al.*, 2013), Rb (Zhang *et al.*, 2019) or PTEN & BRCA (Löhmußaar *et al.*, 2020) mutations to TP53 mutation dramatically improves STIC and HGSOc formation efficiencies in mice. PTEN or Rb loss here could represent the loss of gatekeeper function required for hyperproliferation, but may not necessarily reflect the natural progression of HGSOc, as PTEN and Rb are altered in only 8% and 10% of HGSOc cancers, respectively (TCGA *et al.*, 2011). Overall, identification of the cell-of-origin will enable targeting the driver mutation to the correct cells-of-origin of HGSOc, generating more physiologically relevant tumour models to study disease initiation.

3.5 Chapter Summary

Ovarian Cancer is the fifth most common cancer in women. Survival rates have remained persistently low over the last few decades. This trend is driven primarily by one of EOC's type II tumours, namely HGSOC, the most common, malignant and lethal histotype. HGSOC is characterized by ubiquitous TP53 mutations, low somatic mutational load, extensive CNAs and dysregulation in BRCA and HR repair pathways. TP53 loss is likely the driver of CNAs in HGSOC. BRCA loss cooperates with TP53 loss to promote early onset of HGSOC in predisposed HGSOC patients. HGSOC molecular subtyping identified clusters that correlate with poor prognosis and survival, but more research is needed to understand whether this is attributable to the tumour or to components of its microenvironment. HGSOC and BLBC share many common features in aetiology, predisposition, genomic landscape, progression and treatment.

Several reports have recently implicated the FT in HGSOC pathogenesis. The current working hypothesis is that secretory cell outgrowths, called SCOUTs, accumulate TP53 mutation and subsequently DNA damage, to give rise to non-invasive p53 signature lesions. These progress to STICs and seed to the ovary or peritoneum by a yet unknown mechanism, leading to HGSOC. Upon advanced progression, HGSOC establishes metastatic colonies in more distant anatomical locations. Most major studies extensively profiled advanced HGSOC, but not early HGSOC. This is due to limitations in early detection and availability of early stage tumour samples. Hence, there is currently a blackhole in our understanding of the processes between initiating TP53 mutations and development of HGSOC. There are also limitations in STIC detection, so the association between STIC and HGSOC is probably underappreciated. Nonetheless, HGSOC with and without STIC show no major differences, suggesting shared origins and pathogenesis.

Although the tissue-of-origin of HGSOC is now known, the cell-of-origin remains obscure. STIC lesions are enriched in the fimbria, a FT region in which stem-like cells have been reported. There is evidence, in the oviduct and other FRT tissues, to suggest that these stem-like cells may be Wnt-active. Interrogation of the role of FT stem cells in HGSOC initiation has not been possible, owing to the fact that FT stem cells have not been identified yet. Effective research on stem cell biology necessitates the utility of a model that is capable of reliably re-constructing the stem cell niche *in vitro*. Current FT preclinical models suffer from deficiencies in dimensionality, tractability and longevity. Among these, organoids are emerging as a powerful tool that bridges the gap between inadequate and simplistic *in vitro* models, and expensive highly-technical mouse models. Although a

recent player, organoid culture has already been employed to address critical questions in the field. However, no studies have thus far harnessed the utility of organoids to make progress in FT stem cell research. As we saw for other tissues, organoid-based and mouse technologies will probably be instrumental in identification and characterisation of FT stem cells.

Identifying FT stem cells will make it possible to interrogate their role in HGSOC initiation. There is consensus in the field that the single most effective route towards improving HGSOC survival remains the detection of patients at early stages of the disease. This necessitates an understanding of tumour initiation mechanisms, which are currently lacking. FT stem cells may be the missing link in this story.

CHAPTER 4

AIMS & OBJECTIVES

AIM

The broad aim of this thesis was to identify and characterize FT stem cells at a basic level. The planned objectives were as follows:

OBJECTIVE 1: establish and characterise FT organoids

- (a) To optimize the establishment, propagation and cryopreservation of FT organoid culture
- (b) To confirm concordance between FT tissue and derived organoids at the morphological, transcriptional and protein levels, using established and novel markers of FT cell types
- (c) To test if organoids harbour cells that are multipotent and generate monoclonal and differentiated cells

OBJECTIVE 2: identify multipotent FT stem cells in organoids

- (a) To understand the requirements of self-renewal and differentiation of FT stem cells
- (b) To genetically label putative stem cells within organoids, and isolate them for organoid formation efficiency (OFE) analysis and further characterisation by bulk and single cell approaches

OBJECTIVE 3: understand how FT stem cells are endogenously maintained

- (a) To understand the biochemical nature and range of signals that maintain FT stem cells
- (b) To identify niche-supporting cells that may emanate these signals to FT stem cells
- (c) To test the functional consequence on FT stem cells, of blocking or elevating signals from niche-supporting cells
- (d) To isolate and characterise niche-supporting cells in FT organoids

CHAPTER 5

MATERIALS & METHODS

MATERIALS & METHODS

Please refer to the appendices below for the following

Appendix A: List of reagents and their source / catalogue numbers

Appendix B: List of antibodies and their source / catalogue numbers

Appendix C: List of all qPCR primers used and their sequences

Appendix D: List of siRNA's, their source and sequences

5.1 Human Clinical Samples

Fallopian tube tissue samples were obtained from patients undergoing cancer surgery at the Department of Gynaecological Oncology, Churchill Hospital, Oxford University Hospitals, United Kingdom. Patients were appropriately informed and consented before sample collection. Cases were recruited as part of the Gynaecological Oncology Targeted Therapy Study 01 (GO-Target-01, Research Ethics Approval #11-SC-0014, Berkshire NRES Committee), as well as under the Oxford Centre for Histopathology Research (OCHRe) / Oxford Radcliffe Biobank (ORB) research tissue bank ethics reference 19/SC/0173.

5.2 Mouse Tissue Samples

All mouse tissues were harvested and provided by the Biomedical Sciences Facility, University of Oxford.

Mouse colonies were maintained in this certified and licensed animal facility and in accordance with the United Kingdom's Home Office Animals (Scientific Procedures) Act 1986. All personnel handling animals hold Home Office-issued Personal Licenses. Tissues were obtained from female mice, strain C57BL/6 and aged 7-12 weeks. Oviduct, mammary (breast) and epidermal (skin) tissue were harvested and used in this work. Skin tissue was harvested from the shaved tip of mouse tail bone as previously described (Mascre *et al.*, 2012).

5.3 Tissue Sectioning

A small piece of primary human or mouse tissue was resected and embedded in Fisher Healthcare Tissue-Plus Optimum Cutting Temperature (OCT). This was frozen at -80 °C, sectioned into 10 µm sections using the CryoStar NX50 (Thermo Scientific) cryostat, mounted on regular glass slides (SuperFrost Plus, VWR International) and immediately stored at -80 °C. This was done for a number of patients / mouse samples and a small biobank of fresh frozen slides was established for use in Immunofluorescence staining (IF) and RNA Fluorescent *In Situ* Hybridization (ISH).

5.4 Cell Lines & Culture

Cell lines employed in this project are outlined in Table 7 below. Unless otherwise stated, cells were grown in culture medium containing 10% Fetal Bovine Serum (FBS) and 50U/ml Penicillin-Streptomycin (Pen-strep). Culture medium was replaced every 3-5 days, and cultures were passaged every 8-10 days. SKOV3 cells, HEK 293 and L-Wnt3a cell lines were obtained from the American Type Culture Collection (ATCC), while the HEK 293T HA-RSPO1-Fc cell line was obtained from Amsbio.

	SKOV3	HEK 293	L-Wnt3a	HEK 293T HA-R-Spondin1-Fc
Organism	Homo sapiens	Homo sapiens	Mus musculus	Homo sapiens
Tissue	Ovary, ascites	Embryonic kidney	Connective tissue	Embryonic kidney
Disease	adenocarcinoma	None	None	None
Morphology	Epithelial	Epithelial	Fibroblast	Epithelial
Culture property	Adherent	Adherent	Adherent	Adherent
Overexpression	None	None	Wnt3a protein, obtained from CM	Murine RSPO1 protein with an N-terminal HA epitope tag and a C-terminal murine IgG2a Fc fragment
Base Culture medium	McCoy's medium	DMEM medium	DMEM / F12 medium	DMEM medium for growth of cells CD293 medium for RSPO1 expression

Table 7: Cell lines used and their characteristics. FBS = Fetal Bovine Serum (used at 10%), P/S = Penicillin & Streptomycin (used at 50 U/ml for Penicillin and 50 ug/ml Streptomycin), CM = conditioned medium.

1. Expression of Wnt activating proteins

RSPO1 protein expression was performed using the HEK 293T HA-R-Spondin1-Fc cell line, according to the supplier's protocol. Briefly, cells were cultured in DMEM/FBS until 80% confluency, using 30x T175 flasks. Serum-free CD293 expression medium was added and incubated for 7-10 days. The supernatant was harvested, pelleted to get rid of debris and sterile-filtered. This is RSPO1 CM. It was either stored long-term or used in protein purification, using Pierce Protein A Agarose kit (ThermoFisher Scientific). Purified and concentrated RSPO was stored at -20 °C for a period of 6 months. New batches were made every 6 months.

For Wnt3a protein expression, L-Wnt3a cell line was used. Cells were seeded at 1:10 in culture flasks for 3-4 days. The medium was harvested (this is batch 1) and fresh medium was added to cells for another 3 days. Culture medium supernatant was collected (this batch 2 Wnt3a CM) and

the two CM batches were mixed at a ratio of 1:1 to yield Wnt3a CM. This was pelleted to get rid of debris and cells, sterile-filtered and stored long-term at 4 °C.

WNT7A protein expression was performed using the pcDNA.Wnt7A construct, a gift from Marian Waterman (Addgene plasmid #35914), as well as the pcDNA.WNT7A-V5 construct, a gift from Xi He (Addgene plasmid #43816). HEK cells were reverse transfected with these constructs for 24 hrs. Next day, fresh medium was added and allowed to condition for 3 days. Fresh medium was added and allowed to incubated for a further 3 days. Both batches were combined, pelleted to remove cells, 0.22 µm filter sterilized, aliquoted and stored at – 20 °C for up to 6 months.

All proteins above were functionally validated using the TOPFlash assay (section 5.11). Refer to Results chapters for validation data (Figure 26 for Wnt3a and RSPO1, Figure 59 for WNT7A).

5.5 Tissue Dissociation & Primary Culture

Using sterile scissors / tweezers and working under sterile conditions, primary tissue was washed several times with cold DPBS to remove remnant blood. If possible, human tissue was cut longitudinally to expose the FT epithelium. Tissue was placed in pre-warmed Digestion medium containing 2 mg/ml Trypsin (Sigma), 0.5 mg/ml DNase I (Sigma) and 100 U/ml Collagenase Type I (Invitrogen). This was incubated in a 37 °C warm room in rotation for 45 min to 1 hour.

After that, tube containing tissue was briefly vortexed to detach loosely attached cells and filtered through a 70, 100 or 250 µm cell strainer. Using forceps, de-cellularized tissue was placed into a fresh cell strainer and pressed on using the sterile plunger of a syringe, to squeeze out medium. Cells were pelleted by centrifugation at 300 g / 5 min / 4 °C, washed with cold DPBS and used for downstream analysis.

For standard 2D culture, isolated cells were resuspended in BM2 culture medium containing Advanced DMEM/F12 (ThermoFisher), 12 mM HEPES (ThermoFisher), 5% FBS (GIBCO), 1% Penicillin/Streptomycin (GIBCO), 100 ng/ml EGF (ThermoFisher) and 10 µM Y-27632 (ROCK inhibitor, Sigma), as described (Kessler *et al.*, 2015). On the following day, cells were washed with pre-warmed DPBS to flush out unattached cells (erythrocytes, leukocytes, etc) and fresh medium was supplied to allow epithelial cells to proliferate.

For patient tumour samples, the processing protocol was similar to the above, with the exception that digestion was performed using Tumour Digestion Kit (Miltenyl Biotech). Digestion was performed for 2 hrs at 37 °C.

5.6 Organoid Culture

1. Establishment

Organoid cultures were established from primary tissues in two ways. The first method involved direct organoid culture of cells isolated from human or mouse tissue. The second method entailed growing isolated cells in 2D culture for 3-7 days before establishing the organoid culture (see section 5.5). The latter method was employed for genetic manipulation using siRNA or viral transduction.

To establish human FT or mouse oviduct, breast and skin organoid culture, processed cell pellet was resuspended in Extracellular Matrix (ECM, Matrigel, Corning) and plated as 50 µl drops on pre-warmed plates. Plates were incubated upside down at 37 °C for 20 min to allow Matrigel polymerization with cells encapsulated within its 3D environment. Cells were finally overlaid with pre-warmed Organoid Medium containing BM2 medium (no FBS), supplemented with 100 ng/ml human Noggin (Peprotech), 100 ng/ml human Fibroblast Growth Factor-10 (FGF-10, Peprotech), 1% N2 supplement (ThermoFisher), 2% B27 supplemented (ThermoFisher), 1 mM Nicotinamide (Sigma), 1 mM N-acetyl L-cysteine (Sigma) and RSPO1, which was obtained either commercially (human RSPO1; Peprotech) or produced in-house using a HA-RSPO1-Fc 293T cell line (section 5.4).

For human FT organoid culture, A83-01 (Tocris) and Forskolin (Tocris) were routinely added at 5 µM and 10 µM (Figure 27), unless otherwise stated. Y-27632 was added for the first 2-3 days of organoid culture. For certain experiments that required testing the effect of Wnt3a, Wnt3a conditioned medium was generated using L-Wnt3a cells (ATCC). Refer to Figure 26 for data on Wnt3a expression and functional validation using Luciferase-based Wnt-reporter (TOPFlash) assay.

2. Maintenance

Human FT organoids: Organoids were passaged every 10-14 days. Briefly, organoids were harvested by incubation in Organoid Harvesting Solution (Cultrex) at 4 °C in motion, for 30-60 min. This solution uses a non-enzymatic chemicals to depolymerize Matrigel and yield intact organoids. After collecting

organoids in a 15 ml falcon tube, organoids were pelleted by centrifugation at 300 g / 5min / 4 °C. The organoid pellet was either mechanically sheared using a p200 pipette or resuspended in 1x TrypLE (containing Y-27632 to prevent anoikis) for 5-10 min at room temperature. Mechanical fragmentation was used for routine passage. Trypsinization was used for FACS sorted samples and to assay organoid formation efficiency. Finally, organoids were split at a ratio of 1:2-1:4 and expanded in a well of a 24, 12 or 6-well plate, or on 6 cm dishes for larger experiments (Figure 23).

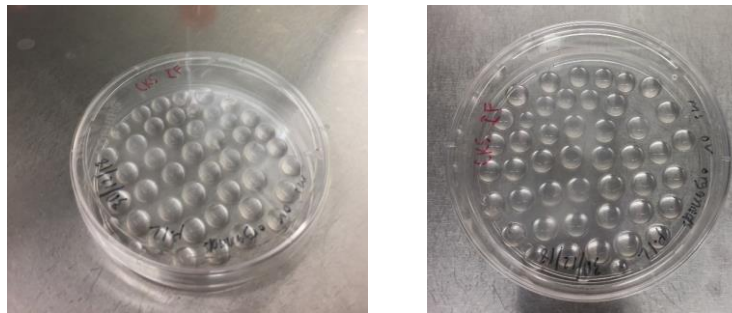


Figure 23: Organoids expanded in 60mm dishes

Mouse organoids: Oviduct, mammary or epidermal organoids were passaged by washing with warm DPBS and incubation in a warm solution of TrypLE + Y-27632 for 30 min at 37 °C, as described (Xie *et al.*, 2018). A p1000 pipette tip was used to mechanically fragment dissociated cells and solubilize Matrigel. Resulting pellet was washed and split 1:15-1:20 to form organoids as described.

3. FACS sorting & Organoid Culture

For FACS sorting, trypsinized cells were resuspended in Organoid FACS Buffer containing Organoid Medium components without recombinant proteins (EGF, Noggin, FGF-10, RSPO1). Non-specific antibody staining was inhibited by incubation with FcR Blocking Reagent (Miltenyi Biotec) for 10 min in a dark fridge. Further antibody incubation was performed using CD45-FTIC and EpCAM-APC (Biolegend), in a 100 µl volume.

4. Organoid RNA extraction & RT-qPCR

For RNA extraction, organoids were harvested as described. Pelleted organoids were washed and mechanically fragmented in cold DPBS using a p200 pipette tip. Washed and fragmented organoids were thereafter pelleted, resuspended in 350 µl RLT buffer (Qiagen) and transferred into a 1.5 ml Eppendorf tube.

The tube was incubated for 15 min at room temperature in rotation and then vortexed for 1 min. A 20G needle was used to further homogenize the cells if necessary. The sample was then stored at -80 °C for later use or immediately utilized for RNA extraction according to the Qiagen RNeasy Plus Micro Kit. Extracted RNA was tested for concentration (using NanoDrop) and quality (using TapeStation). Up to 2 ug of extracted RNA was used to generate cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) or TaqMan™ Reverse Transcription Reagents (Invitrogen). RT-qPCR was set up using the SYBR® Green PCR Master Mix (ThermoFisher) and performed using the StepOnePlus RT-PCR machine (ThermoFisher).

5. Cryopreservation & Organoid Biobank

Organoids were harvested as described and washed by cold DPBS. Upon mechanical fragmentation with a pipette, organoids were embedded in Recovery Cell Culture Freezing Medium (ThermoFisher), transferred to a labelled cryovial and gradually frozen at -80 °C overnight using Mr Frosty Isopropanol Freezing Container (ThermoFisher). Next day, frozen cryovials were transferred to Liquid Nitrogen storage.

Upon thawing, organoids were resuspended in 9 ml DMEM/F12, pelleted, washed with cold DPBS and cultured in a well of a 24-well plate, as described.

5.7 Lentiviral Transduction of Organoids

1. Virus packaging

To generate lentiparticles, HEK cells were transfected in a T25 flask or a 6 cm dish with the lentiviral construct, envelope plasmid (VSV-G) and packaging plasmid (Gag/pol) using Lipofectamine 3000. This was incubated for 4-6 hrs under serum-free conditions. Transfection medium was replaced with fresh culture medium for 72 hrs. Cells were imaged to confirm transduction. Viral supernatant was collected and centrifuged at 500 g / 10 min / 4 °C to pellet cells and debris. Supernatant was collected and passed through a 0.45 µm filter. Envelope and packaging constructs used were pMD2.G (Addgene #12259) and psPAX2 (Addgene #12260) respectively, both gifts from Didier Trono.

Lentivirus particles were concentrated using the Lenti-X Concentrator (Takara). Briefly, 1 volume of Lenti-X Concentrator solution was added to 3 volumes of viral supernatant. This was

incubated on ice for 30 min and then centrifuged at 1,500 g / 45 min / 4 °C, leaving a concentrated viral pellet that was resuspended in BM1 medium and frozen in aliquots at -80 °C.

2. Viral Transduction

Target cells were prepared a few days prior to transduction. Target cells used were cell lines, 2D patient-derived primary FT culture or mouse organoids that were dissociated and cultured in 2D.

Transduction medium comprised a thawed virus aliquot and 8 ug/ml polybrene in low-volume culture medium. Transduction was allowed to proceed overnight, before replacing with fresh medium for a further 48 hrs. Upon microscopic confirmation of transduction, target cells were dissociated and selected by FACS sorting. Organoid culture was established from transduced cells and expanded to yield a human FT organoid line or a mouse oviduct organoid line. For cell lines, transduced cells were expanded in standard 2D culture as described.

5.8 Single cell RNA-sequencing of Wnt-reporter organoids

The above-mentioned lentivirus transduction protocol was employed to genetically engineer organoid lines containing a stably-integrated fluorescent reporter of Wnt signaling. This was successfully achieved for organoids from multiple patients, as well as mouse oviduct organoids. The lentiviral construct used was 7TGC, which was a gift from Roel Nusse (Fuerer & Nusse, 2010; Addgene #24304). Please refer to Results chapters (Figure 46) for construct map.

Single cell RNA sequencing (scRNA-seq) was performed on human Wnt-reporter organoid lines. Low passage (p.2 to p.4) organoids were harvested on Day 8 from 3 confluent wells of a 6-well plate, using Organoid Harvesting Solution supplemented with Y-27632 and RNasin Plus RNase inhibitor (Promega). Single cell dissociation was performed as described above and organoids (now single cells) were resuspended in Organoid FACS Buffer containing RNase inhibitor, 2 mM EDTA and 1% RNase-free BSA (Sigma). Cells were passed through a 30 µm cell strainer and single cell FACS sorting performed using the MA900 Sony Sorter.

Cells were sorted into 96-well plates containing 4 µl lysis buffer supplemented with 0.1 µl RNase inhibitor (Clontech), 1.9 µl 0.4% Triton X-100, 1 µl 10 µM 5'-biotinylated oligo-dT30VN (IDT) and 1 µl 10 mM dNTP (Thermo Scientific). Cells were sorted at one cell per well, with bulk controls

(10 cells) and empty well controls (0 cells) included for each plate. Plates were snap frozen on dry ice and stored at - 80 °C before single-cell RNA sequencing.

Single cell cDNA synthesis and library generation were performed by Zhiyuan Hu according to the SMART-seq2 protocol (Picelli *et al.*, 2014) and as described (Hu *et al.*, 2020). Briefly, cells were lysed by removing plates from - 80 °C and heating at 72 °C for 3 min. Plates were then placed at 4 °C before adding the reverse transcription mix containing 5'-biotinylated TSO (Qiagen). PCR products were cleaned up using 0.8:1 Ampure XP beads (Beckman Coulter) with Biomek FxP Laboratory Automation Workstation (Biomek). Quality of single-cell cDNA was tested using TapeStation, as well as by single cell qPCR for GAPDH or ACTB using the QuantiNova SYBR Green PCR Kit (Qiagen). cDNA concentration was measured using Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen) on the CLARIOstar Plate Reader (BMG Labtech). Wells with Ct values of GAPDH or ACTB below 20 were selected as wells with a good quality cDNA.

Libraries from single cell cDNA were generated using miniaturized Nextera XT (Illumina) protocol (Mora-Castilla *et al.*, 2016) with Mosquito HTS (ttplabtech), in 384-well Endure plate (Life Technology). Library sequencing was performed by Novogene. Library preparation and data analysis were performed by Zhiyuan Hu.

5.9 Immunofluorescence Staining

Fresh frozen OCT-embedded tissue sections, including human FT sections and mouse oviduct, breast and skin tissues were prepared as described (sections 5.1-5.3). Organoids derived from these tissues were prepared by passage and growth for 7-12 days on an 8-well chamber slide (Thistle Scientific).

Fixation was performed for 15-20 min using methanol-free Paraformaldehyde diluted in DPBS (4% for tissue sections and 2% for organoids, to avoid Matrigel collapse). To reduce staining background, samples were washed three times (10 min each) with PBS containing 0.4M Glycine (Sigma). Glycine binds to the free, charged and reactive ends of unreacted fixative agents like PFA, avoiding non-specific antibody capture and reducing background. For non-cell surface marker staining, samples were permeabilized for 10 min using Permeabilization buffer containing PBS and Triton X-100 at 0.2% (tissue section) or 0.5% (organoids). All washing steps were done using Wash Buffer, comprising 0.2% Triton X-100 and 0.05% Tween-20 in PBS. This was done three times (5 min each for tissue sections, 10-15 min each for organoids).

Blocking was done in 5-10% Normal Donkey Serum (Sigma) prior to antibody staining. Blocking was performed for 1 hr for tissue sections and 2-3 hrs for organoids. Primary antibody incubation was performed overnight. On the following day, samples were washed and secondary antibodies + DAPI (Sigma) added. Secondary antibody incubation was performed for 1 hr for tissue sections and 2-3 hrs for organoids. Finally, samples were washed and mounted in Vectashield Mounting Medium (Vector Laboratories). Images were obtained using the Zeiss 780 Inverted Confocal Microscope.

5.10 RNA *In situ* Hybridization

RNA *In situ* Hybridization was performed using the RNAScope® Multiplex Fluorescent V2 Assay (ACD) and as described (F. Wang *et al.*, 2012). Fresh frozen human or mouse tissue sections were retrieved from - 80 °C freezer and immediately fixed in 4% PFA for 20-30 min at room temperature. Organoid sections were derived by dissociating organoids, washing and employing Cytospin technology to attach dissociated cells on a glass slide. Slides were then immediately fixed in 4% PFA.

After PBS washes, slides were dehydrated in an ascending series of Ethanol incubations (50%, 70% and then 100%, 5 min each) at room temperature. ImmEdge Hydrophobic PAP Pen (Vector Laboratories) was used to create a thick water-repellent barrier around tissue sections on the slide. Upon drying, endogenous peroxidase activity was blocked by treatment with Hydrogen Peroxide for 10 min. Sections were then washed with PBS, treated with Protease IV reagents for 30 min and washed again. If required, RNase treatment was performed after Hydrogen Peroxide and before protease treatment.

Following sample prep, all incubation steps were performed at 40 °C in a humidified environment using the RNAScope HyBEZ II Hybridization Oven. All washing steps were done twice, 2 min each, using the RNAScope wash buffer. Tissue sections were incubated with positive control, negative control or target-specific probes for 2 hrs. Sections were then washed and stored overnight in RNase-free 5X SSC Buffer (ThermoFisher). The following day, pre-amplifier and amplifier binding was performed by incubating sections with Amp1 (30 min), Amp2 (30 min) and Amp3 (15 min) reagents, with intercalated washing steps. Finally, HRP-based fluorescent signal development was done by incubation with HRP reagent (15 min), TSA-conjugated fluorophores (30 min) and HRP blocker reagent (15 min). If more than one target was investigated, this series of HRP-based reactions was repeated to develop the HRP-based signal using a different fluorophore for each target. Finally,

slides were counterstained with 100 ng/ml DAPI (Sigma) and mounted using Gold Antifade Mounting medium (ACD). Slides were left overnight at room temperature to dry. On the following day, slides were imaged for the punctate signal characteristic of RNA, using a Zeiss 780 Inverted Confocal microscope.

5.11 Wnt-reporter (TOPFlash) Assay

The TOPFlash Assay is a luciferase-based transcriptional Wnt/ β -catenin reporter assay. Constructs used in this project were the M50 Super 8x TOPFlash (Addgene #12456) and M51 Super 8x FOPFlash (Addgene #12457). Both were gifts from Randall Moon (Veeman *et al.*, 2003). TOPFlash construct encodes firefly luciferase under the control of 7 x TCF elements, activatable by β -catenin/TCF binding. FOPFlash construct encodes a mutant version of these WREs. A Renilla luciferase construct was used as endogenous control, driven under a general promoter. The TOPFlash assay was used for functional validation of in-house generated and commercial Wnt signaling reagents. WNT7A constructs were used, as detailed (section 5.4). FZD3, 5 and 6 were also used. pRK5-mFzd3-1D4 (Addgene #42265), pRK5-mFzd5-1D4 (Addgene #42267) and pRK5-mFzd6-1D4 (Addgene #42268) were a gift from Chris Garcia & Jeremy Nathans.

To conduct the assay, HEK 293 cells were reverse transfected with TOPFlash construct (100 ng) and Renilla construct (5 ng) using Lipofectamine 3000. 100K-200K cells were plated per well of a 24-well plate and incubated for 48 hrs. After that, Wnt signaling reagents, proteins, conditioned medium or small molecule inhibitors were introduced to the cells in fresh medium and incubated overnight. Next day, Firefly / Renilla luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). In brief, cells were washed once with warm DPBS and lysed in 100 μ l 1X Passively Lysis Buffer for 20-30 min at room temperature on a shaker. At this point, lysates were either used immediately for luciferase measurements or frozen at -20 $^{\circ}$ C for less than a week.

To measure luciferase activity, 5 μ l of lysates was transferred to a white and opaque 96-well plate and injected into an automated dual injector GloMax Luminometre (Promega). The luminometer dispensed 25 μ l of pre-prepared Luciferase Assay Reagent II substrate, measured Firefly Luciferase activity, dispensed 25 μ l of Stop&Glo reagent to quench Firefly luciferase activity and measure Renilla luciferase activity. TOPFlash readings were normalized to Renilla readings, and all readings were normalized to untreated sample.

5.12 siRNA Transfection

All siRNA's were purchased from Dharmacon (Horizon). For siRNA transfection, primary cells or cell lines were plated on a 6-well plate and incubate for 1-3 days to reach 50-70% confluency. Cells were then transfected with either non-targeting or target-specific siRNA using Lipofectamine 3000 or Lipofectamine RNAiMAX (ThermoFisher). Final [siRNA] was 40 nM. Next day, fresh culture medium was introduced, and this was incubated for a further 48 hrs. Cells were then utilized for downstream molecular analysis.

5.13 Western Blotting (WB)

1. Cell lysis

Cell lysis was strictly performed on ice. In brief, cells were washed twice with cold PBS and scraped in 60-70 μ l in-house made lysis buffer (see Table 8) containing 1:100 Phosphatase Inhibitor Cocktail 3 (Sigma) and 1:100 Halt Protease Inhibitor (ThermoFisher).

Reagent	Amount
MilliQ water	325 ml
HEPES	2.38 g
5M NaCl	15 ml
NP-40 10%	50 ml
Sodium deoxycholate	1.25 g
Glycerol	50 ml
Sodium Fluoride	840 mg
Beta-glycerophosphate	1295 mg
100 mM Sodium Orthovanadate	5 ml
0.5M EDTA	500 μ l
0.5M EGTA	2.5 ml

Table 8: Composition of WB lysis buffer. The buffer was adjusted to pH 7.5 and frozen long-term at -80°C . Working aliquots were stored at 4°C .

Lysates were then collected in pre-cooled 1.5 ml Eppendorf tubes and incubated in rotation for 30 min at 4 °C, to allow complete lysis of cells. Lysates were centrifuged at 17,000 *g* / 5 min / 4 °C to pellet membranes and debris. Supernatant (containing proteins) was collected and 30 µl of it was transferred into a 1.5 ml eppendorf tube containing 12 µl 4X Sample Buffer (ThermoFisher) and 5 µl 10X Sample Reducing Agent (ThermoFisher).

2. SDS-PAGE electrophoresis

Lysates were boiled for 5-10 min at 95 °C and immediately placed on ice for 5 min to allow condensation of evaporated sample. Finally, the full volume of lysates was loaded on precast 4-12% Bis-Tris or 3-8% Tris-acetate gels (ThermoFisher) as specified, in a tank containing 444 ml 1X MOPS buffer or 1 x Tris-Acetate buffer, respectively (ThermoFisher).

Proteins in lysates were resolved using SDS-PAGE electrophoresis, at 160 V for 1 hr. Resolved proteins were transferred from gel to a Nitrocellulose membrane using the Invitrogen iBlot2 Dry system (Invitrogen). Membranes were blocked for 1 hr in Blocking Buffer containing 5% semi-skimmed milk and 0.1% Tween 20 detergent in 1 x TBS solution. After blocking, membranes were incubated in primary antibodies (diluted in Blocking Buffer) overnight at 4 °C on a shaking platform. On the following day, membranes were washed three times (5 min each) in TBS buffer + 0.1% Tween 20, then incubated in HRP-conjugated or IRDye-conjugated secondary antibodies (diluted in blocking buffer) for 1 hr at room temperature on a shaking platform. After 3 x washes, protein bands were imaged using LiCOR infrared-detecting scanner or developed using ECL-based detection method.

3. Removal of Oligosaccharides from glycosylated proteins

PNGase F was used to conduct cleavage of sugar complexes from proteins, as detailed in NEB protocol. PNGase F is an amidase that cleaves and releases most N-linked oligosaccharides from glycoproteins (Maley *et al.*, 1989). The PNGase F enzyme and its buffers were obtained from New England Biolabs. For denaturing, 1-20 µg of protein lysate was mixed with 9 µl water and 1 µl of 10X Glycoprotein Denaturing Buffer. This was heated at 100 °C for 10 min. Denatured sample was cooled on ice and centrifuged for 10 sec. To perform the cleavage reaction, 2 µl of 10X GlycoBuffer 2, 2 µl of 10% NP-40, 6 µl water and 1 µl PNGase F enzyme were mixed. This was incubated at 37 °C for 1 hr, after which the cleavage was assessed by mobility shifts in WB.

4. Protein Lambda Phosphatase treatment

Lambda Protein Phosphatase is an Mn^{2+} dependent protein phosphatase that catalyses the dephosphorylation of phosphorylated serine, threonine and tyrosine residues on proteins (Cohen & Cohen, 1989). The Protein Lambda Phosphatase and its buffers were obtained from New England Biolabs. To perform the phosphatase reaction, 30 μl protein lysates were mixed with 4 μl 10X NEBuffer for Protein MetalloPhosphatase (PMP), 4 μl of 10 mM MnCl_2 solution and 1 μl Lambda Protein Phosphatase. The reaction mixture was incubated at 30 $^{\circ}\text{C}$ for 30 min, after which the phosphatase action was assessed by mobility shifts in WB.

5.14 Chapter Summary

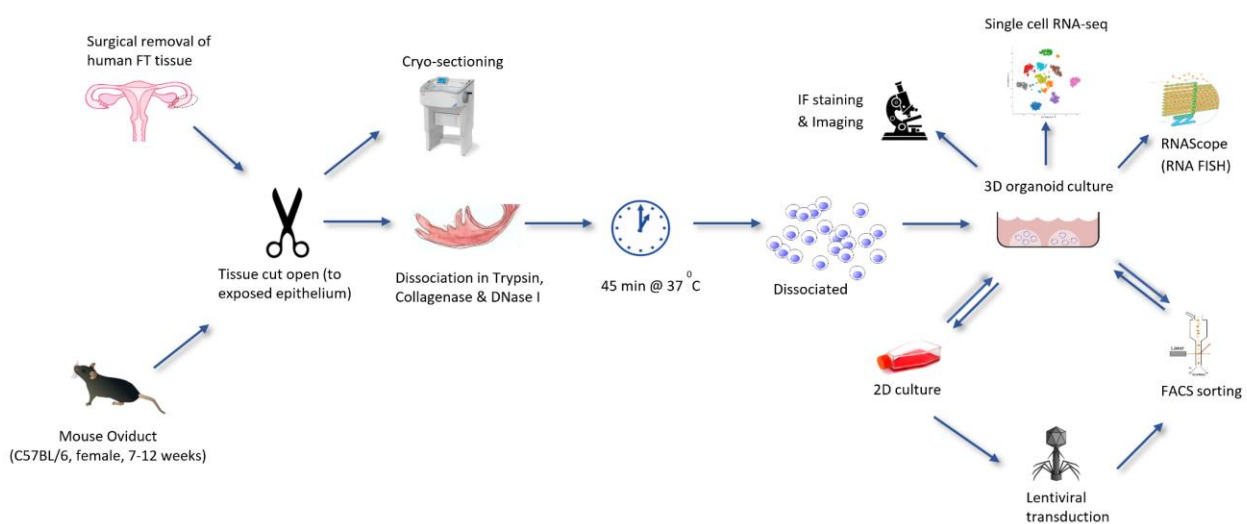


Figure 24: Graphical summary of techniques used to characterise organoids

5.15 APPENDIX A: List of Reagents

REAGENT	SOURCE
CULTURE MEDIUMS AND REAGENTS	
DPBS, no calcium, no magnesium	ThermoFisher (#14190094)
TrypLE Select Enzyme (10X)	ThermoFisher (#A1217702)
DMEM, high glucose, pyruvate	ThermoFisher (#41966052)
McCoy's 5A (Modified) Medium	ThermoFisher (#26600080)
Fetal Bovine Serum, qualified, heat inactivated, E	ThermoFisher (#10500064)
Penicillin Streptomycin (Pen Strep)	ThermoFisher (#15070063)
DH5 alpha competent bacterial cells	New England Biolabs (#C29871)
Stbl3™ competent bacterial cells	ThermoFisher (#C737303)
Opti-MEM I Reduced Serum Medium	ThermoFisher (#51985026)
Recovery Cell Culture Freezing Medium-50 mL	ThermoFisher (#12648010)
Lipofectamine 3000 Transfection Reagent	ThermoFisher (#L3000008)
Lipofectamine RNAiMAX Transfection Reagent	ThermoFisher (#13778030)
siGLO Red Transfection Indicator	Dharmacon (#D-001630-02-05)
Neg-50 Frozen Section Medium	ThermoFisher (#6502)
Pierce 16% Formaldehyde (w/v)	ThermoFisher (#28908)
Microscope slide, Superfrost plus	VWR (#631-0108)
VECTASHIELD anti-fade mounting medium	Vector Laboratories (#H-1000)
Immedge Hydrophobic Pen	Vector Laboratories (#H-4000)
8 well chamber slide, TC treated	Ibidi, supplier ThistleScientific (#IB-80826)
Dual-Luciferase Reporter Assay System	Promega (#E1910)
Brefeldin A (BFA)	ThermoFisher (#00-4506-51)
Monensin	ThermoFisher (#00-4505-51)
Lambda Protein Phosphatase	New England Biolabs (#P0753S)
PNGase F	New England Biolabs (#P0704S)
Lenti-X Concentrator	Takara Bio (#631231)
Polybrene Reagent	Millipore UK (#TR-1003-G)
Lithium Chloride	Sigma-Aldrich (#203637-10G)
RECOMBINANT PROTEINS & SMALL MOLECULE INHIBITORS	
CHIR99021 (3 μ M)	Bio-Techne (#4423/10)
IWP-2 (2 μ M)	Bio-Techne (#3533/10)
LGK-974 (2 μ M)	Strattech Scientific (#S7143-SEL)

REAGENT	SOURCE
XAV-939 (5 μ M)	Strattech Scientific (#S1180-SEL-10 mg)
PRI-724 (10 μ M)	Abcam (#ab229168-5mg)
Surrogate Wnt (0.5 nM)	UProtein Express (#N001 - 0.025 mg)
IgG 2919 (FZD5 inhibitor)	Gift from The Sidhu Lab, University of Toronto
IgG 2921 (FZD5 inhibitor)	Gift from The Sidhu Lab, University of Toronto
Anti-LGR5 IgG	BD Bioscience (#562732)
Isotype Control for anti-LGR5 IgG	BD Bioscience (#553986)
Anti-LGR6 IgG	Origene (#TA340562)
Wnt3a (conditioned medium)	In-house generated
WNT4 (conditioned medium)	In-house generated
WNT7a (conditioned medium)	In-house generated
Rspo1	In-house generated
Valproic Acid (1 mM)	Bio-Techne (#2815/100)
DAPT (10 μ M)	Bio-Techne (#2634/10)
Estrogen (100 nM)	Bio-Techne (#2824/100)
Progesterone (1 μ M)	Bio-Techne (#2835/100)
Wnt3a Protein, human, recombinant	Bio-Techne (#5036-WN-010)
Wnt3a Protein, human, recombinant	AMSbio (#AMS.rhW3aL-002-stab)
Wnt7a Protein, human, recombinant	Abcam (#ab129138)
Wnt Protein Stabilizer	AMSbio (#AMS.bWps)
Jagged - 1 (188 - 204), Notch Ligand, DSL Peptide	Eurogentec (#AS-61298)
REAGENTS FOR DISSOCIATION OF PRIMARY TISSUE	
Trypsin from Bovine pancreas	Sigma-Aldrich (#T9935)
Collagenase, Type I	ThermoFisher (#17018029)
Dnase I grade II	Sigma-Aldrich (#000000010104159001)
Red blood Cell Lysis Solution	Miltenyi Biotech (#130-094-183)
Tumor Dissociation Kit, human	Miltenyi Biotech (#130-095-929)
ORGANOID CULTURE	
Advanced DMEM/F-12, no phenol red	ThermoFisher (#21041033)
HEPES	ThermoFisher (#15630056)
Penicillin Streptomycin (Pen Strep)	ThermoFisher (#15070063)
Epidermal Growth Factor (EGF), human, recombinant	ThermoFisher (#PHG0311)
Noggin, human, recombinant	Peprtech (#120-10C)
Fibroblast Growth Factor-10 (FGF-10), human, recombinant	Peprtech (#100-26)
Human RSPO1	Peprtech (#120-38-100)

REAGENT	SOURCE
Purified mouse Rspo1	In-house generated
N-2 Supplement	ThermoFisher (#17502048)
B-27 Supplement	ThermoFisher (#12587010)
Nicotinamide	Sigma-Aldrich (#N0636-100G)
N-Acetyl L-Cysteine	Sigma-Aldrich (#A9165-5G)
Forskolin (FSK)	Bio-Techne (#1099/10)
A83.01	Stemcell Technologies (#72022)
Y-27632 (ROCK inhibitor)	Bio-Techne (#1254/10)
Cultrex Organoid Harvesting Solution	Bio-Techne (#3700-100-01)
Matrigel (phenol red-free)	Corning, supplier SLS (#356237)
SINGLE CELL RNA-SEQUENCING	
AMPure XP	Beckman Coulter (#A63881)
KAPA HIFI HotStart ReadyMix PCR kit	Roche Diagnostics (#07958935001)
SuperScript II Reverse Transcriptase	ThermoFisher (#18064071)
Bovine Serum Albumin (BSA, nuclease and protease-free)	Merck Chemicals Ltd (#126609-5GM)
Sequencing generated libraries	Novogene
PROTEIN EXPRESSION AND PURIFICATION	
L Wnt3a cell line	ATCC (#CRL-2647)
HA-R-Spondin1-Fc 293T Cells	AMSBio (#3710-001-01)
CD293 medium	ThermoFisher (#11913019)
GlutaMAX-1 (100X)	ThermoFisher (#35050-061)
Pierce Disposable Columns	ThermoFisher (#29925)
Pierce Protein A IgG Binding Buffer	ThermoFisher (#21001)
Pierce IgG Elution Buffer	ThermoFisher (#21004)
Pierce Protein A Agarose	ThermoFisher (#20333)
WESTERN BLOTTING REAGENTS	
NuPAGE Novex 4-12% Bis-Tris Gels (1.5-mm thick, 10-well)	ThermoFisher (#NP0335BOX)
NuPAGE Novex 3-8% Tris-Acetate Protein Gels	ThermoFisher (#EA0378BOX)
The iBlot 2 Transfer Stacks	ThermoFisher (#IB23001)
NuPAGE LDS Sample Buffer	ThermoFisher (#NP0007)
Sample Reducing Agent	ThermoFisher (#NP0009)
Antioxidant	ThermoFisher (#NP0005)
Milk	Sigma-Aldrich (#70166-500G)
InstantBlue Protein Stain	Strattech Scientific (#ISB1L-EXP-1L)
SeeBlue Plus2 Pre-stained Protein Standard	ThermoFisher (#LC5925)

REAGENT	SOURCE
RNA EXTRATION AND QPCR	
RNeasy Mini Kit	Qiagen (#74104)
RNeasy Plus Micro Kit	Qiagen (#74034)
TaqMan Reverse Transcription Reagents	ThermoFisher (#N8080234)
High-Capacity cDNA Reverse Transcription Ki	ThermoFisher (#4368814)
SYBR Green PCR Master Mix	ThermoFisher (#4309155)
PowerUp SYBR Green Master Mix	ThermoFisher (#A25780)
MicroAmp Fast Optical 96-Well Reaction Plate, 0.1	ThermoFisher (#4346907)
StepOnePlus™ RT PCR System	ThermoFisher (#4376600)
RNA IN SITU HYBRIDIZATION (RNAScope)	
Multiplex Fluorescent Detection Kit v2	Bio-Techne (#323110)
SSC solution (20X)	ThermoFisher (#AM9763)
ACD HybEZ™ II Hybridization System	Bio-Techne (#321720)
Rnase A	Qiagen (#19101)
Probe (3-plex human Positive Control)	Bio-Techne (#320861)
Probe (3-plex mouse Positive Control)	Bio-Techne (#320881)
Probe (3-plex Negative Control)	Bio-Techne (#320871)
Probe (human WNT7A)	Bio-Techne (#408231)
Probe (human AXIN2)	Bio-Techne (#400241-C2)
Probe (human LGR5)	Bio-Techne (#311021-C3)
Probe (mouse Wnt7a)	Bio-Techne (#401121)
Probe (mouse Axin2)	Bio-Techne (#400331-C2)
Probe (mouse Lgr5)	Bio-Techne (#
TSA Plus Fluorescein System	PerkinElmer (#NEL741001KT)
TSA Plus Cyanine 3 System	PerkinElmer (#NEL744001KT)
TSA Plus Cyanine 5 System	PerkinElmer (#NEL745001KT)
ANTIBIOTICS	
Penicillin Streptomycin (Pen Strep)	ThermoFisher (#15070063)
Ampicillin	Sigma-Aldrich (#A5354)
Kanamycin	Abcam (#ab146574)
Puromycin	ThermoFisher (#A1113803)
Zeocin	ThermoFisher (#R25001)
Geneticin (G418)	ThermoFisher (#10131035)
Doxycycline	Sigma (#D9891)

Table 9: APPENDIX A (List of Reagents).

5.16 APPENDIX B: List of antibodies

ANTIBODY	HOST	SOURCE	DILUTION
DAPI	-	Sigma (#D9542-1MG)	100 ng / ml
PRIMARY ANTIBODIES USED IN IMMUNOFLOUORESCENCE STAINING			
Pan KRT	Ms mAb	Abcam (#ab7753)	1:250
EpCAM-FITC	Ms	Biolegend (#324203)	1:100
EpCAM	Rb pAb	Sigma (#HPA026761)	1:200
E-cadherin	Ms mAb	BD Biosciences (#610181)	1:200
Occludin	Rb pAb	Invitrogen (#71-1500)	1:100
α -tubulin	Ms mAb	Sigma (#T5168)	1:2000
Active β -catenin	Ms mAb	Merck Millipore (#05-665)	1:300 for IF
Ki67	Rb mAb	Cell Signaling (#9027)	1:100
c-Myc	Rb pAb	Cell Signaling (#9402)	1:100
Pax8	Rb pAb	Proteintech (#10336-1-AP)	1:50
KRT7 or CK7	Ms mAb	Millipore	1:10
TUBB4	Ms	Sigma (#T7941)	1:100
CAPS	Rb pAb	Sigma (#HPA043520-100UL)	1:200
Phospho-Histone3 (Ser10)	Rb pAb	Cell Signaling (#9701)	1:200
Phospho-Histone3 (Ser10)	Ms mAb	Cells Signaling (#9706)	1:100
KRT17 or CK17	Rb pAb	Cambridge Bioscience (#HPA045062)	1:50
CD44	Rat mAb	Affymetrix eBiosciences (#14-0441-82)	0.5 ug
Wnt7a (for IF)	Rb pAb	Proteintech (#10605-1-AP)	1:50
KRT14 or CK14	Ms mAb	Abcam (ab7800)	1:200
KRT14 or CK14	Rb pAb	Biolegend (#905301)	1:500
mKrt5	Rb pAb	Covance, Biolegend (#PRB-160P)	1:200
hKRT5	Ms mAb	ThermoFisher (#MA5-12596)	1:200
HA tag	Rb pAb	Abcam (#ab9110)	1:1,000
V5 tag	Ms mAb	Abcam (#ab27671)	1:500
PRIMARY ANTIBODIES USED IN WESTERN BLOTING			
Wnt7a (for WB)	Rb pAb	Abcam (#ab100792)	1:800 for WB
HA tag	Rb pAb	Abcam (#ab9110)	1:1,000
V5 tag	Ms mAb	Abcam (#ab27671)	1:500

ANTIBODY	HOST	SOURCE	DILUTION
GAPDH	Ms mAb	Proteintech (#60004-1-Ig)	1:1000
Active β -catenin	Ms mAb	Merck Millipore (#05-665)	1:1000 for WB
FLUOROPHORE-CONJUGATED ANTIBODIES USED IN FACS			
FcR blocking Reagent, human	-	Miltenyi Biotec (#130-059-901)	1:10
FcR blocking Reagent, mouse	-	Miltenyi Biotec (#130-092-575)	1:10
EpCAM-APC, human	Ms mAb	Miltenyi Biotec (#130-091-254)	1:10
EpCAM-APC, mouse	Rat	Miltenyi Biotec (#130-102-234)	1:10
CD45-FITC, human	Ms mAb	Biolegend (#304006)	1:50
CD45-FITC, mouse	Rat	Miltenyi Biotec (#130-116-500)	1:50
CD44-APC	Rat mAb	ThermoFisher (#17-0441-82)	0.5 μ g
SECONDARY ANTIBODIES USED IN IMMUNOFLUORESCENCE STAINING			
Donkey anti-Mouse secondary antibody, Alexa Fluor 488	Donkey	ThermoFisher (#A21202)	1:200
Donkey anti-Rabbit secondary antibody, Alexa Fluor 488	Donkey	ThermoFisher (#A21206)	1:200
Donkey anti-Rat secondary antibody, Alexa Fluor 488	Donkey	ThermoFisher (#A21208)	1:200
Donkey anti-Mouse secondary antibody, Alexa Fluor 546	Donkey	ThermoFisher (#A10036)	1:200
Donkey anti-Rabbit secondary antibody, Alexa Fluor 546	Donkey	ThermoFisher (#A10040)	1:200
Donkey anti-Mouse secondary antibody, Alexa Fluor 647	Donkey	ThermoFisher (#A31571)	1:200
Donkey anti-Rabbit secondary antibody, Alexa Fluor 647	Donkey	ThermoFisher (#A31573)	1:200
SECONDARY ANTIBODIES USED IN WESTERN BLOTTING			
HRP-conjugated secondary antibody	Goat anti-rabbit	ACTIVE MOTIF (#15015)	1:10,000

ANTIBODY	HOST	SOURCE	DILUTION
Fluorescence-conjugated secondary antibody	Goat anti-rabbit	LI-COR IRDye 800CW (green)	1:10,000
Fluorescence-conjugated secondary antibody	Goat anti-mouse	LI-COR IRDye 680RD (red)	1:10,000

Table 10: APPENDIX B (List of Antibodies). *Ms* = mouse, *Rb* = rabbit, *mAb* = monoclonal antibody, *pAb* = polyclonal antibody

5.17 APPENDIX C: List of qPCR primers and sequences

GENE	FORWARD PRIMER	REVERSE PRIMER
MYC	TTTCGGGTAGTGGAACCA	CACCGAGTCGTAGTCGAGGT
PAX8	TGCCTCACAACCTCCATCAGA	CAGGTCTACGATGCGCTG
KRT7	CTGCCTACATGAGCAAGGTG	GGGACTGCAGCTCTGTCAAC
CAPS	AGGGTGTGTGCAGGAAGTG	GGTCCAGCTTGCCAAATG
CCDC17	TGTGGGACCTGTGACATGGT	ACGCCCTGGTGTCTTGTG
FOXJ1	CAACTTCTGCTACTTCCGCC	CGAGGCACTTTGATGAAGC
LGR6	GCCTGAAAATCCTGATGCTG	ACCAGGGAGATGAGGTTGG
CD44	GACAAGTTTTGGTGGCACG	CACGTGGAATACACCTGCAA
Ki67	CCTGTACGGCTAAAACATGGA	GCTGGCTCCTGTTACGTA
LGR5	CAGCGTCTTCACCTCCTACC	GTTTCCCGCAAGACGTAAC
GFP	AAGGGCATCGACTTCAAGG	TGCTTGTCGGCCATGATATAG
FZD3_1	AATATGGACGTGTCACACTTCC	GGATATGGCTCATCACAATCTGG
FZD3_2	GTTTCATGGGGCATATAGGTGG	GCTGCTGTCTGTTGGTCATAA
FZD5_1	CATGCCCAACCAGTTCAACC	CGGCGAGCATTGGATCTCC
FZD5_2	CCGTTCTGTGCAAGTGTC	GAAGCGTTCATGTCGATGAG
FZD6_1	ATGGCCTACAACATGACGTTT	GTTTACGACAAGGTGGAACCA
FZD6_2	GAGCAAGTGAACAGGATTACCT	ATTCTGGTCGAGCTTTTGC
WNT7A	TGCCCCGACTCTCATGAAC	GTGTGGTCCAGCACGTCTTG
Wnt7a (mouse)	CCTTGTTGCGCTTGTTCTCC	GGCGGGGCAATCCACATAG
AXIN2	AGTGTGAGGTCCACGGAAAC	CTGGTGCAAAGACATAGCCA
Axin2 (mouse)	TGTCCAGCAAACTCTTC	CTTCTCTTGAAGGACCTGA
β -catenin (Total)	AAAGCGGCTGTTAGTCACTGG (Exon 2)	CGAGTCATTGCATACTGTCCAT (Exon 3)
β -catenin (exogenous)	GACTACAAAGACGATGACGACAAG (Flag tag)	CGAGTCATTGCATACTGTCCAT (Exon 3)
β -catenin (endogenous)	TCGAGGACGGTCGGACT (5' UTR)	ATTGTCCACGCTGGATTTTC (5' UTR)
KRT5 (mouse)	TCTGCCATACCCCATCTGT	CCTCCGCCAGAACTGTAGGA
GAPDH	GCAAATTCATGGCACCG	TCGCCCCACTTGATTTTGG
HPRT (mouse)	AAGTTTGTTGTTGGATATGC	CATCTTAGGCTTTGTATTGG

Table 11: APPENDIX C (List of qPCR primers and sequences). All qPCR primers were chemically synthesized and provided by Sigma-Aldrich. Unless otherwise stated, all qPCR primers target the human version of the gene. All sequences are in the direction 5' – 3'

5.18 APPENDIX D: List of siRNA's and sequences

Target gene	Type	Catalogue #	siRNA sequences
Non-Targeting	siGENOME	D-001210-05-20	Sequence: UGGUUUACAUGUCGACUAA
β -catenin (CTNNB1)_09	ON-TARGETplus	LQ-003482-00-0002	Sequence: GAUCCUAGCUAUCGUUCUU
β -catenin (CTNNB1)_10	ON-TARGETplus	LQ-003482-00-0002	Sequence: UAAUGAGGACCUAUACUUA
β -catenin (CTNNB1)_11	ON-TARGETplus	LQ-003482-00-0002	Sequence: GCGUUUGGCUGAACCAUCA
β -catenin (CTNNB1)_12	ON-TARGETplus	LQ-003482-00-0002	Sequence: GGUACGAGCUGCUAUGUUC
Non-Targeting	siGENOME SMARTpool	D-001206-13-05	Sequence 1: UAGCGACUAAACACAUCAA Sequence 2: UAAGGCUAUGAAGAGAUAC Sequence 3: AUGUAUUGGCCUGUAUUAG Sequence 4: AUGAACGUGAAUUGCUCAA
WNT7A	siGENOME SMARTpool	M-008543-01-0005	Sequence 1: GCGCAAGCAUCAUCUGUAA Sequence 2: UCAAGAAGCCACUGUCGUA Sequence 3: CAACGAGGCAGGCCGAAAG Sequence 4: GAACUGCUCUGCACUGGGA
Frizzled-3 (FZD3)	ON-TARGETplus SMARTpool	L-005502-00-0005	Sequence 1: CCAAUACUCCUAUCAUAA Sequence 2: ACAGAUACUCCAGGCAUA Sequence 3: GUUCGAAGCUAUGGAGAU Sequence 4: UGAUUGAUGUCACAAGAUU
Frizzled-5 (FZD5)	ON-TARGETplus SMARTpool	L-005504-00-0005	Sequence 1: GCAUGUGGUGGCCUGCUA Sequence 2: GCACAUGCCCAACCAGUUC Sequence 3: AAUACGGUGCCCAUGUG Sequence 4: GAUCCGCAUCGGCAUCUUC

Frizzled-6 (FZD6)	ON-TARGETplus SMARTpool	L-005505-00- 0005	Sequence 1: CCAGAGAGACCAUUUAUUAU Sequence 2: UCGCAAAUCUGGAAUGUUC Sequence 3: GAAGGAAGGAUUAGUCCAA Sequence 4: CAGUGAAAGUCGAAGAGUA
β -catenin (CTNNB1)	ON-TARGETplus SMARTpool	LQ-003482-00- 0002	Sequence 1: GAUCCUAGCUAUCGUUCUU Sequence 2: UAAUGAGGACCUAUACUUA Sequence 3: GCGUUUGGCUGAACCAUCA Sequence 4: GGUACGAGCUGCUAUGUUC

Table 12: APPENDIX D (List of siRNA sequences). All siRNA's were obtained from Dharmacon (GE Healthcare). SMARTpool siRNA's were used as a 4-in-1 pool, targeting different regions of the same mRNA.

CHAPTER 6

ESTABLISHING & CHARACTERISING
FALLOPIAN TUBE ORGANOIDS

ESTABLISHING & VALIDATING FT ORGANOID

6.1 Introduction

HGSOC is a public health burden and the main driver of high mortality in ovarian cancer. Research into HGSOC initiation has witnessed a paradigm shift in the last decade, with numerous studies documenting precursor lesions in the distal part of the FT. This FT region is reportedly enriched in cells with longevity, multipotency and stem-like character. Interrogation of the role of FT stem cells in HGSOC initiation has been hampered by lack of knowledge about FT stem cells, and lack of reliable FT models.

Identifying FT stem cells necessitates the employment of a model that can reconstruct the stem cell niche *in vitro*, and allow it to be tractably and functionally investigated. Although organoid culture was optimised for FT tissue (Kessler *et al.*, 2015), the reported protocol is poorly optimised for reasons previously outlined (section 3.4.2). A major limitation is that the reported protocol is not amenable to isolation of specific cell populations or to single cell approaches. This precludes isolation and characterization of putative stem cells.

In this chapter, I further optimise the conditions for FT organoid culture. In line with Objective 1 of the thesis, I test the fidelity of organoids in modelling FT tissue. The testing of this fidelity is further stretched to examine, in organoids, the presence of novel FT cell types predicted by scRNA-seq of fresh hFT cells. Finally, the presence of multipotent stem cells will be investigated by single cell culture.

6.2 Optimizing FT Organoid Culture

In the last two decades, a number of FT models have been established (Table 5). Each model suffers from a drawback that precludes investigating FT stem cells. Primary 2D culture methods of patient-derived tissue is the most frequently employed, and is thought to be more physiologically relevant than cell lines, as it derives from patient material.

1. Limitations of primary 2D culture

I began by considering this culture system for modelling FT biology. Table 5 lists some of the general limitations associated with this culture system, including dimensionality and lack of representation of cellular diversity. This culture system is epithelial upon establishment (Figure 25 A-B) but loses its epithelial character over time. This happens within days in the absence of Y-27632 (Figure 25 C-E), or within 5 weeks in the presence of Y-27632 (Figure 25F). Y-27632 is a selective ROCK inhibitor, used as an anoikis inhibitor and pro-survival factor in ESC culture (Li *et al.*, 2008), as well as in organoids.

By 2-3 weeks in 2D culture, cells lose organoid-forming ability (data not shown). Ciliated cells are not seen in 2D-cultured FT cells, casting doubt on its physiological relevance. Work from the host lab has compared, at the single cell level, the transcriptional profiles of FT cells freshly obtained versus cells 2D-cultured using this method. In addition to absence of ciliated cells and putative stem cells, we showed that 2D-cultured cells lack the heterogeneity seen in the secretory cell compartment (Hu *et al.*, 2020). Collectively, these data suggest that 2D culture is not a reliable and faithful model of FT tissue.

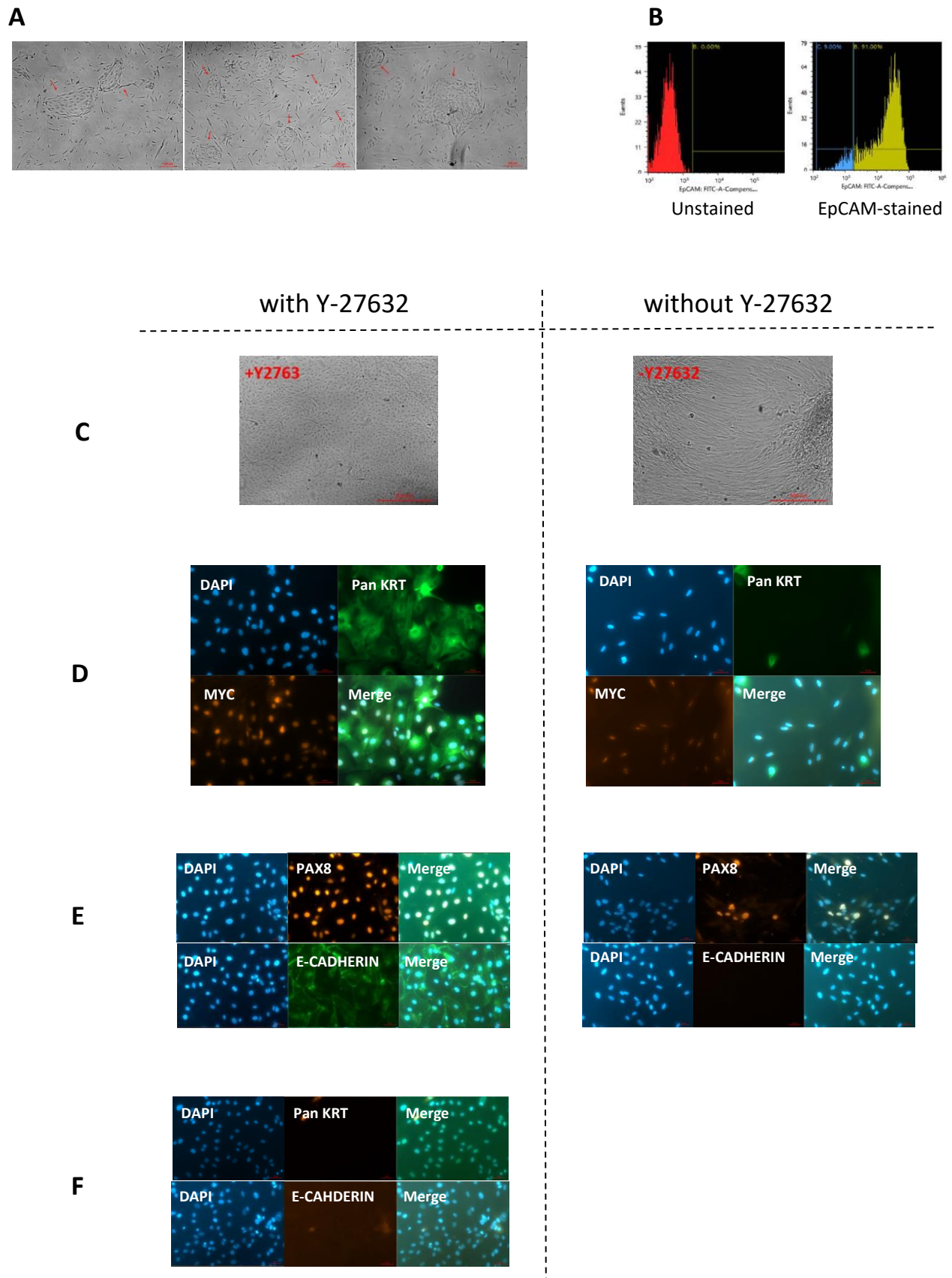


Figure 25: Primary FT 2D culture loses epithelial character in vitro. (A) Representative images of FT 2D culture on day 3. Red arrows point to emerging 'islands' of epithelial cells. (B) At day 7, cells have high epithelial purity (over 90%) as estimated by flow analysis. (C) Upon withdrawal of Y-27632, cells lose epithelial morphology and resemble fibroblasts (right panel). (D-E) Cells with no Y-27632 also lose expression of epithelial (pan keratin and E-cadherin) as well as FT-specific (PAX8 and MYC) markers. (F) If Y-27632 is not withdrawn, cells lose epithelial character by Day 37.

2. Expression and purification of organoid culture proteins

Thus, it became necessary to optimise organoid culture as a reportedly more superior model of FT tissue. Activation of Wnt signaling is critical for self-renewal of ASCs in organoid-based cultures of many tissues (Clevers, 2016). This includes FT organoids (next chapter). Most studies use purified RSPO1 protein or RSPO1 conditioned medium (CM). As shown later (Figures 44 and 45), the latter is not ideal and interferes with stem cell self-renewal. To establish the organoid culture, RSPO1 protein was expressed and purified in-house (Figure 26 A-B), and its activity was validated using the Wnt-reporter (TOPFlash) assay (section 5.11 for technical details). I utilize purified RSPO1 as a routine component of organoid medium, unless otherwise stated. Wnt3a protein, which is currently the only culture-stable Wnt ligand widely used in organoid culture, is also produced to test its relevance to FT biology. Wnt3a alone or RSPO1 alone are insufficient to activate Wnt signaling, but together the pair potentially activate the pathway (Figure 26-D).

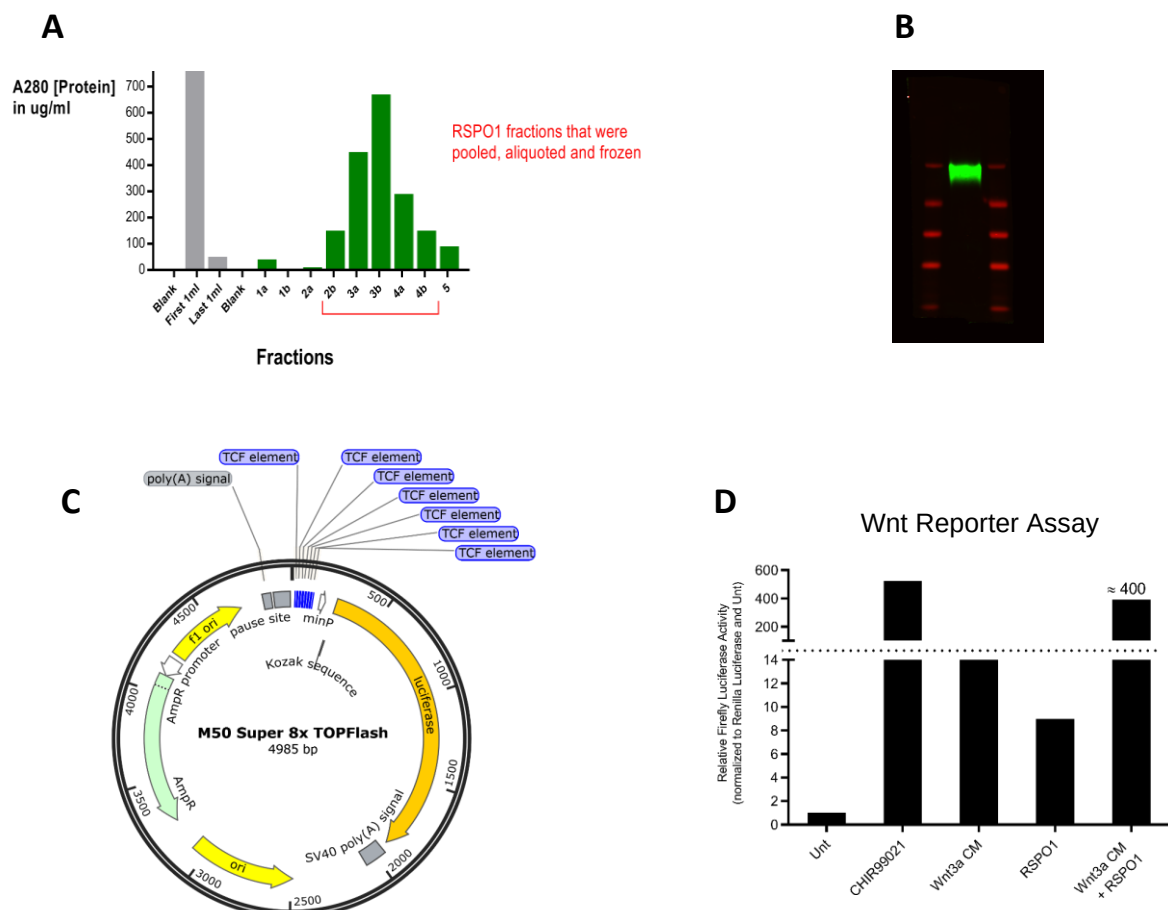


Figure 26: RSPO1 expression and purification. (A) RSPO1 protein purification using Protein A Agarose Affinity columns. (B) WB using HA tag antibody detects HA-tagged purified RSPO1. (C) TOPFlash plasmid used to transfect HEK cells. TOPFlash contains TCF/LEF WRE elements that drive firefly Luciferase expression. Luminescence was detected using the GloMax luminometer. (D) Activity of expressed Wnt3a and RSPO1 validated by the TOPFlash assay. Unt = Untreated, CHIR99021 is a potent GSK-3 β inhibitor and activator of Wnt signaling, used here as a positive control. CM is conditioned medium. CHIR99021 is used at 3 μ M, Wnt3a CM at 25% and RSPO1 at 500 ng/mL. (A), (B) and (D) are representative of three biological replicates.

3. Optimizing FT organoid culture

Next, the purified proteins were used to establish organoid culture using the reported method (Kessler *et al.*, 2015). This presented challenges outlined in Table 13.



	Reported protocol
Establishing organoids	✓
Passaging organoids	✗*
Re-establishment after cryopreservation	✗*
Organoid regeneration after single cell dissociation	✗
Organoid regeneration after FACS of existing organoids	✗
Organoid establishment after FACS of freshly dissociated tissue	✗
Organoid growth after single cell FACS sorting	✗

Table 13: Limitations of the reported protocol for hFT organoid culture. A number of criteria require to be met before a robust organoid culture method can become established and reproducible. Apart from establishing organoids, none of the other criteria were met. * denotes criteria that were met in 2 other publications, but were not reproducible in this thesis. No studies reported the ability to grow hFT organoids after single cell dissociation or FACS isolation, whether in bulk or single cell culture. This indicates that culture conditions are not sufficiently optimised to cater for stem cell self-renewal and autonomous stem cell growth.

As organoids are propagated by a minority of self-renewing stem cells, I reasoned that lack of organoid growth might be due to inadequate maintenance of FT stem cells within organoids. This might signify that stem cells could be promptly differentiating and failing to generate organoids. There is some indication in the literature that robust Wnt activation might be important for FT organoid regeneration. Reported approaches for Wnt activation were tested (Table 14) but failed to instigate improvements in the protocol.

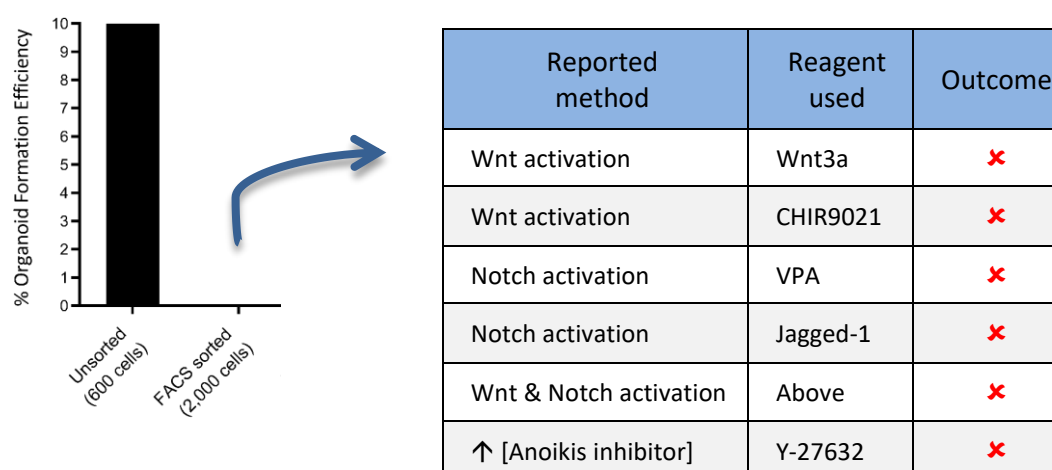


Table 14: Optimisation of organoid regeneration after single cell dissociation. Left panel shows the %OFE of unsorted cells from this patient. Upon FACS sorting and culture of the same cells using the reported protocol, organoids do not survive. This could be due to inadequate maintenance of stem cells in organoid culture (see chapter 7). Wnt activation by various reported methods were unsuccessfully trialled.

TGF- β signaling promotes stem cell differentiation (Sakaki-Yumoto *et al.*, 2013), and its inhibition is important for organoid regeneration. To this end, Noggin is a key component of human-derived organoid cultures from various organs, including the FT (Kessler *et al.*, 2015). Noggin inhibits BMP-activated TGF- β signaling, that is mediated by Smads 1/5/8. Noggin does not, however, inhibit TGF- β signaling through Smads 2/3. I reasoned this could be driving stem cell differentiation and arrest of organoid growth. To overcome this, A83.01 was utilized as a selective inhibitor of TGF- β type I receptors, effectively shutting down this route of TGF- β activation.

Furthermore, Forskolin was tested to promote cell survival. Forskolin is an activator of Adenylyl Cyclase. It increases intracellular cAMP levels and promotes survival, including of patient-derived HGSOC cells (Ince *et al.*, 2015), which have probably originated from the hFT. A83.01 and Forskolin treatment promoted organoid regeneration after single cell dissociation (Figure 27). The pair greatly improved organoid passaging and regeneration from single cells derived from organoids or from fresh tissue (data not shown), as well as from single cells plated in single wells (shown later, Figure 31). Organoid growth after cryopreservation also benefitted from this, although it has not been as robustly reproducible as other criteria outlined in Table 13.

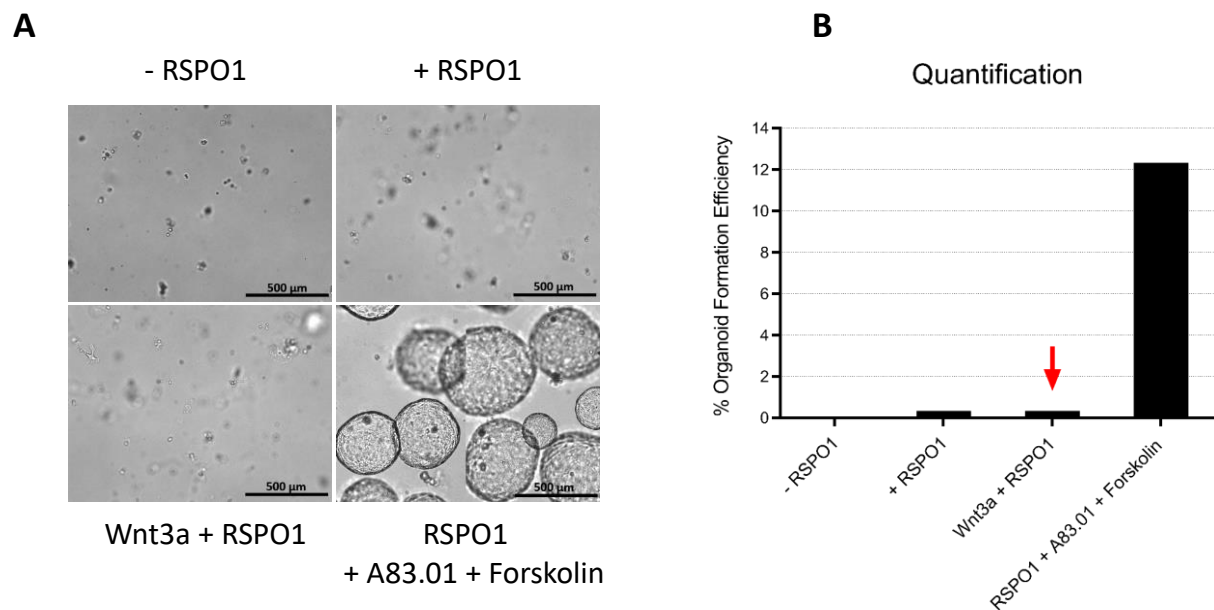


Figure 27: A83.01 and Forskolin promote regeneration of hFT organoids after single cell dissociation.

(A) Cells were dissociated from p.1 organoids using TrypLE, and immune-labelled. 1,200 CD45- EpCAM+ cells were sorted directly into ECM (Matrigel). A83.01 and Forskolin restore OFE to levels seen in unsorted cells (Table 14). **(B)** Quantification of images shown in (A). Note that the previously reported protocol, which uses SB431541 at 0.5 μ M (red arrow), fails to promote organoid regeneration. Importantly, A83.01 and Forskolin do not non-specifically induce organoid regeneration, and do not promote stem cell-independent cell division (shown later, Figure 40). A83.01 and Forskolin were incorporated into routine organoid medium, at 5 μ M and 10 μ M, respectively. Data representative of 3 patient replicates. A83.01 alone is sufficient to instigate organoid growth after single cell dissociation (shown later, Figure 40). Forskolin was shown to improve organoid passage (Huch et al., 2015) and survival of FT-derived cancer (Ince et al., 2015). Effect of Forskolin on organoid passage was not tested in this thesis.

4. Organoids are spherical and express epithelial and FT-specific markers

After successful establishment and propagation of organoid culture, a basic characterisation was performed to confirm the relevance of human FT (hFT) organoids to hFT tissue. Organoids emerge only from epithelial cells (Figure 28A), are spherical in structure (Figure 28 B-C) and display invaginations characteristic of hFT tissue (Figure 28 D-E). Notably, organoids have a strictly monolayered cellular arrangement, which is perturbed in tumour organoids (Figure 28F) and was shown to dysregulate upon insertion of TP53 and BRCA mutations into otherwise healthy mouse oviduct organoids (Löhmußsaar et al., 2020).

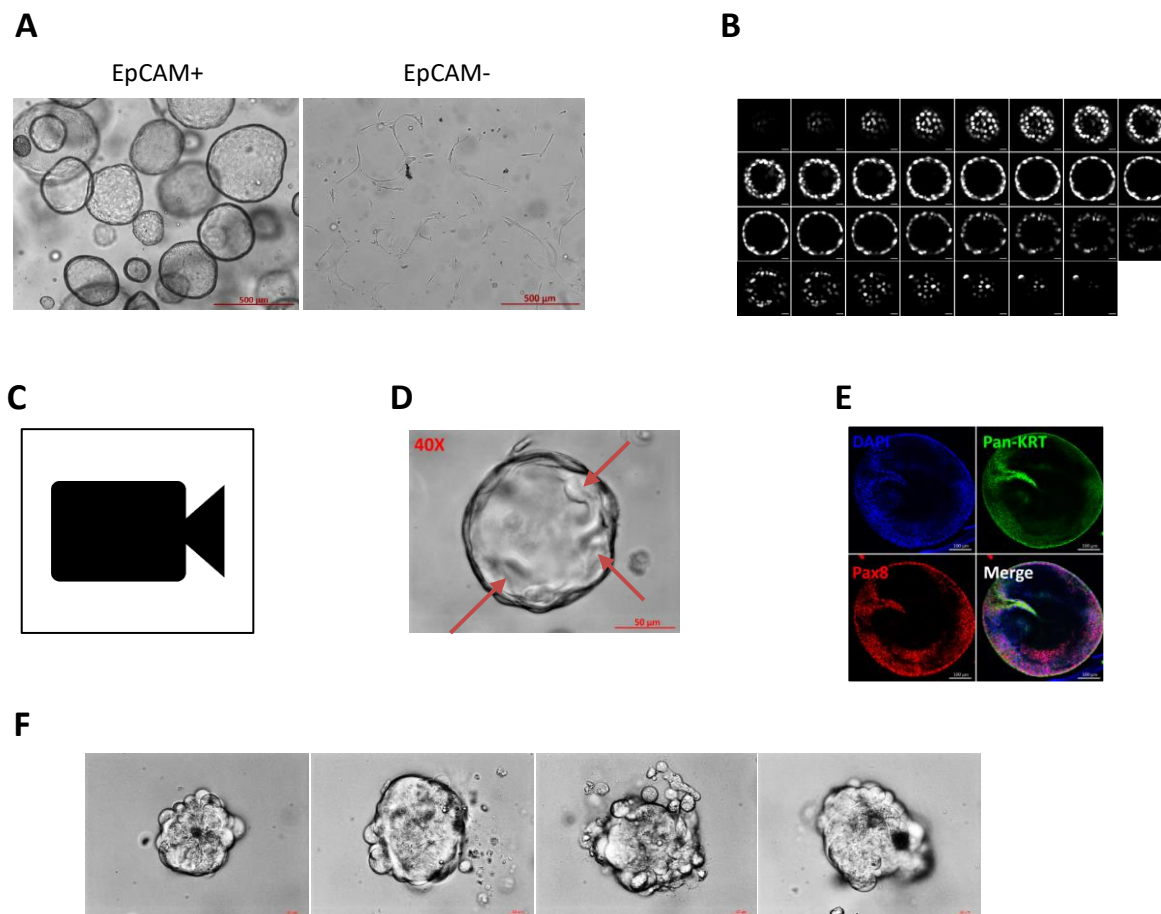


Figure 28: Morphology of FT organoids. (A) Organoids have a spherical structure. Organoids do not emerge from non-epithelial (EpCAM-) cells. (B) Optical Z-stack sections of a live organoid after nuclear staining with Hoechst, showing a monolayered cellular arrangement with a hollow interior. (C) See movie files accompanying this thesis. The movies show a rotating 3D reconstruction of the upper semi-sphere of a live organoid, confirming monolayered spatial organization and a hollow interior. Blue staining is Hoechst, red is phalloidin, showing cell edges. (D) Mature organoids contain internal foldings and invaginations characteristic of FT tissue. Invaginations are pointed to by red arrows. (E) Invaginations clearly observed upon IF staining. KRT = keratin. (F) Organoids established from the metastatic omentum tumour deposits of a HGSOc patient. Note the marked disruption in the monolayered cellular arrangement.

Organoids also express epithelial (Figure 29A) and FT-specific markers of secretory and ciliated cells (Figure 29B), confirming their FT origin. A small fraction of cells in organoids are proliferative (Figure 28C). Although proliferation may be driven by TA cells or by de-differentiated cells upon injury, long-term proliferation or self-renewal under homeostatic conditions is restricted to stem cells. For instance, in intestinal organoids established from Lgr5-EGFP mice, proliferation markers exclusively localize to GFP+ Lgr5+ cells (Yin *et al.*, 2013). Thus, these proliferative cells in FT organoids may represent or enrich for FT stem cells.

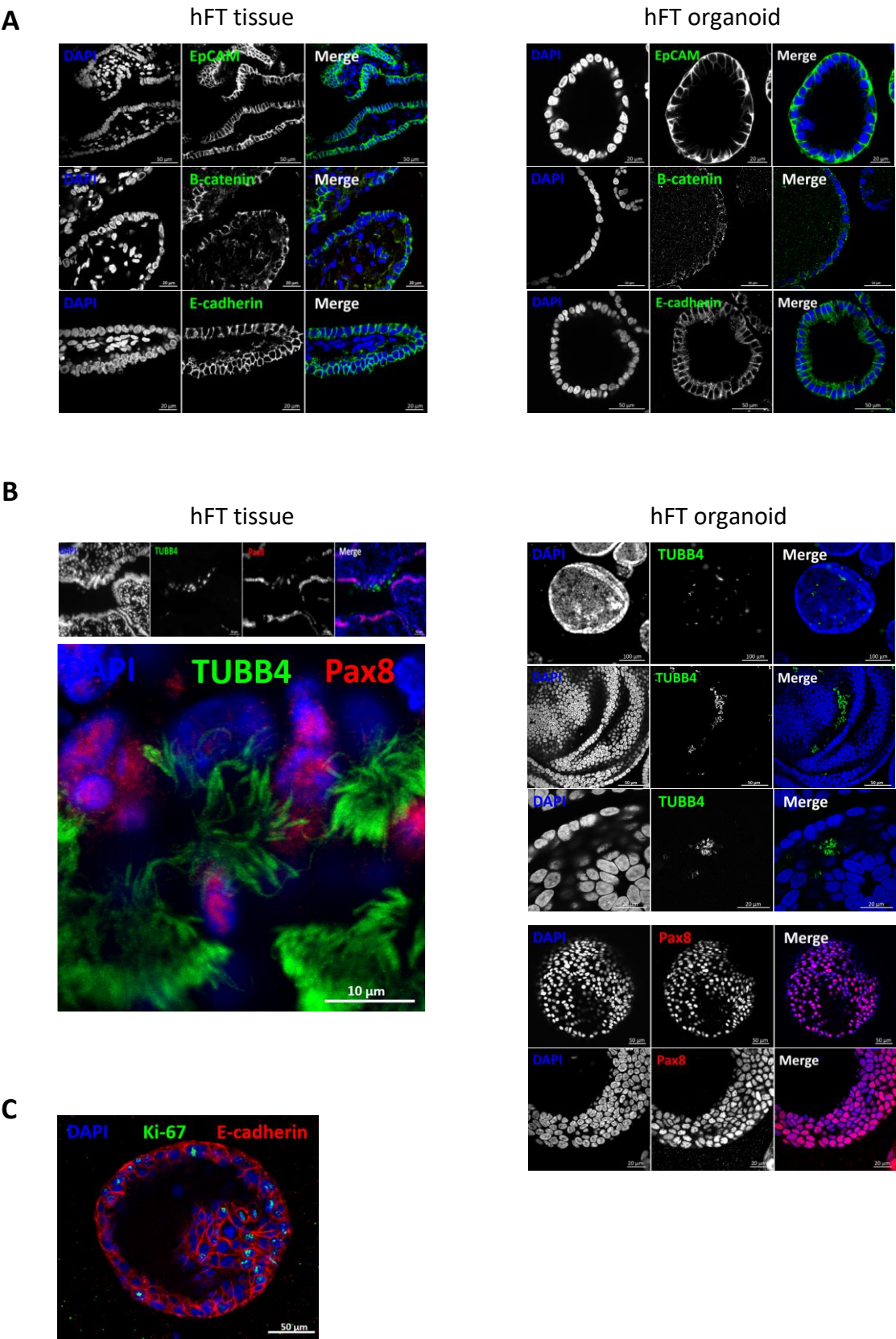


Figure 29: Side-by-side comparison of hFT tissue and hFT organoids. (A) Organoids express epithelial markers exemplified by EpCAM, β -catenin and E-cadherin staining. **(B)** Organoids express PAX8 and TUBB4, markers of FT secretory and ciliated cells, respectively. **(C)** Few cells within organoids are proliferative.

Growth of organoids from mouse Oviduct (mOV) tissue was also optimized (data not shown), based on a previous report (Xie *et al.*, 2018). It is confirmed here that mOV organoids are spherical (Figure 30A), contain proliferative cells (Figure 30B) and express FT-specific markers (Figure 30C).

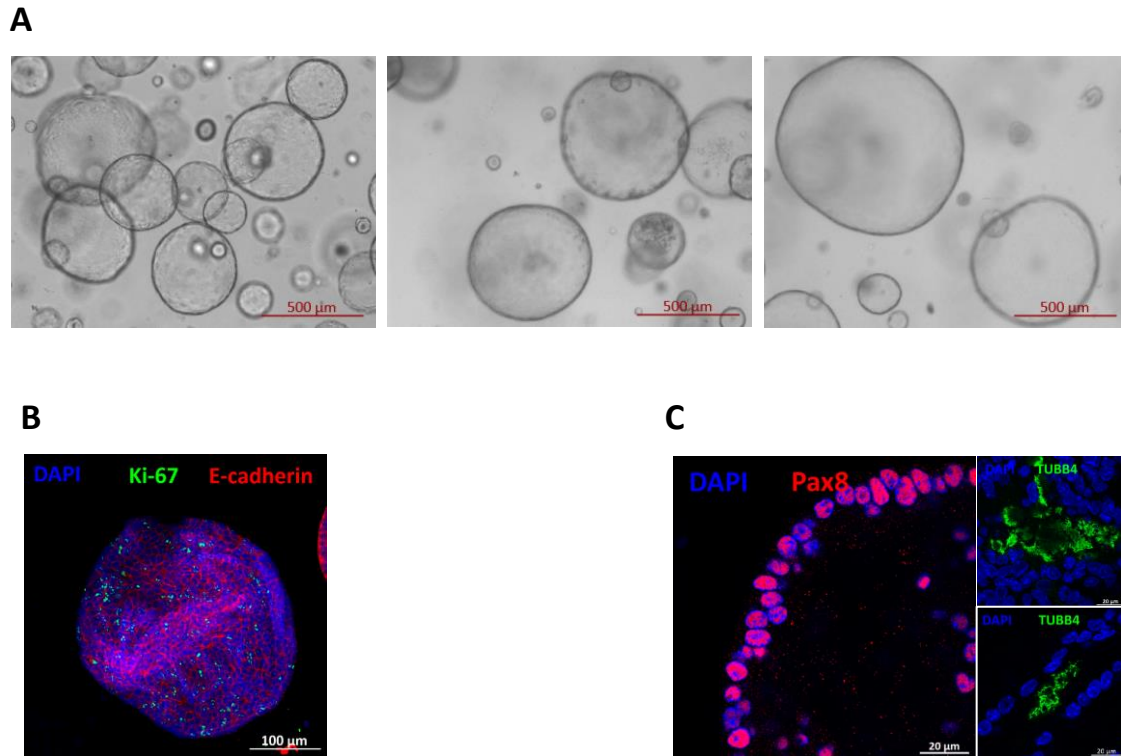


Figure 30: Validation of mOV organoids. Like hFT organoids, mOV organoids are morphologically spherical (A) and express epithelial (B) and FT-specific (C) markers. Ciliated cells emerge after a week in culture. This was also observed for hFT organoids (Kessler *et al.*, 2015). The culture medium of human and mouse organoids was the same, with the exception of the human-specific supplements A83.01 and Forskolin.

5. Organoids emerge from a single multipotent cell

Organoids are multicellular structures containing differentiated progeny. It can be argued that *in vitro* culture conditions promote cell division of normally quiescent and differentiated cells, resulting in cell types proliferating to yield their own kind. To rule this out, and to confirm the presence of multipotent stem cells in organoids, single cells were plated into single culture wells and tracked for a period of over 1 month. A small proportion of cells, 0.5-1%, were able to form organoids (Figure 31A) containing differentiated cells (Figure 30B). This confirms the existence of yet unidentified FT stem cells. It may be argued that this only happens in single cell culture, and that in bulk culture organoids may form clumps of well-organized cells. When single GFP+ or RFP+ FT cells were mixed and grown, emerging organoids were shown to be completely green or completely red, not both

(Kessler *et al.*, 2015). This rules out the notion that organoids are clumps of cells, and definitively establishes organoids as monoclonal structures emerging from a single multipotent stem cell.

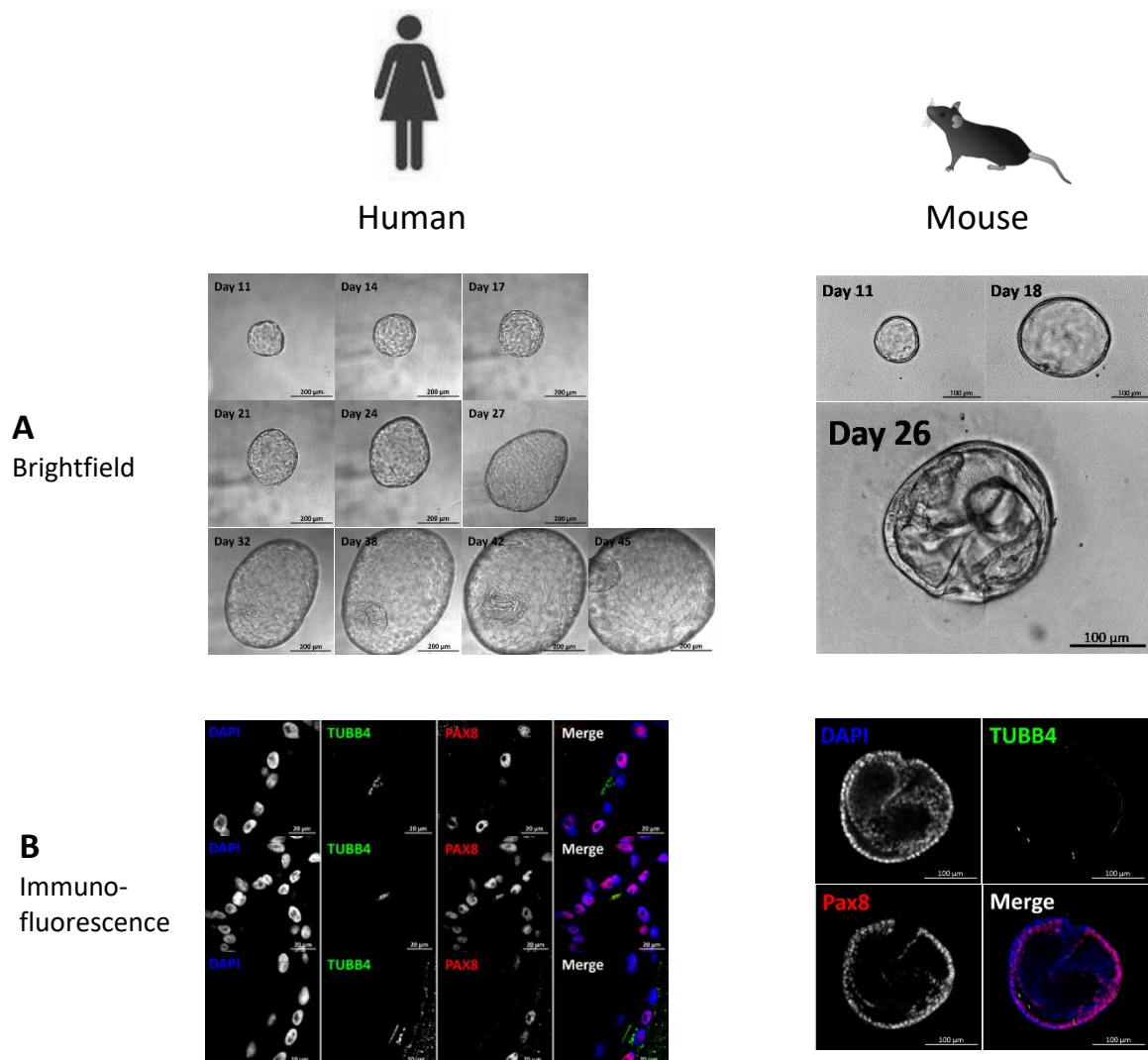


Figure 31: hFT and mOV organoids emerge from a single multipotent stem cell. (A) Sorted CD45- EpCAM+ cells were plated at a limiting dilution of 0.7 cells per well on 96-well plates. 0.5-1% of cells formed organoids that gradually increased in size and complexity. **(B)** These monoclonal organoids contained Pax8+ secretory cells and TUBB4+ ciliated cells, evidencing multipotency of the organoid-forming cell.

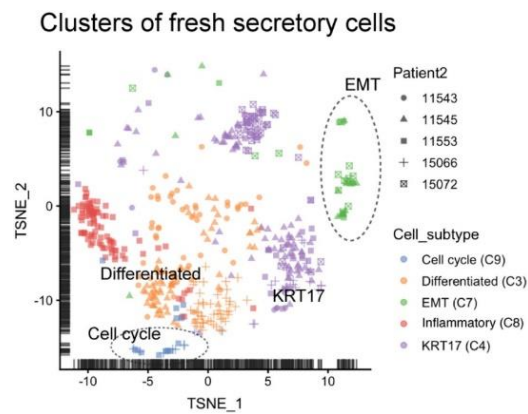
6.3 Organoids as *in vitro* models of the FT

Upon optimization of organoid culture, I sought to investigate how reliable organoids are as a model of tissue biology. Comparative bulk transcriptomic analyses showing physiological relevance of organoids to FT tissue was previously reported (Löhmussaar *et al.*, 2020; Kopper *et al.*, 2019). The first single cell atlas of fresh hFT tissue was published by the host lab (Hu *et al.*, 2020). The findings unmasked a previously unappreciated heterogeneity in FT cell types, and below I show validation data for some of these observations. Importantly, and as an extension of the comparative analyses between hFT tissue and organoids, I was interested to see if organoids reflect the detection of these novel cell types / markers in FT tissue.

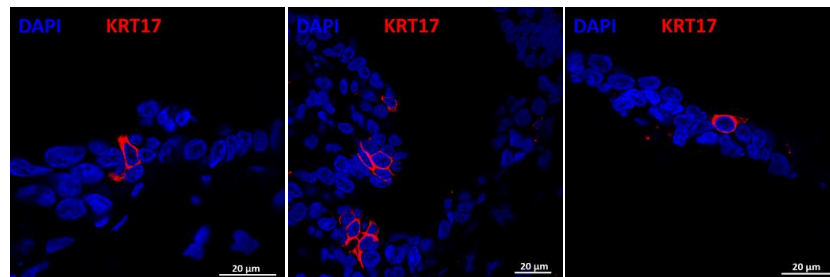
1. KRT17 marks a novel cluster of FT secretory cells

Among the newly identified secretory cell types in hFT tissue, KRT17 marks an interesting cluster of cells (Figure 32A). KRT17+ cells upregulate MHC class II genes, a number of cytokeratins as well as aldehyde dehydrogenases. KRT17+ cells are hypothesized to play a role in FT immune biology, and could be important for HGSOc. KRT17+ cells were detected in hFT tissue (Figure 32B) as well as organoids (Figure 32C). Thus, organoids present an opportunity for isolating these cells, characterising them deeper and undertaking functional research on their interactions with immune cells under health conditions, during tumourigenesis and upon pathogenic infection.

A
Predictions from
scRNA-seq data



B
hFT tissue



C
hFT organoids

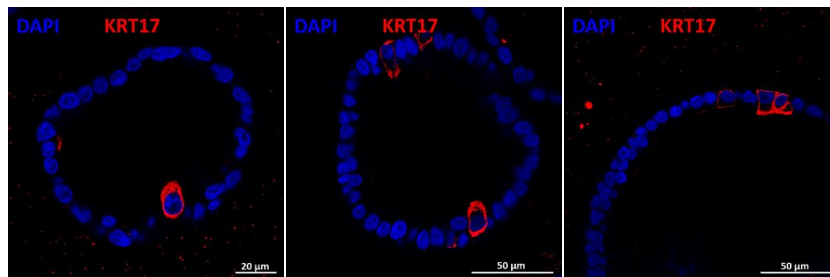


Figure 32: Validation of KRT17 cluster in the hFT. (A) KRT17 marks a cluster of cells with elevated expression of ALDH and MHC class II genes. KRT17+ cells were validated at the protein level in tissue sections (B) and organoids (C). [(A) is the work of Zhiyuan Hu. (B) and (C) were generated by this thesis. All are published in Cancer Cell at Hu et al. (2020)].

2. KRT7 and CAPS are novel markers of FT cell types

Currently, very few markers exist to identify different cell types in the FT epithelium (section 3.3.1). Cell type biomarkers are useful tools in research, for isolation and characterization of their functions in a tissue context. The aforementioned study identified a number of novel and specific markers highly enriched in FT secretory or ciliated cells (Figure 33A). The putative markers were validated in hFT tissue (Figure 33B), and organoids faithfully reproduce this (Figure 33C).

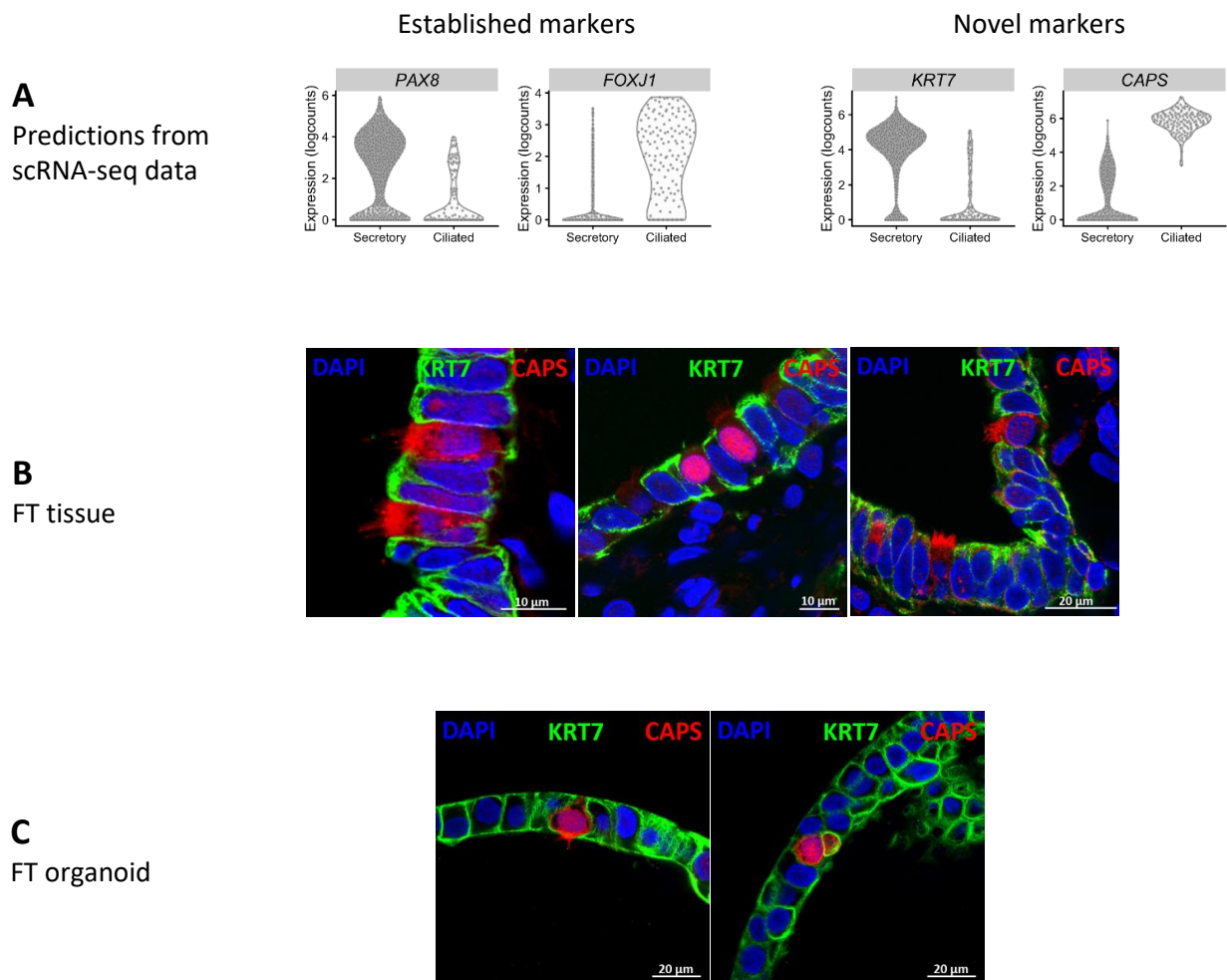


Figure 33: Validation of novel markers of FT cells. (A) KRT7 is a marker of secretory cells. CAPS marks ciliated cells. Both markers have been validated in hFT tissue (B) and organoids (C). Note that CAPS localization can be nuclear, cytoplasmic or on cilia. [(A) is the work of Zhiyuan Hu, (B) and (C) were generated by this thesis. All are published in *Cancer Cell* at Hu et al. (2020)].

KRT7 marks secretory cells. CAPS marks ciliated cells. Both markers were not mutually exclusive, and double-positive cells were detected (Figure 34). Given that putative stem cells are secretory in nature (this thesis; Ghosh *et al.*, 2017), this double-positive population could represent an intermediate cell population *en route* to ciliogenesis. Interestingly, the double-positive cells were more enriched in organoids relative to FT tissue (data not shown). This is consistent with the hFT tissue as a slowly cycling organ, while hFT organoids are dynamic and in a constant state of self-renewal and differentiation. Organoids thus present a versatile tool that can be harnessed to understand differentiation mechanisms in the hFT. This is relevant to cancer, as cells blocked in differentiation have been implicated in tumour initiation (Chen *et al.*, 2010; Hellner *et al.*, 2016), including in HGSOc initiation where early precursor lesions (SCOUTs, section 3.3.2) are documented

to be expansive outgrowths of secretory cells that are blocked in their ability to differentiate to ciliated cells.

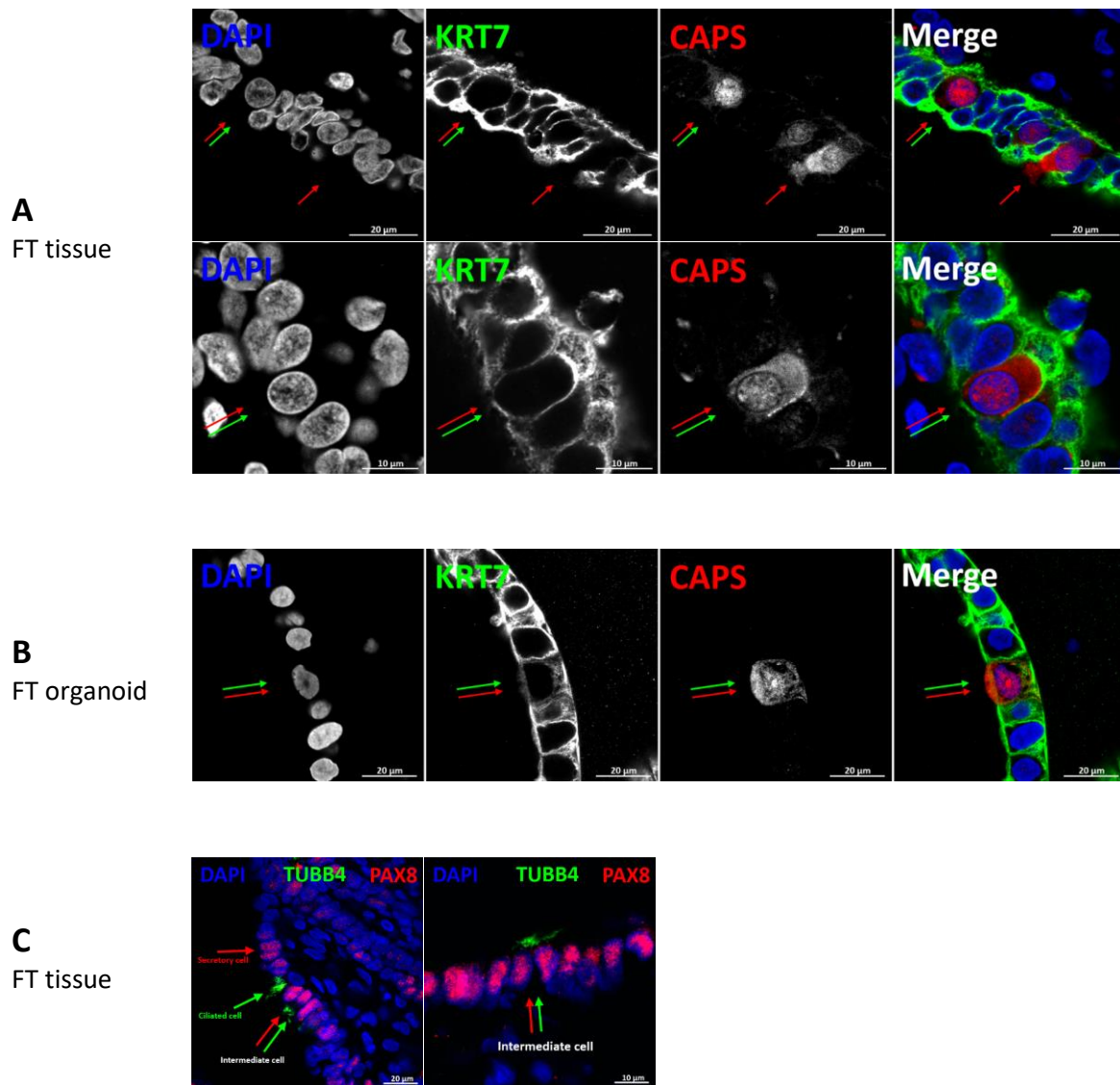


Figure 34: KRT7+ CAPS+ double-positive cells. These were detected in hFT tissue (**A**) and hFT organoids (**B**). Note in (**B**) that the CAPS+ cell has reduced KRT7, and is perhaps en route to losing other secretory markers and differentiating to a ciliated lineage. (**C**) Double-positive cells detected using established markers of FT cell types. These are rare, and less common than KRT7+ CAPS+ cells.

Comparing the established and novel ciliated cell markers, these do not completely overlap (Figure 35). CAPS is a calcium-binding protein involved in ion transport. It can be seen to localize to the cilia of ciliated cells (Figure 33B), as well as to the nucleus and cytoplasm. This could indicate a role for calcium signaling in FT ciliary function. Again, although beyond the remit of this thesis, FT organoids present an opportunity to investigate these hypotheses in live ciliated cells within a 3D environment.

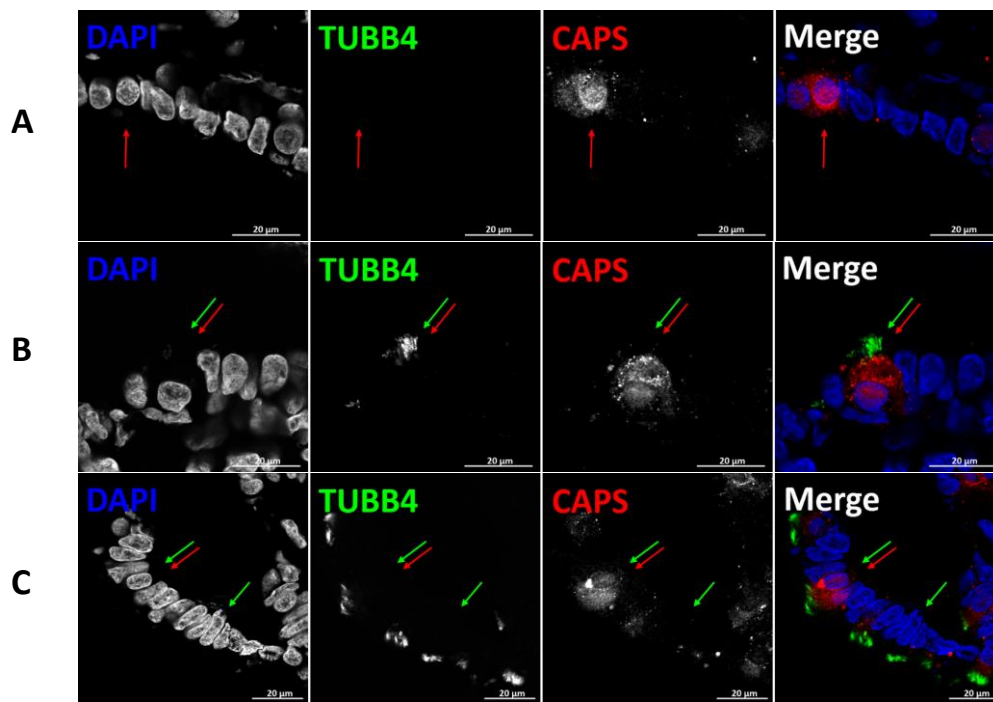


Figure 35: CAPS does not mark all ciliated cells. TUBB4 is a marker of mature ciliated cells. **(A)** CAPS may mark cells that are not yet ciliated (TUBB4-). **(B)** CAPS and TUBB4 co-localize in ciliated cells, as predicted by the scRNA-seq data. **(C)** CAPS may be absent from mature ciliated (TUBB4+) cells. Population (A) is rare (data not shown), suggesting that CAPS could be an early marker of ciliogenesis, disappearing in mature TUBB4+ ciliated cells.

3. Basal FT cells are not stem cells

In addition to secretory and ciliated cells, a third cell type is observed in the FT. These cells, called basal or peg cells, localize basally within epithelial sheets. The identity of these cells has remained a bone of contention over decades (Peters, 1986; Ferenczy *et al.*, 1972). A report has proposed these basal cells to be stem cells marked by CD44 (Paik *et al.*, 2012), showing enhanced colony formation ability, although by a modest factor of 5. Others have argued that these are lymphocytes with no epithelial character.

Figure 36 shows these cells. They are clearly proliferative (Figure 36A) and epithelial (Figure 36 A-B). Interestingly, these cells also express general immune marker CD45 and T-lymphocyte marker CD3 (Figure 36 C-D). As reported by Paik *et al.* (2012), these cells are also CD44+ (Figure 36C). Further characterization in the host lab revealed these cells to be lymphocyte-like, expressing CD3, CD69 and CD103 (Hu *et al.*, 2020). One can appreciate the contention these basal cells have stirred, given the unconventional mix of epithelial and immune markers they express. These immuno-epithelial cells were also detected in hFT and mOV organoids (Figure 36 E-F). In organoids, basal cells are extremely

rare (less than 0.1% as quantified by FACS) and tend to disappear after the first few passages. I speculate they require specific immune factors for maintenance, or at least co-culture with other non-epithelial compartments. Like KRT17+ cells, these cells may be important in FT immune biology (see Discussion, section 9.1.2). For the purpose of this thesis, the frequency of these cells and their absence from organoid culture disqualifies their candidacy as FT stem cells, at least in organoids.

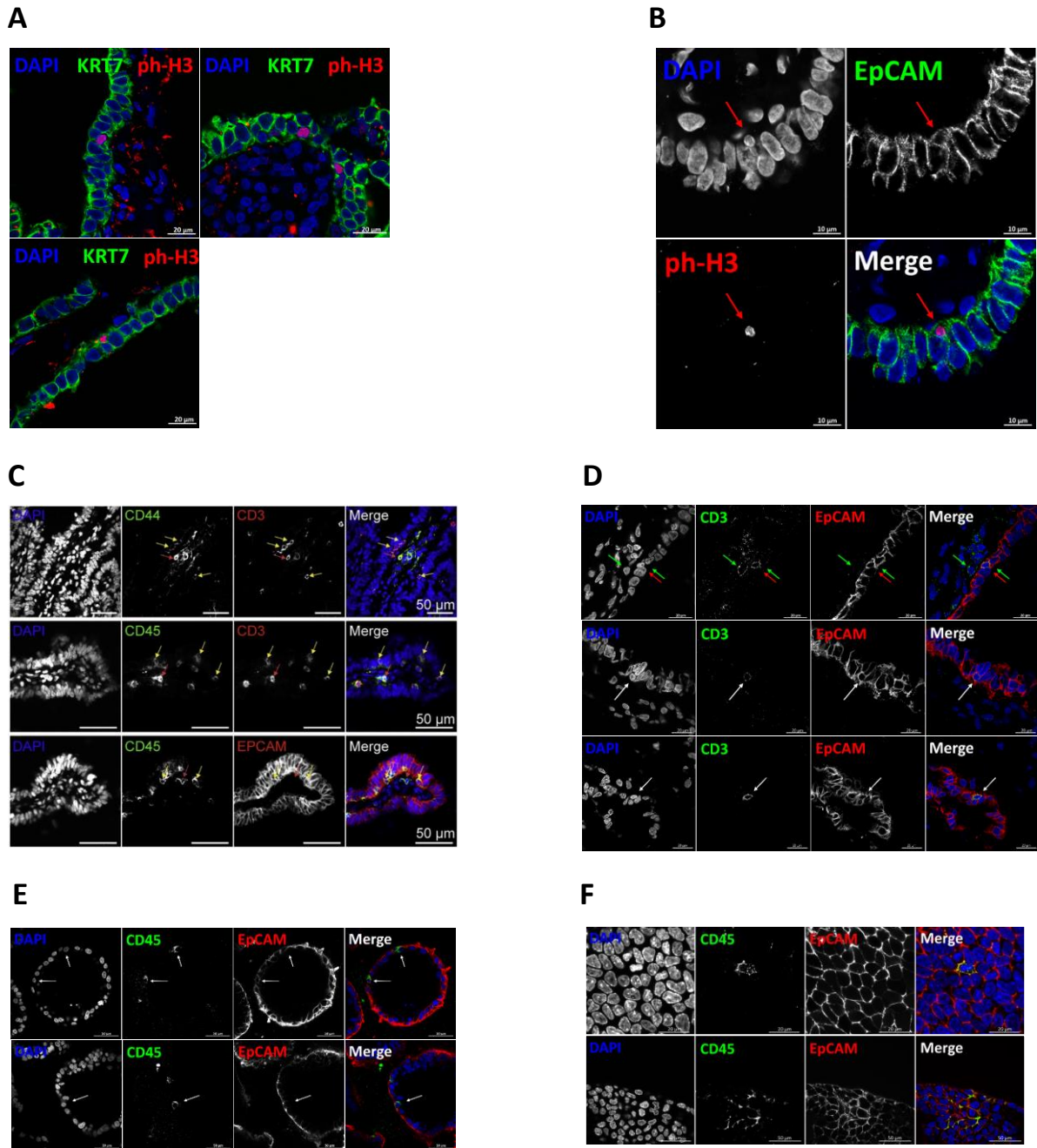


Figure 36: Basal proliferative FT cells are immune cells with epithelial markers. (A-D) performed in hFT sections; (E-F) in organoids. **(A)** Basal cells are KRT7+ and proliferative, as shown by phospho-Histone H3 (S10) staining. **(B)** In addition to KRT7, basal cells also express EpCAM, suggesting they may be epithelial. **(C)** These cells express CD45, a leukocyte marker, and CD44, as reported (Paik et al., 2012). **(D)** Basal cells express CD3, a marker of T-lymphocytes. Note in the top image that CD3+ cells can be seen in the stroma (green arrow), but within the epithelial sheet, CD3+ cells are also EpCAM+ (green and red arrows). These immune-epithelial cells are detectable in hFT organoids **(E)** and MOV organoids **(F)**. [Some of these images were published in Cancer Cell (Hu et al., 2020). (D) is the work of Nina Wietek].

4. KRT5 and KRT14 in the FT

KRT5 and KRT14 are markers of basal cells, stem cells or both in various tissues, including the mammary gland (Van Keymeulen *et al.*, 2011), skin (Mascré *et al.*, 2012), lung (Q. Liu *et al.*, 2019), taste buds (Ren *et al.*, 2014), bladder (Papafotiou *et al.*, 2016), oesophagus (DeWard *et al.*, 2014) and other tissues. Single cell work in the host laboratory identified a cluster of KRT5-expressing and KRT14-expressing cells (Figure 37) in hFT tissue. Although the pair are generally co-expressed in the aforementioned tissues, these cells are distinct in the FT. Given what is known about KRT5/14+ cells in other tissues, I asked whether these cells might have stem cell properties or, as documented in taste buds (section 1.4.12), mark Wnt-active stem cells.

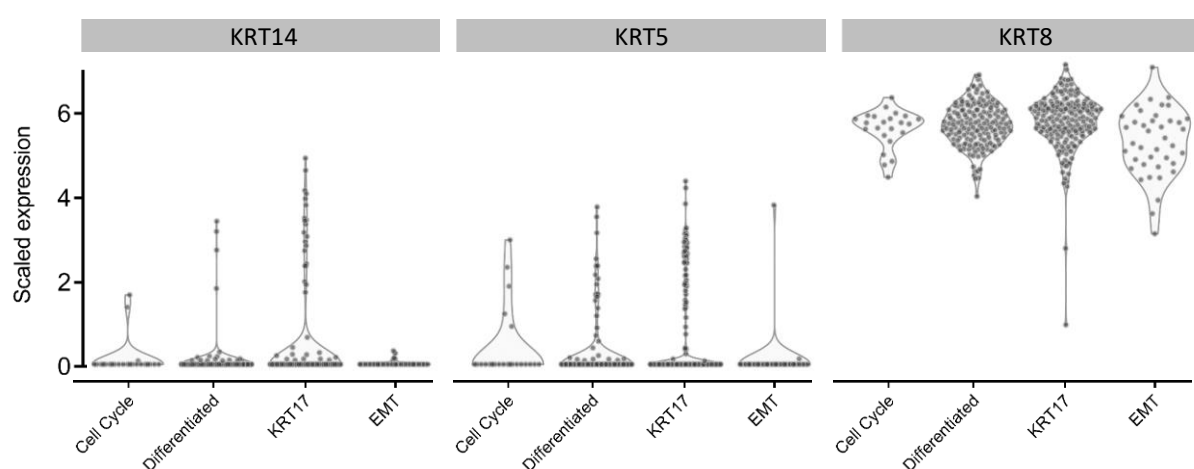


Figure 37: Rare KRT5-expressing and KRT14-expressing cells are detected in hFT tissue. Both are enriched in KRT17+ cluster, but mark distinct cells. KRT8 is a luminal marker and ubiquitously expressed by FT cells, confirming their luminal nature. X-axis denotes identified clusters. [Data in this figure was obtained from single cell dataset generated by Zhiyuan Hu].

To validate the presence of these cells at the protein level, mammary and skin tissues were employed as positive control tissues. Organoid protocol was optimised for both (data not shown). As predicted, KRT5+ cells exist and mark rare cells in the hFT and mOV tissues (Figure 38). Organoids faithfully reproduce these cells. However, contrary to predictions from scRNA-seq data, KRT14+ cells did not exist in hFT or mOV tissue (Figure 39). Again, organoids faithfully reflect what is seen in tissues. KRT5+ cells do not mark cells with multipotent potential (shown later, Figure 48D), indicating they do not represent FT stem cells.

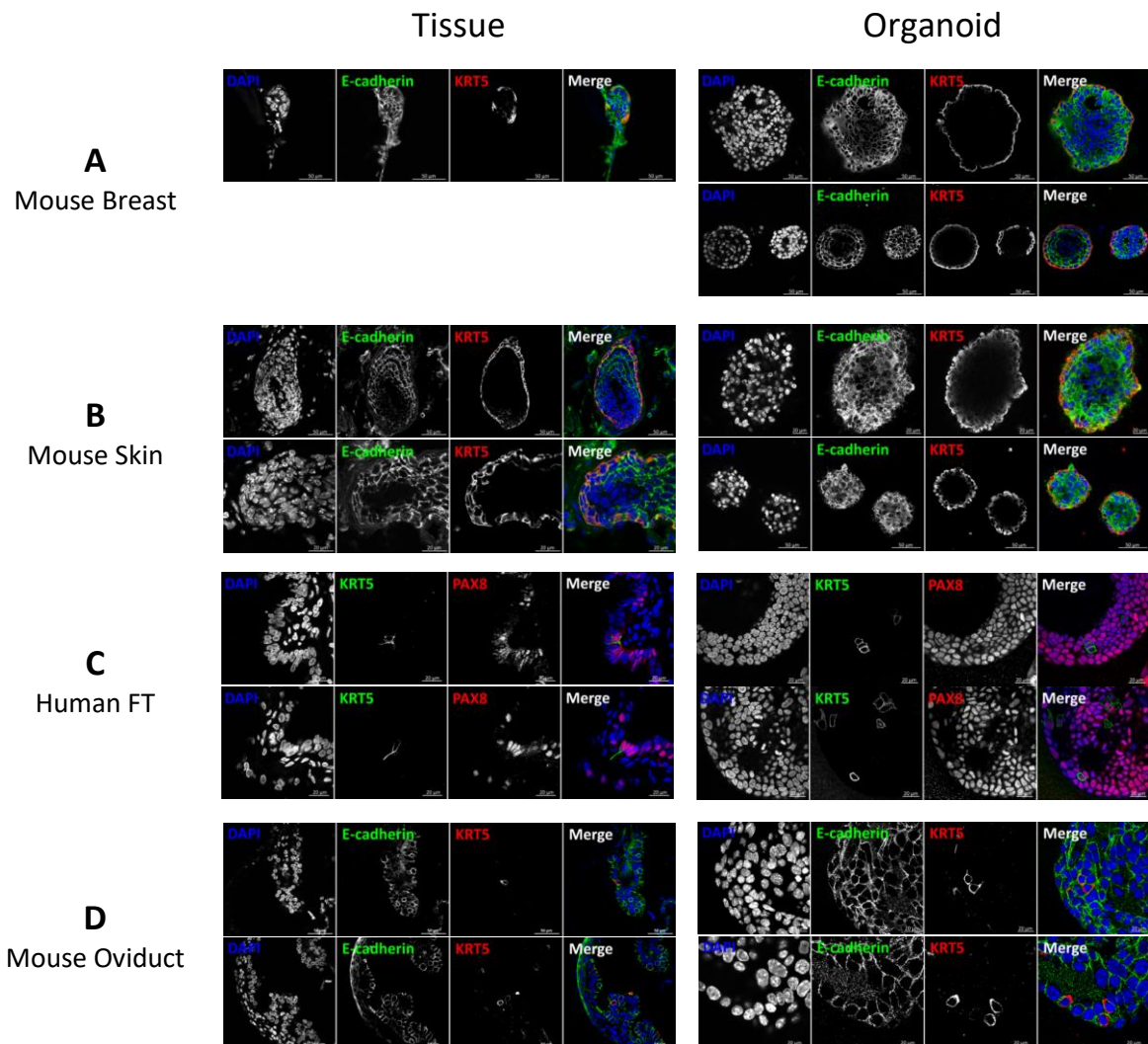


Figure 38: KRT5+ cells in the FT/oviduct. Mouse breast (**A**) and skin (**B**) were employed as positive controls for KRT5+ cells. Note the strictly basal localization of KRT5 in these tissues, as well as their derived organoids. As predicted by scRNA-seq from hFT cells, rare KRT5+ cells are detected in hFT (**C**) and mouse oviduct (**D**). Notably, organoids reproduce this, faithfully modelling the tissues they derive from.

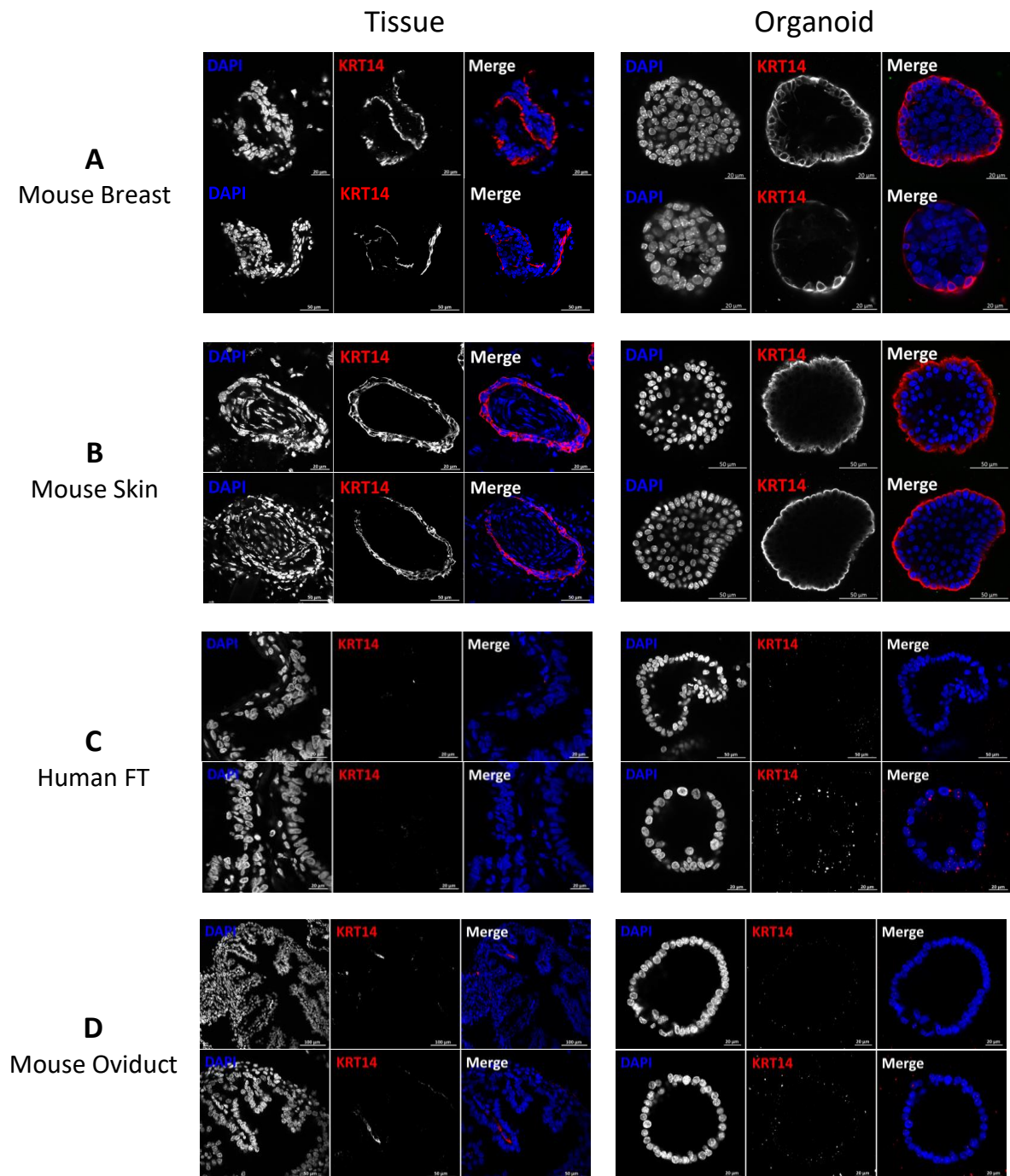


Figure 39: KRT14 is not expressed in the FT/oviduct. Mouse breast (**A**) and skin (**B**) were employed as positive controls for KRT14+ cells. Contrary to predictions by the single cell data, KRT14 is not expressed at the protein level, in the hFT (**C**) and mouse oviduct (**D**). Again, organoids faithfully reproduce this.

6.4 Chapter Summary

Identification of FT stem cells necessitates the employment of a culture system that can reconstruct the stem cell niche *in vitro*, and allow it to be tractably and functionally investigated. Traditional 2D culture of patient material does not sustain epithelial character of FT cells *in vitro*. Biased culture conditions eliminate putative stem cells, ciliated cells and the heterogeneity seen in the secretory cell compartments of hFT tissue. Organoid culture for hFT was reported, but the reported protocol is poorly optimised. A83.01 and Forskolin overcome limitations of the reported protocol and promote organoid regeneration after single cell dissociation, enabling isolation and functional characterisation of specific cells within organoids.

In this chapter, organoid culture was optimized for hFT and mOV organoids. Structurally, organoids are spherical, have a hollow interior, a monolayered cellular arrangement and exhibit internal foldings and invaginations characteristic of FT tissue. Organoids express epithelial and FT-specific markers, evidencing their FT origin. Crucially, organoids emerge from a single multipotent stem cell. KRT17+ cells are a novel cluster of FT cells with immune phenotypes. KRT7 and CAPS are novel markers of secretory and ciliated cells, respectively. Immune-epithelial cells are conceptually intriguing and warrant further investigation. KRT5+ cells are detectable in the FT. These novel cell types, and the interesting questions they pose, are unlikely to represent FT stem cells. However, their presence, predicted by a scRNA-seq study, was validated in hFT and mOV tissues. Organoids impressively reproduce these findings in an *in vitro* setting, presenting an opportunity for further functional characterisation. I conclude that organoids are a robust and superior model of FT biology.

CHAPTER 7

WNT SIGNALING MAINTAINS
FALLOPIAN TUBE STEM CELLS

WNT SIGNALING MAINTAINS FT STEM CELLS

7.1 Chapter Introduction

In the previous chapter, the organoid culture was validated as a faithful model of hFT tissue, and optimised for propagation and cell isolation approaches. The fact that organoids emerge from a single multipotent cell upon single cell culture, and are monoclonal in bulk culture, strongly points to the presence of a stem cell. Similar to organoids of other tissues, FT organoid culture is essentially driven by dynamic stem cell self-renewal and differentiation. This presents an opportunity to characterise FT stem cells.

In line with Objective 2 of the thesis, this chapter will investigate the essential requirements for self-renewal of putative FT stem cells in organoids. Putative stem cells will be genetically marked, isolated and tested for organoid formation efficiency (OFE), a surrogate test of stem cell potential. Finally, candidate stem cells will be transcriptionally characterized using bulk and single cell approaches.

7.2 Role of Wnt Signaling in Organoid Regeneration

Wnt-active cells are ASCs in various tissues (section 1.4). Although FT stem cells have not been identified yet, *in vivo* studies on mouse oviducts (Ghosh *et al.*, 2017) hint at a role for Wnt signaling in homeostatic regeneration. Wnt-active cells, marked by Axin2, contribute to endometrial (Syed *et al.*, 2020) and vaginal (Ali *et al.*, 2020) regeneration. Both are tissues of shared embryonic origin with the oviduct. This points to the possibility that FT regeneration is driven by Wnt-active cells. In this subchapter, I put this hypothesis to test, by investigating the role of Wnt signaling in organoid regeneration.

1. Wnt signaling is essential for organoid regeneration

RSPO1 binds LGR4-6 receptors to potentiate Wnt signaling. It does so by degrading RNF43/ZNRF3, an E3 ubiquitin ligase that targets FZD receptors for degradation (section 1.2.5). To test if Wnt signaling

is required for self-renewal of FT stem cells, RSPO1 was withdrawn from the organoid culture medium. This severely compromises organoid regeneration (Figure 40).

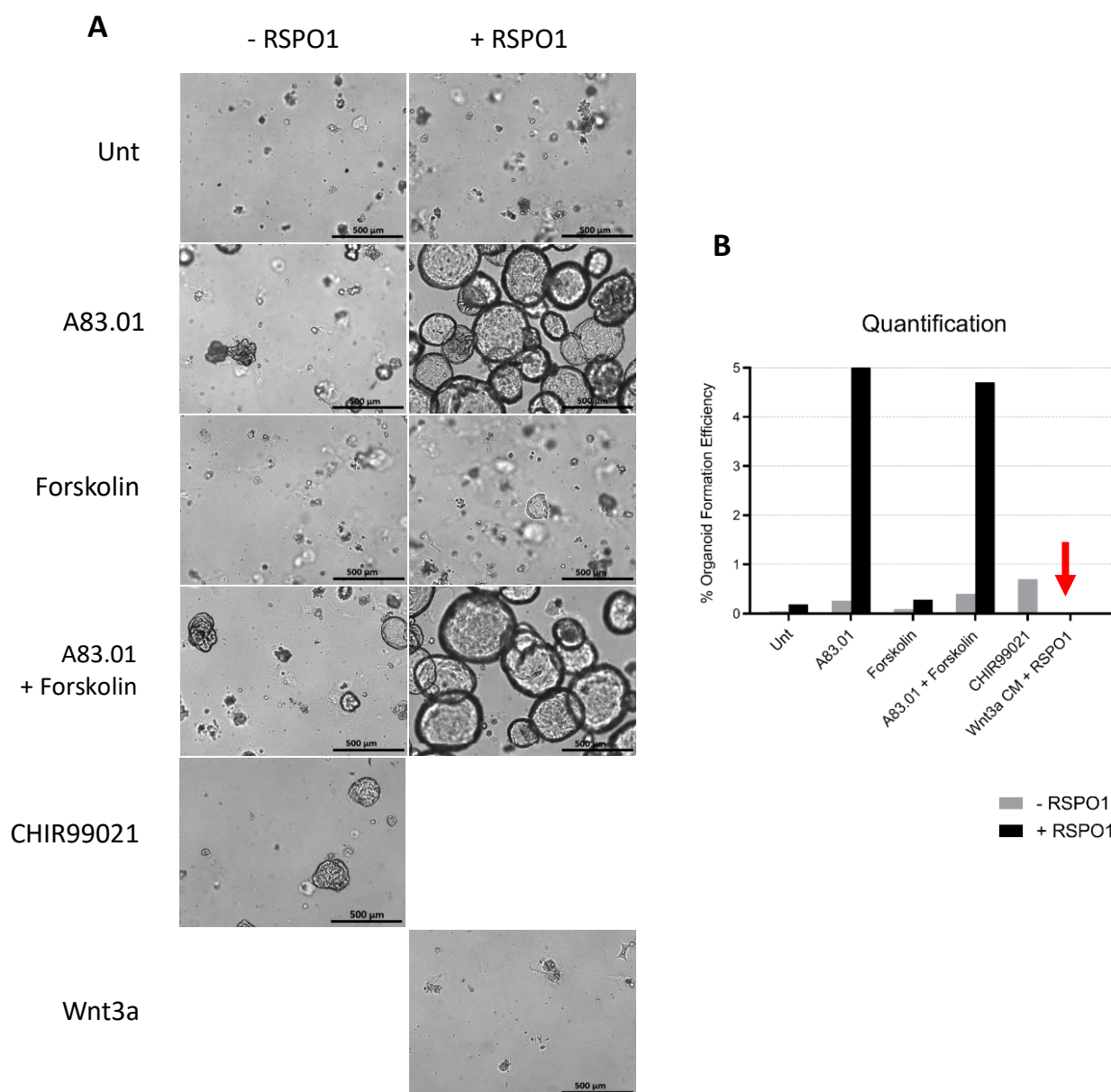


Figure 40: RSPO1 is essential for organoid regeneration. Patient FT tissue was dissociated and immunostained. CD45- EpCAM+ cells were FACS isolated and plated for each sample. RSPO1 dependence emerges upon survival of FACS-sorted stem cells, promoted by A83.01 and Forskolin. **(A)** Images taken on day 12. **(B)** Quantification of images in (A). Note that in the absence of A83.01 and Forskolin, even CHIR99021 treatment is not sufficient to instigate growth of these Wnt-dependent organoids. This suggests that TGF- β inhibition is essential for stem cell self-renewal in organoids. Upon addressing this, organoids emerge and their dependence on Wnt signaling is readily observable. Red arrow points to the culture conditions of the reported method (Kessler et al., 2015). Data is representative of replicates from three different patients.

RSPO1 is only an augmentor of Wnt signaling, preventing the degradation of FZD receptors. RSPO1 is unable to activate Wnt signaling directly, as observed in the Wnt-reporter assay (Figure 26D

and 41A). The data in Figure 40 suggests that RSPO1 might be interacting with an endogenous Wnt source. To confirm this, endogenous WNT secretion was blocked using IWP-2 and LGK-974. Both are small molecule inhibitors of Porcupine, the acyl transferase essential for endogenous Wnt processing and secretion (section 1.2.2). IWP-2 and LGK-974 were validated using the Wnt-reporter assay (Figure 41A).

IWP-2 and LGK-974 disrupt Wnt signaling upstream of the pathway and target Wnt-expressing cells, which are in many cases different to Wnt-active cells. To ensure direct targeting of the Wnt pathway within Wnt-active cells, XAV-939 and PRI-724 were also tested on organoids. As discussed (section 1.3.2), XAV-939 is a small molecule inhibitor of the poly-ADP-ribosylating enzymes called Tankyrases. Tankyrases destabilize AXIN and encourage its ubiquitination and degradation (Huang *et al.*, 2009). XAV-939 inhibits Wnt signaling by stabilizing AXIN and the β DC complex, increasing the rate at which β -catenin is targeted for degradation. XAV-939 increases intracellular AXIN concentrations, and its IC_{50} for Tankyrases 1 and 2 is 11 nM and 4 nM, respectively (Huang *et al.*, 2009). On the other hand, PRI-724 (section 1.5.5) is a small molecule that interferes with β -catenin's binding to its transcriptional partner CBP, thereby abrogating Wnt signaling. As such, LGK-974, XAV-939 and PRI-724 inhibit canonical Wnt signaling at upstream, midstream and downstream nodes of the pathway. The inhibitory effects of XAV-939 and PRI-724 on Wnt signaling were confirmed using the Wnt-reporter assay (Figure 41B).

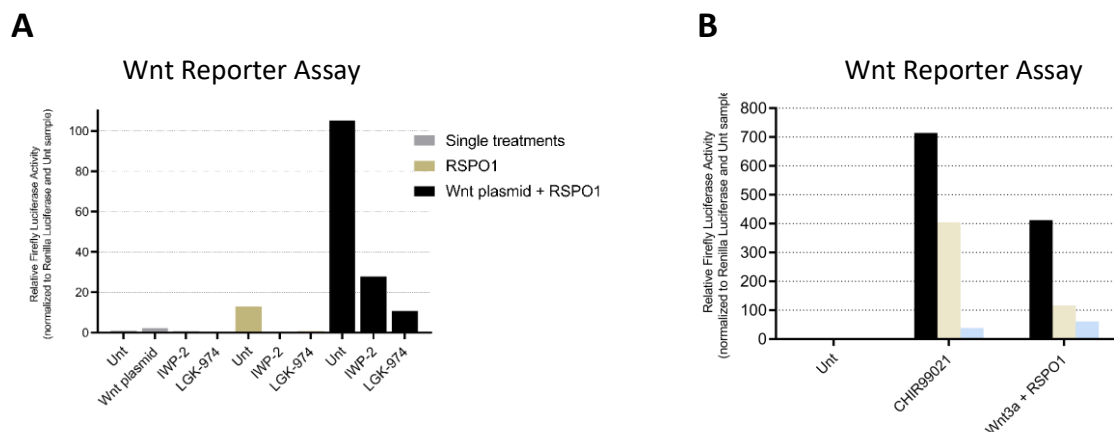


Figure 41: Validation of Wnt signaling inhibitors. (A) IWP-2 and LGK-974 block endogenous Wnt processing and secretion. Both are selective Porcupine inhibitors. **(B)** XAV-939 and PRI-724 inhibit Wnt signaling at midstream and downstream nodes of the pathway, respectively. XAV-939 is a tankyrase inhibitor, while PRI-724 disrupts β -catenin's interaction with CBP. Data representative of two biological replicates. Throughout the thesis, CHIR99021 (3 μ M), IWP-2 (3 μ M), LGK-974 (2 μ M), XAV-939 (5 μ M) and PRI-724 (10 μ M) were used at the indicated concentrations.

Blocking endogenous WNTs abolishes organoid regeneration (Figure 42), even in the presence of RSPO1. This confirms that RSPO1 cooperates with a yet unknown Wnt ligand to drive Wnt signaling and organoid regeneration. From Figures 40 and 42, it can be hypothesized that multipotent organoid-forming cells express both the WNT receptor (one of the FZDs) and the RSPO1 receptor (LGR4, 5 or 6), with one of these being the limiting factor in dictating multipotency, which is confined to 3-5% of cells as seen in Figures 40 and 42.

Interestingly, the OFE of RSPO1-treated samples in high confluency organoid cultures is 3-5% (Figure 42B, patients 1 and 2). This drops to under 1% in low confluency cultures (Figure 42B, patient 3). CHIR99021-treated samples maintain similar OFEs in both high and low confluency cultures. This suggests that at confluency, close proximity of cells enables a single Wnt-expressing cell to support more than one multipotent stem cell, satiating all possible stem cells to generate organoids to levels seen in CHIR99021-treated samples. At lower confluency this is not the case, and some stem cells are exhausted in the absence of an available Wnt ligand, leading to a drop in OFE. This notion is supported by Figure 42B (patient 3), where the drop in OFE of a low confluency culture is rescued by CHIR99021 treatment. This corroborates the notion that reduced Wnt ligand presentation to FT stem cells is responsible for the reduced OFE in low confluency cultures.

Importantly, CHIR99021-induced organoid regeneration is due to Wnt activation and not a side effect of GSK-3 β inhibition. This is confirmed by the data in Figure 42C. Of note, CHIR99021 is a soluble and permeable inhibitor that activates Wnt signaling in up to 50% of organoid cells (shown later, Figure 47 B-C). However, only 3-5% of these CHIR99021-treated cells progress to generate organoids, similar to the number of organoid-forming cells in RSPO1-treated samples. This indicates that cells expressing the Wnt and RSPO1 receptors have a monopoly in generating organoids, even when Wnt activation is ectopically upregulated in all other cells. Thus, stemness and organoid regeneration are strictly confined to multipotent stem cells, and plasticity in stemness does not appear to exist in hFT organoids under these conditions. This also indicates that Wnt signaling is essential but not sufficient for maintenance of FT stem cells.

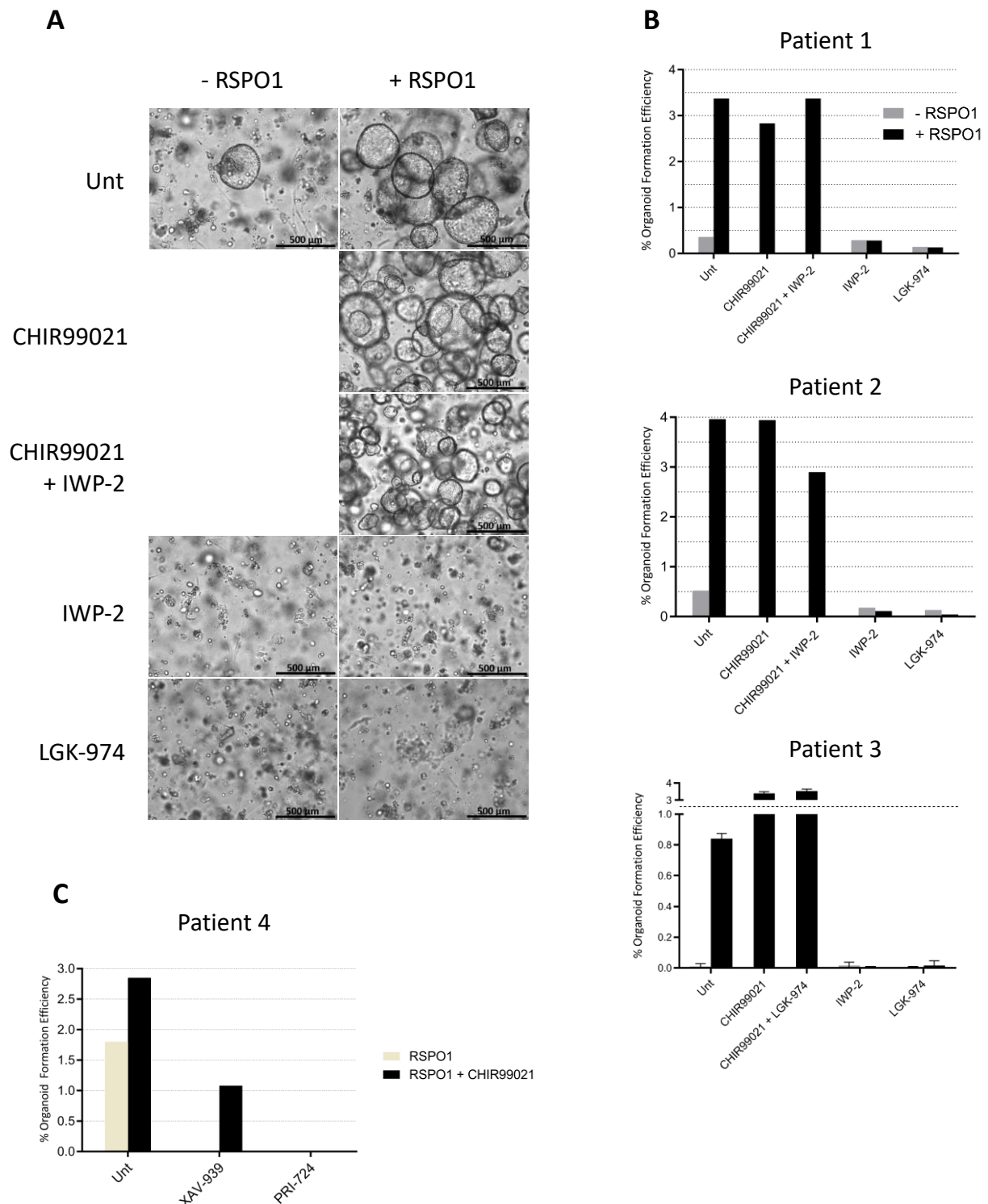


Figure 42: RSPO1 cooperates with an endogenous Wnt ligand to drive organoid regeneration. (A) Representative images on day 8. (B) Quantification shown for three patient replicates, conducted separately and constituting biological replicates. 19K cells, 12K cells and 7.4K cells were plated per well, for patients 1, 2 and 3, respectively. Patient 3 experiment was conducted in three technical replicates. Treatments were begun on plating day. Note that CHIR99021 activates Wnt signaling downstream of Wnt ligands. CHIR99021 + IWP-2/LGK-974 samples show that lack of organoid regeneration in IWP-2/LGK-974 treated samples is not due to inhibitor toxicity. (C) Inhibition of Wnt signaling 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 not due to non-specific targets of GSK-3 β .

2. Regeneration of mOV organoids differs from that of hFT organoids

In organoid cultures of various tissues, mouse organoids are generally more self-sufficient in stem cell self-renewal requirements compared to their human counterparts. For instance, human intestinal organoids require WNT3A supplementation for growth (Matano *et al.*, 2015). Mouse intestinal organoids, on the other hand, propagate using endogenously secreted Wnt3a and only utilize externally supplemented RSPO1 (Sato *et al.*, 2011). However, both are Wnt-dependent, and growth of both is inhibited upon blocking endogenous Wnts (Sato *et al.*, 2011). For the hFT, endogenous Wnt ligands and exogenously supplied RSPO1 are essential in driving self-renewal and propagation of organoids.

In a stark difference, regeneration of mOV organoids neither increases with Wnt activation, nor abrogates upon blocking endogenous Wnts (Figure 43 A-D). However, inhibiting Wnt signaling at more downstream points of the pathway inhibits growth of mOV organoids, as reported (Xie *et al.*, 2018). The data suggests that Wnt signaling may be required for regeneration of mOV organoids, but is not driven by Wnt ligands. However, blocking endogenous Wnts (using LGK-974) does abolish Wnt signaling in mOV organoids (shown later, Figure 47B). Therefore, the role of Wnt signaling is unclear in this context. In any case, this is a stark difference to human organoids. Although hFT tissue is the major focus of this thesis, such inter-species differences warrant further investigation, as currently mouse oviducts are used as a model of hFT and HGSOc initiation.

It can be speculated that other pathways are more relevant for mOV organoids. Notch signaling is often intertwined with Wnt signaling biology, forming the Wntch signaling module (section 1.4.2). Notch signaling may promote or inhibit Wnt signaling, depending on context. Here, inhibition of Notch signaling reduces mOV regeneration (Figure 43E). This is consistent with previous reports which showed that Notch signaling is important for self-renewal of FT stem cells in organoids, and that Notch inhibition promotes organoid differentiation to a ciliated cell fate (Kessler *et al.*, 2015; Xie *et al.*, 2018).

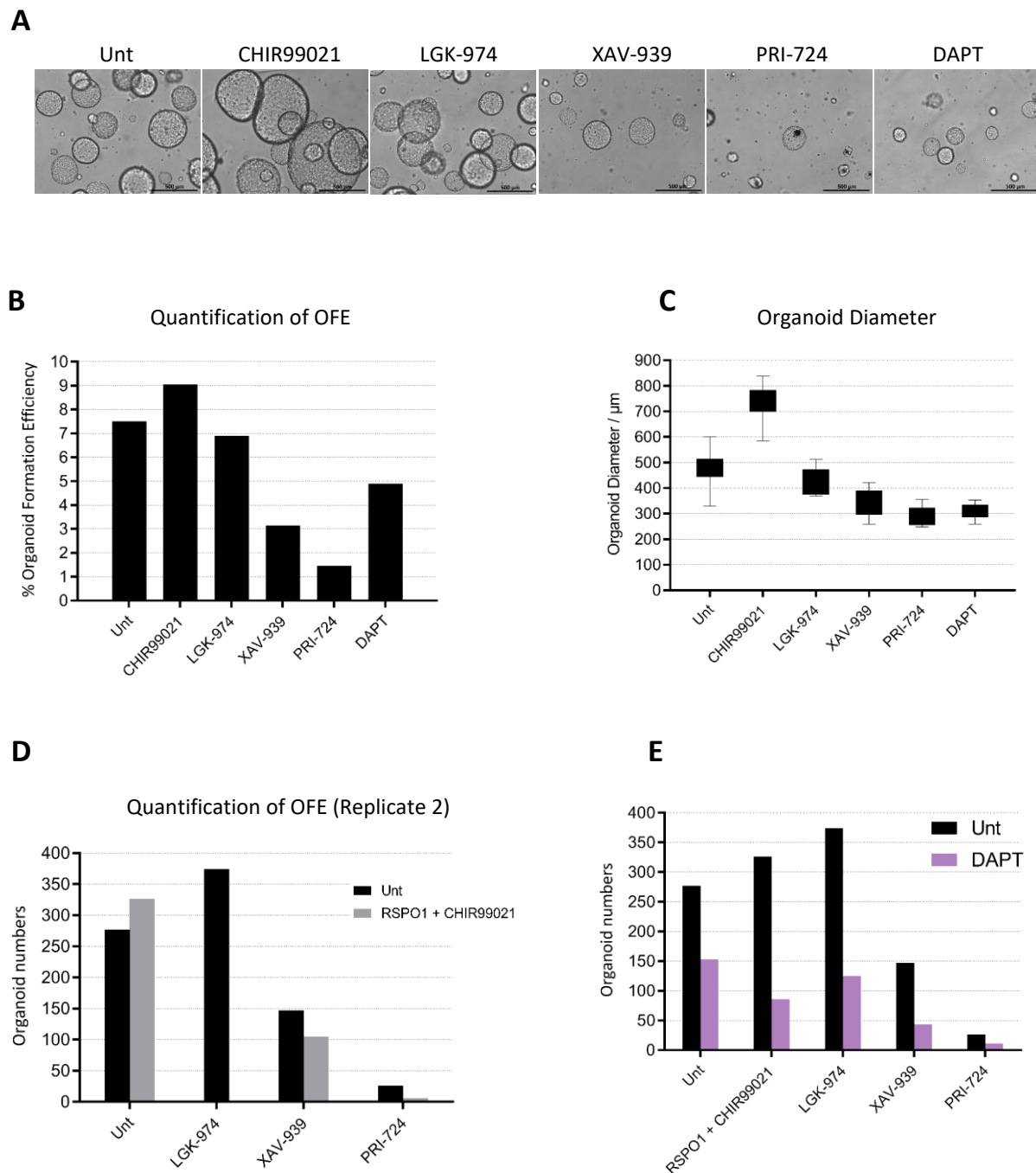


Figure 43: Wnt signaling, but not Wnt ligands, is essential for regeneration of mOV organoids.

(A) Wnt and Notch inhibitors compromise growth of mOV organoids, which are unaffected by Wnt activation (CHIR99021) or blocking of Wnt secretion (LGK-974). **(B)** Quantification of images shown in (A). **(C)** Wnt signaling promotes organoid size, but not organoid numbers as shown in (B). Diameter shown is average of the 10 largest organoids per sample. **(D)** Biological replicate for the data in (B), showing that mOV organoids are sensitive to Wnt pathway inhibition at midstream and downstream nodes, but resistant to blocking Wnt secretion upstream of the pathway. **(E)** Notch inhibition compromises regeneration of mOV organoids. Equal cell numbers plated for (D) and (E). Initial cell numbers were not determined. Base medium is OM – RSP01. Treatments were initiated on plating day, for 9-11 days.

3. Widely used Wnt3a CM is not functional in FT organoids

As the only culture-stable Wnt ligand, Wnt3a conditioned medium (CM) is widely used in organoid culture of various tissues, to activate Wnt signaling and drive stem cell self-renewal and organoid propagation. Wnt3a CM is a routine supplement in hFT organoid culture (Kessler *et al.*, 2015; Hoffmann *et al.*, 2020, 2019; Kopper *et al.*, 2019; Kessler *et al.*, 2019), as well as in mOV organoid culture (Löhmußaar *et al.*, 2020). To test its activity in FT organoids, Wnt3a CM was expressed using L-Wnt3a cell line (section 5.4.1), and its activity validated using the Wnt-reporter assay (Figure 26). Interestingly, Wnt3a was unable to rescue the growth inhibition of WNT-blocked organoids (Figure 44). In fact, it interferes negatively with stem cell self-renewal. For mOV organoids, FBS within Wnt3a CM promotes proliferation that is possibly stem cell-independent (Figure 45).

In human intestinal organoids, Wnt3a CM rescues WNT-blocked organoids (Sato *et al.*, 2011). Here, there is rationale for using Wnt3a CM, as its induction of Wnt signaling and intestinal organoid regeneration might outweigh the negative effects of the undefined factors contained in the CM. This is not the case for FT organoids, in which purified Wnt3a protein also shows no activity (data not shown). Mouse oviduct organoids grow independently of Wnt signaling. As such, there is no rationale for use of Wnt3a CM in hFT and mOV organoids. The recommendation in organoid cultures is to employ serum-free conditions wherever possible. Completely-defined culture medium precipitates stem cell requirements in a physiologically relevant way, as seen in this subchapter. Serum-free mediums are also important in organoid research for clinical applications (discussed in section 3.4.1).

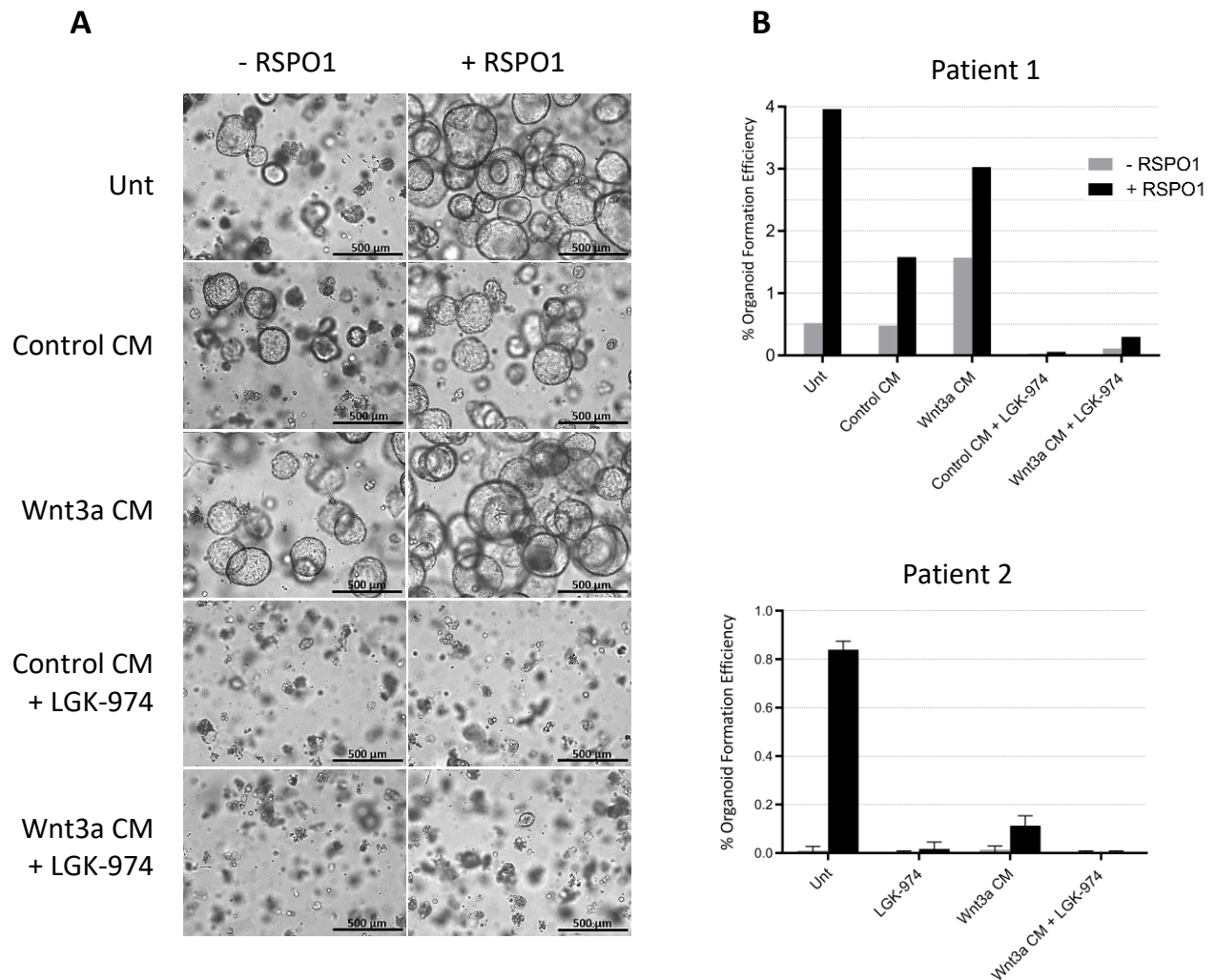


Figure 44: Wnt3a fails to rescue organoid inhibition upon blocking endogenous Wnt secretion. (A) Representative images shown. (B) Quantification shown for two patient replicates. Patient 2 experiment was conducted in three technical replicates and under low confluency conditions. CM is conditioned medium. Activity of the Wnt3a CM batch used here was confirmed by the Wnt reporter assay (see Figure 26).

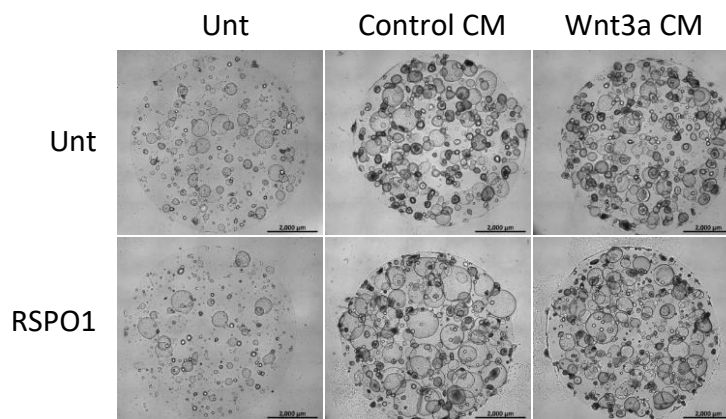


Figure 45: FBS promotes stem cell-independent proliferation in mOV organoids. 2,000 FACS-sorted EpCAM+ cells from mOV organoids were cultured and treated as shown. CM is conditioned medium. Both negative control and Wnt3a CMs contained 10% FBS and were harvested after 3-4 days of incubation with cell lines. Both CMs were used at 25% in organoid medium, so final [FBS] is 2.5%. Negative control CM was obtained upon incubation with HEK293 cells. Wnt3a CM was obtained upon incubation with L-cells.

7.3 Characterising Wnt-active cells

1. Genetic labelling and isolation of Wnt-active cells

The above set of experiments confirm that Wnt signaling drives organoid regeneration, and therefore self-renewal, of FT stem cells. It is plausible, as indicated by *in vivo* studies (Gosh *et al.*, 2017), that putative FT stem cells are Wnt-active. To understand more about the nature and character of these cells, I genetically integrated a fluorescent reporter of Wnt signaling into hFT organoids using lentiviral methods (Figure 46). This generated Wnt-reporter organoid lines, in which cells with activation in Wnt signaling are marked by EGFP. We will refer to these as Wnt-active cells. This is not to be confused with Wnt-expressing cells, which produce the Wnt ligand that is received by Wnt-active cells and are often a different cell type.

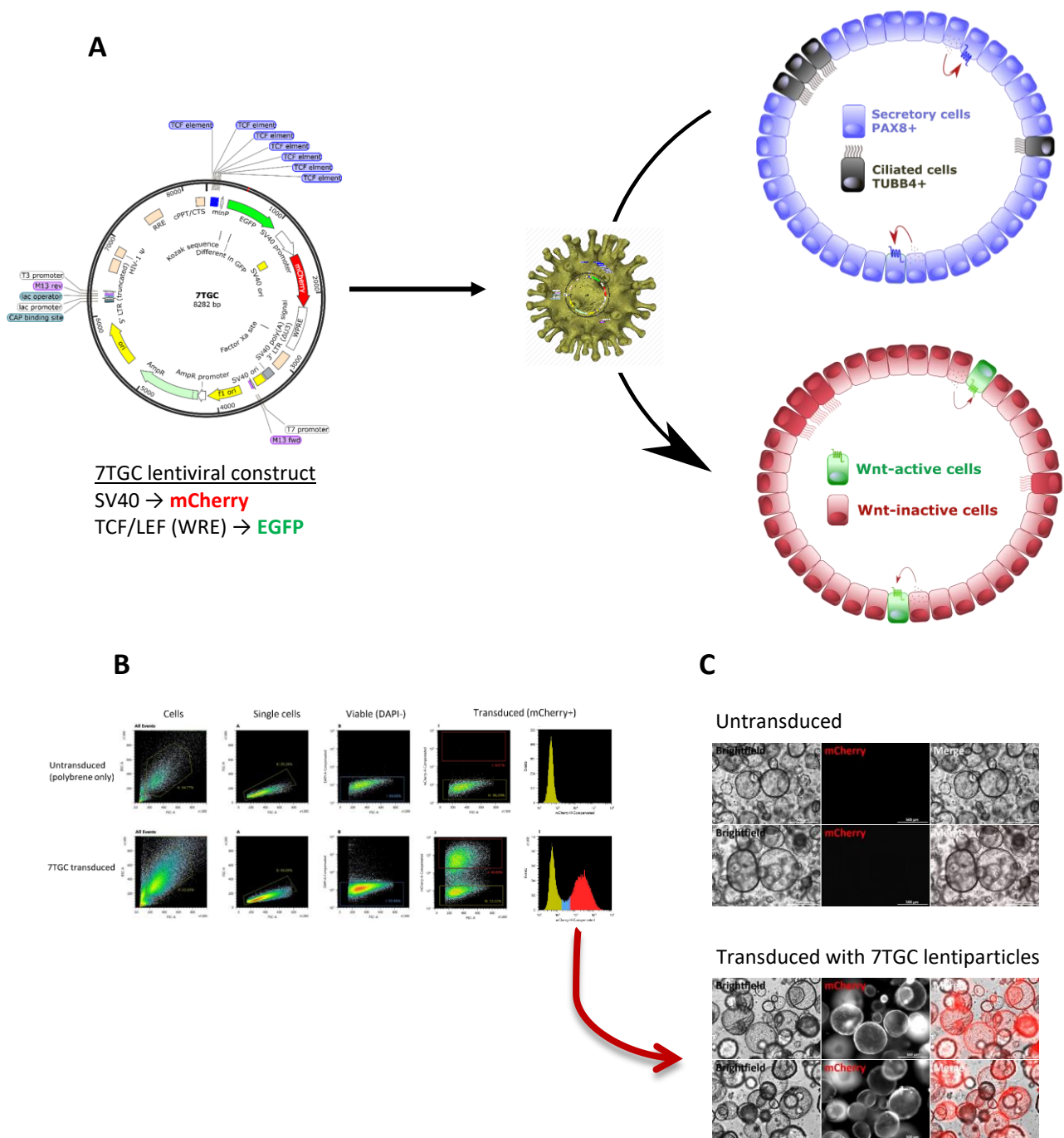


Figure 46: Generating Wnt-reporter organoid lines. (A) Viral lentiparticles were generated from the 7TGC construct. Refer to section 5.7 for technical details. The 7TGC construct expresses mCherry under a general promoter and EGFP under Wnt-reporter elements (WREs, also called TCF/LEF elements). **(B)** 72 hrs after transduction, transduced cells were FACS isolated and cultured using the optimised culture medium (Figures 27 and 40). **(C)** Wnt-reporter organoids after FACS selection and growth. Data in this figure are representative of 4 patient replicates.

The specificity of WRE elements in reporting Wnt signaling within Wnt-reporter organoids was confirmed (Figure 47). The proportion of Wnt-active cells in organoids is 3-5%, reflecting the

proportion of cells with organoid-formation capacity (Figures 40, 42 and 44, show earlier). This proportion reduces to almost 0% upon Wnt inhibition and increases 10-fold upon Wnt activation (Figure 47B). AXIN2, a marker of Wnt activation (Figure 47E) is upregulated in EGFP+ Wnt-active cells (Figure 47D), confirming this construct is a faithful reporter of Wnt signaling. Confocal imaging of live (Figure 48A) and fixed (Figure 48B) organoids confirms the localized activation of Wnt signaling in certain cells within Wnt-reporter organoids. As expected, these cells are PAX8+ secretory cells (Figure 48C). As predicted by qPCR data (Figure 47D), Wnt-active cells and KRT5+ cells are different (Figure 48D).

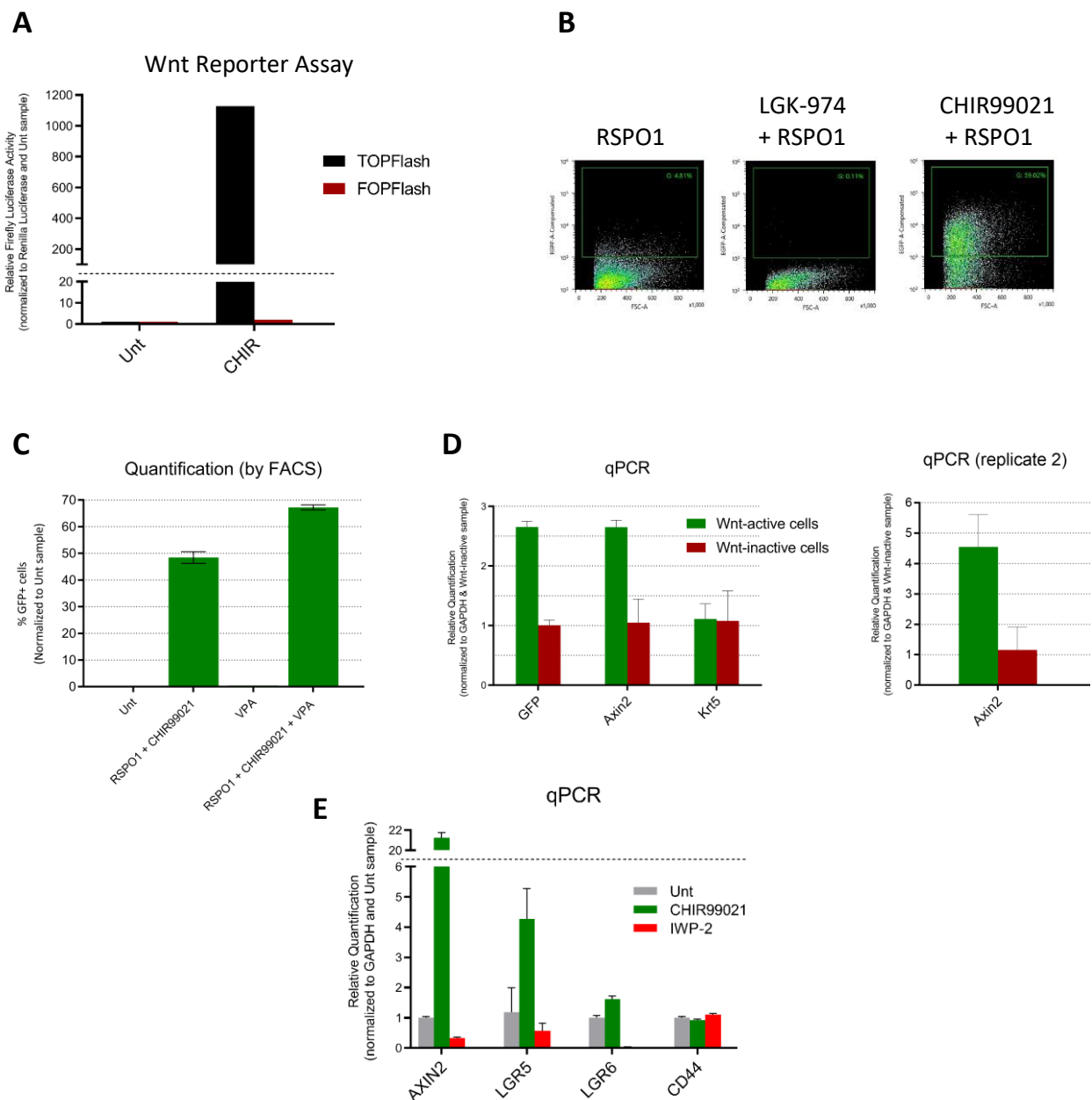


Figure 47: WRE elements specifically report Wnt signaling. (A) Wnt-reporter (TOPFlash) assay confirming specificity of WRE elements. TOPFlash construct contains the same WRE elements within the 7TGC construct. FOPFlash construct is a negative control containing these elements with single nucleotide mutations. (B) % of Wnt-active (EGFP+) cells within Wnt-reporter organoids. Inhibition of Wnt signaling by LGK-974 treatment abolishes EGFP+ cells. Activation of Wnt signaling with CHIR99021 treatment increases % of Wnt-active cells by 10-fold, indicating faithful reporting of Wnt signaling. Treatments were done for 1 week. (C) hFT Wnt-reporter organoids were treated with Wnt activators or none for 7 days. Almost half of organoid cells become Wnt-active (GFP+). Quantification is done for 2-3 technical replicates. This increase is potentiated by Notch activation, using Valproic Acid (VPA). (D) Wnt-active cells have higher levels of endogenous Axin2, a marker of Wnt activation. This was performed in mOV Wnt-reporter organoids (E) Normal hFT organoids were treated with CHIR99021 or IWP-2. AXIN2 and LGR5 are markers of Wnt activation and inhibition.

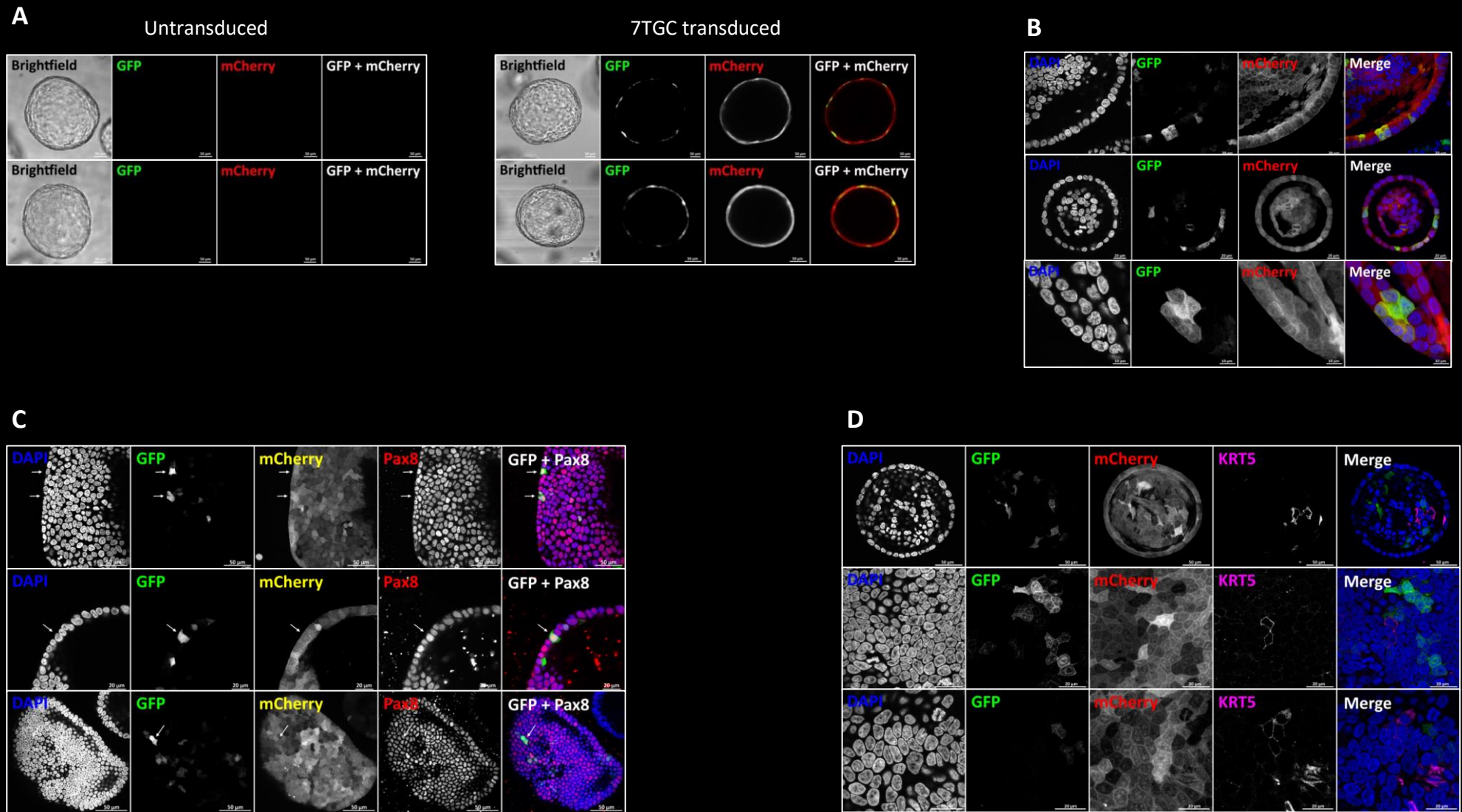


Figure 48: Imaging of hFT Wnt-reporter organoids. (A) Confocal imaging of live Wnt-reporter organoids. Note the localized activation of Wnt signaling in a few Wnt-active (GFP+) cells. (B) Imaging of Wnt-reporter organoids at the cellular level, upon fixation. (C) Wnt-active cells are PAX8+, indicating their secretory cell lineage. Arrows point to Wnt-active cells. (D) KRT5+ cells and Wnt-active cells are different and do not overlap. GFP and KRT5 channels are shown in Merge.

2. Organoid-forming capacity is not exclusive to Wnt-active cells in mOV organoids

hFT Wnt-reporter organoids always contained a number of Wnt-active cells (Figure 48). In contrast, mOV Wnt-reporter organoids may possess or be devoid of Wnt-active cells (Figure 49A). As we saw earlier, regeneration of mOV organoids is independent of Wnt signaling (Figure 43), so the absence of Wnt-active cells here is not surprising. Wnt-active and Wnt-inactive cells were isolated from mOV Wnt-reporter organoids, and subsequently cultured. Each was able to form organoids and reproduce both Wnt-active and Wnt-inactive cells (Figure 49B).

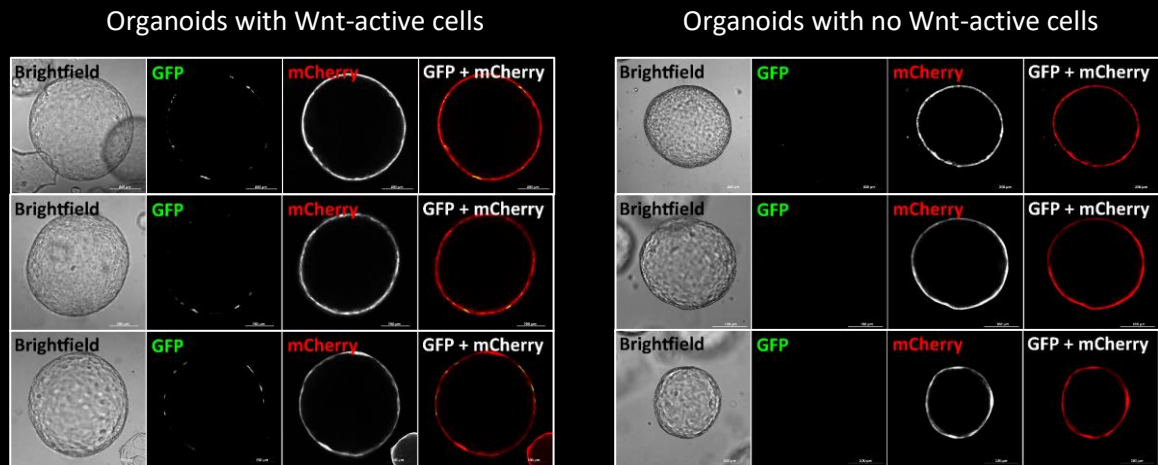
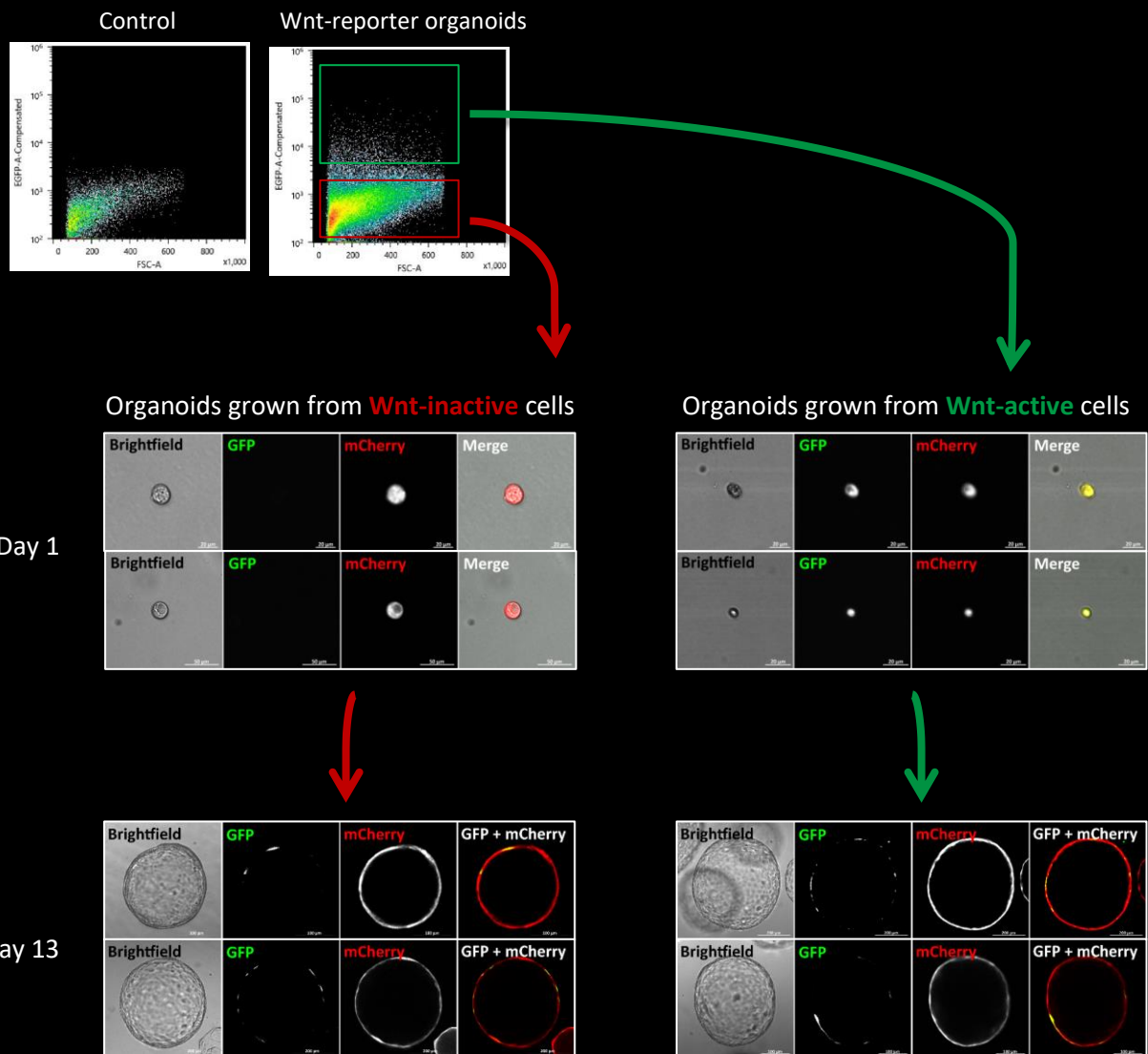
A**B**

Figure 49: Mouse organoids display regenerative plasticity. (A) Live cell imaging of mOV Wnt-reporter organoids. In contrast to hFT Wnt-reporter organoids, mouse organoids may (left panel) or may not (right panel) contain Wnt-active cells. **(B)** FACS isolation and organoid culture of Wnt-inactive or Wnt-active cells. Both are able to regenerate organoids.

In fact, Wnt-active and Wnt-inactive cells form organoids at equivalent efficiencies in complete organoid medium (Figure 50A) as well as in different mediums (Figure 50B). Interestingly, Wnt-inactive cells are more sensitive to RSPO withdrawal (Figure 50B) or endogenous Wnt blocking (Figure 50C) than Wnt-active cells. To confirm that Wnt signaling is not required, all Wnt-activatable cells were induced using Surrogate Wnt (S-Wnt), a synthetic FZD-LRP5/6 hetero-dimerizer and extracellular activator of Wnt signaling. Please see section 8.3.1 for more information, and Figure 66 for validation of S-Wnt activity. Quantification shows that 77% of cells in mOV organoids upregulate Wnt signaling upon Surrogate Wnt treatment, suggesting the majority of cells here express the Wnt receptor (Figure 50E). This is in comparison to the 5% of cells activating the pathway under standard conditions. Upon excluding all Wnt receptor-expressing cells, non-Wnt expressing cells are still able to generate organoids (Figure 50F). Overall, this data corroborates earlier findings that Wnt signaling may be dispensable for regeneration of mOV organoids.

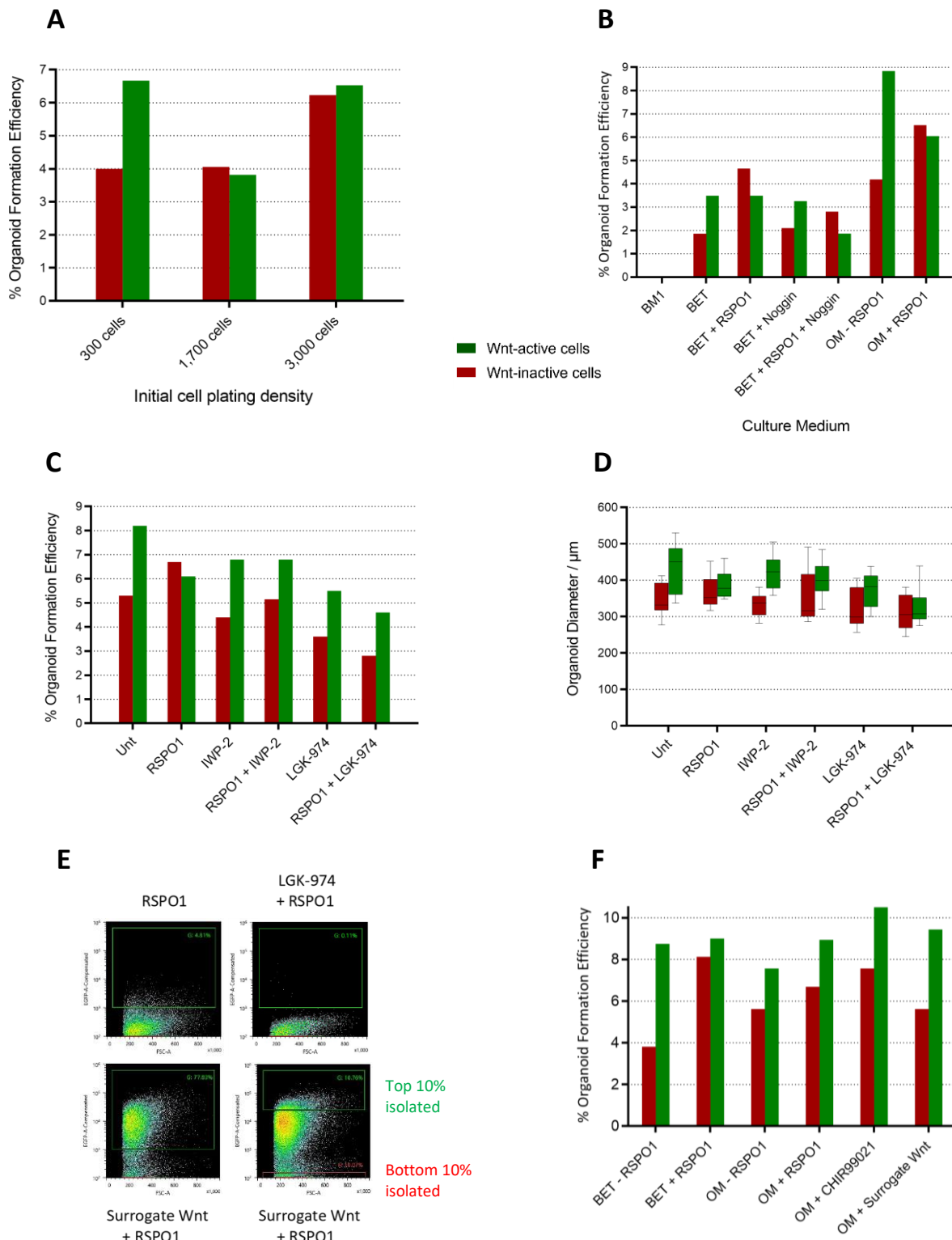


Figure 50: Wnt-active and Wnt-inactive cells from mOV organoids have comparable organoid-forming capacities. (A) In complete organoid medium, Wnt-inactive cells generate organoids as efficiently as Wnt-active cells. (B) Effect of different culture mediums on OFEs. BM1 is Basal Medium 1 (DMEM/F12 + HEPES + P/S). BET is BM1 + B27 + EGF + SB431542). OM is complete organoid medium. Note that Wnt-active cells have double the OFE of Wnt-inactive cells in complete OM medium lacking RSPO1. (C-D) Effect of blocking endogenous Wnt secretion on OFE (C) and organoid size (D). (E) Surrogate Wnt treatment of mOV organoids. Most cells here express Wnt receptors and are capable of Wnt activation. RSPO1 and LGK-974 treatments shown as controls. Top 10% GFP+ cells and Bottom 10% (Wnt-inactive and no expressed Wnt receptor) were isolated to test OFE. (F) Quantification of OFE of isolated cells in (E). Cells containing no Wnt receptors are still able to generate organoids.

3. Single cell profiling of Wnt-active cells

Thus far, hFT organoids have shown a dependence on Wnt signaling for organoid regeneration. Wnt-active cells here are strong candidates for multipotency and stem cell character. Using the protocol previously described (sections 5.7 and 5.8), single Wnt-active and Wnt-inactive cells were isolated and their transcriptomes profiled at the single cell level using SMART-seq2 scRNA-seq protocol (Picelli *et al.*, 2014).

Four clusters of cells emerge from hFT Wnt-reporter organoids (Figure 51A). The majority of Wnt-active cells are seen in cluster 1 (Figure 51B). Interestingly, the Wnt-active cluster is marked by CA125 (Figure 51C), also called MUC16. CA125 is highly overexpressed in HGSOC patients (Bast *et al.*, 1983). CA125 levels are used clinically to diagnose HGSOC patients, as well as track their disease progression and response to therapy (Partridge *et al.*, 2009).

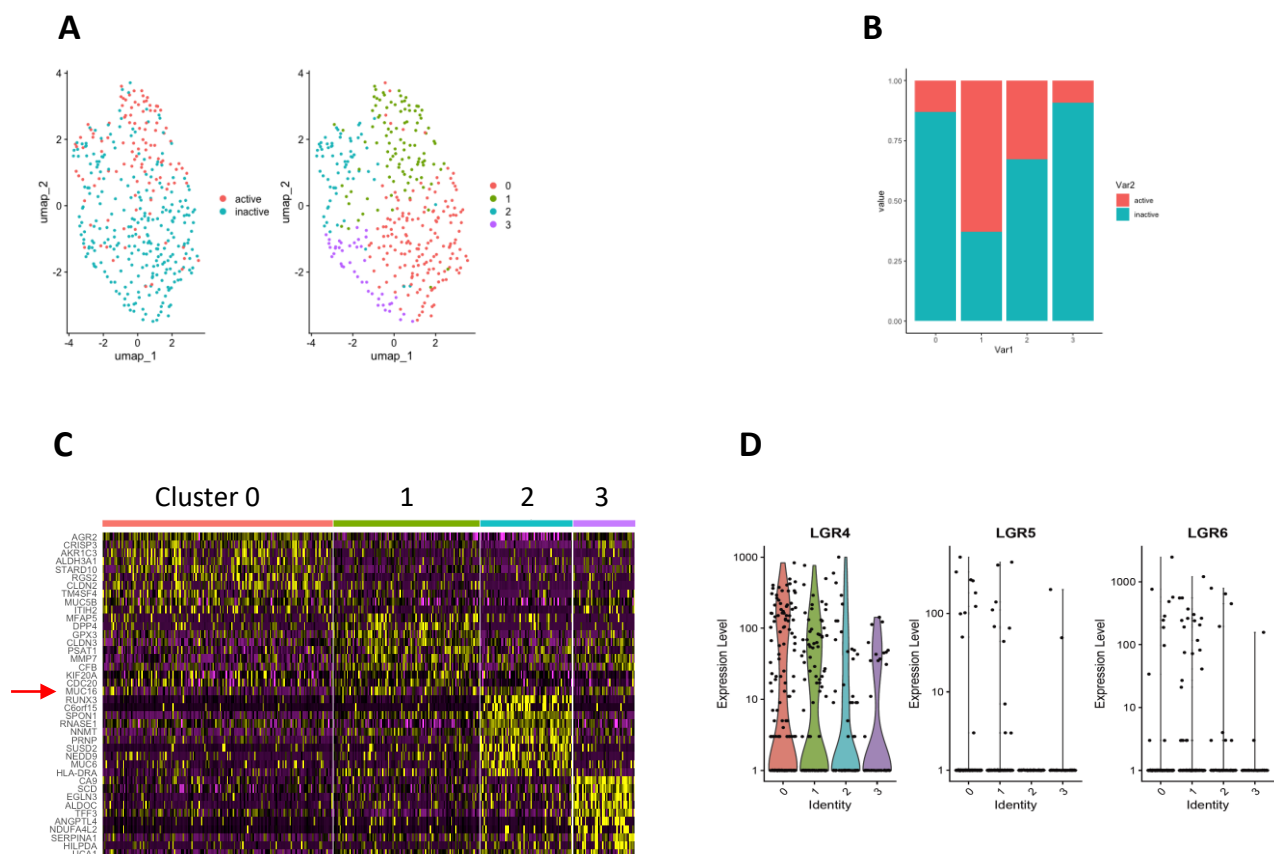


Figure 51: Single cell transcriptomic profiling of hFT Wnt-reporter organoids. (A) De novo clustering of cells after cell cycle correction, showing that hFT organoids possess 4 clusters of cells. (B) Proportion of Wnt-active cells in each cluster. Two-thirds of Wnt-active cells are found in cluster 1, and over a quarter in cluster 2. (C) Marker genes for each cluster. Wnt-active cluster is marked by MUC16 (red arrow). MUC16, also called CA125, is a clinical marker of HGSOC progression and response to therapy. (D) Expression of RSPO1 receptors in organoids. LGR4 expression is diffuse, and % of LGR4+ cells is inconsistent with the frequency of organoid-forming cells, which is much lower. LGR5/6 are the likely RSPO1 receptors, but are not well-captured including in the 77 Wnt-active cells sequenced.

The observation that Wnt-active cells overexpress CA125 may potentially implicate this cell cluster in HGSOc initiation. This data is based on 77 Wnt-active cells from a single patient. Thus, at the time of thesis submission this data was not robust enough. Effective and reproducible characterization of these cells will require augmentation of the data, both in cell numbers sequenced and patient replicates included. This effort is currently ongoing.

Interestingly, although Wnt-activation markers such as AXIN2 and LGR5 are detectable in Wnt-active cells using qPCR (shown later, Figure 68B) and RNA FISH (shown later, Figures 56 and 57), they are not prominent in the single cell data. It is plausible that endogenous levels of AXIN2 and LGR5/6 transcripts are too lowly expressed and more vulnerable to technical dropouts, even in Wnt-active cells. Technical difficulty in capturing AXIN2 expression in single cell data was previously reported from samples in which Axin2 expression was validated using RNA FISH and *in vivo* lineage tracing (Ali *et al.*, 2020). It is reasonable to assume that drop outs may affect other markers of Wnt-active cells, leading to a shallow transcriptomic map of putative FT stem cells.

Therefore, single cell transcriptomic approaches may be limited in capturing and characterizing Wnt-active cells. Wnt signature specifically in hFT organoids is lacking, and this is required to capture FT Wnt-active cells. To address these limitations, ongoing work will characterise Wnt-active cells using bulk RNA-seq analysis. Ongoing work will also generate a stable organoid line expressing a doxycycline-inducible β -catenin construct. For validation, Figure 52 shows establishment of a HEK293 cell line using this construct. The inducible modified β -catenin lacks the first 45 amino acids at the N-terminal (Δ N45), which host residues S33, S37 and T41, phosphorylated by GSK-3 β , as well as S45, phosphorylated by CK1 α (shown earlier, Figure 7). Phosphorylation of β -catenin by these kinases leads to the generation of a 3D motif, the phosphodegron, which acts as a docking site for β -TrCP E3 ubiquitin ligase, targeting β -catenin for subsequent proteasomal degradation. As such, β -catenin Δ N45 is free from regulation by the cell's β DC complex, and should produce a robust signature of Wnt-activation. Acquiring a Wnt signature in this way is more biologically relevant than artificial treatment with inhibitors, such as CHIR99021. Aside from undesirable inhibitor selectivity issues, CHIR99021 targets GSK-3 β , one of the most promiscuous cellular kinases, leading to many gene expression changes independent of Wnt signaling.

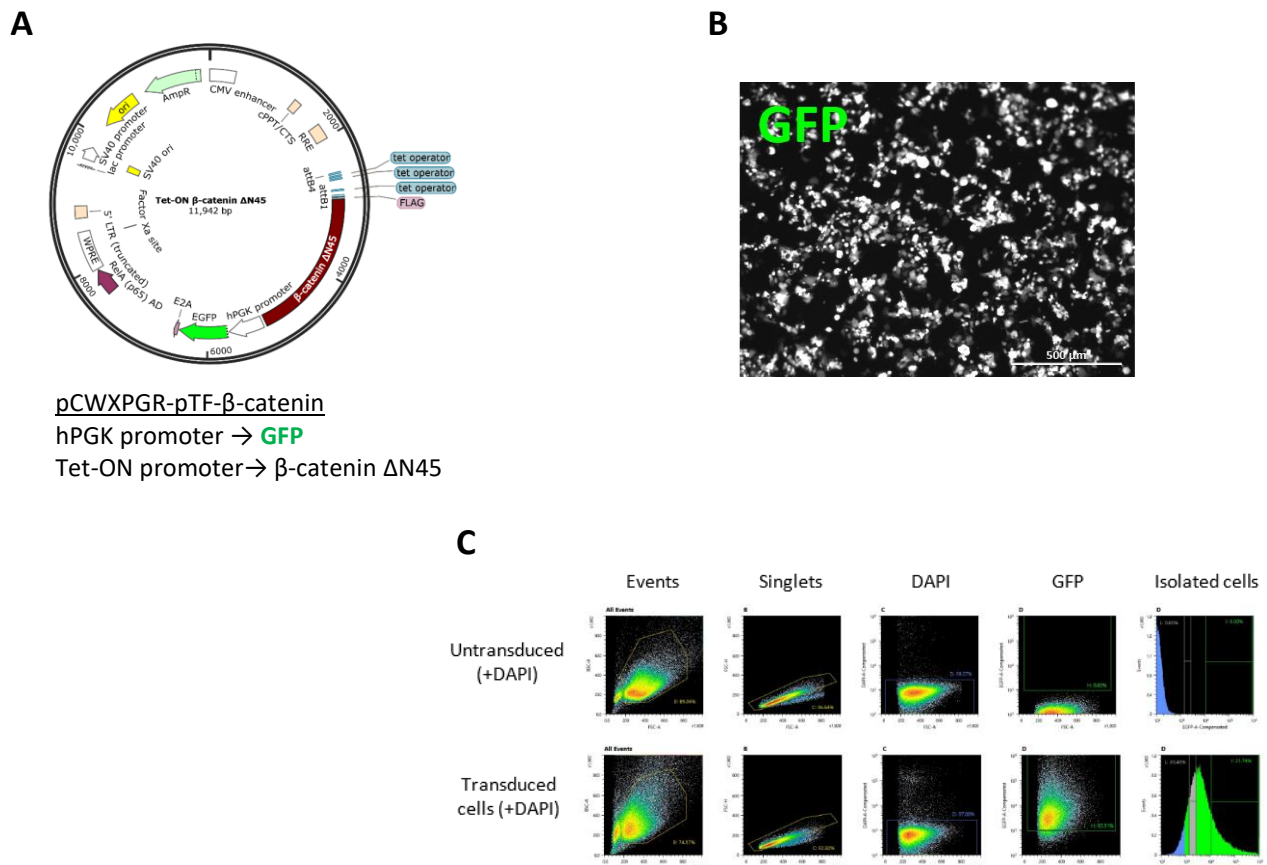


Figure 52: Generating doxycycline-inducible β-catenin ΔN45 HEK293 cells. (A) Map of viral construct used to generate lentiparticles and transduce organoids. The construct features a Tet-ON β-catenin overexpression system, encoding β-catenin that lacks the first 45 N-terminal amino acids, which confer regulation by the βDC complex. **(B)** HEK cells upon transduction with lentiparticles generated by the construct. **(C)** FACS plots showing isolation of GFP^{high} and GFP^{low} cells, corresponding to the highest 20% and lowest 20% of cells in GFP positivity, respectively.

Figure 53 shows validation of the β-catenin ΔN45 construct in HEK cells. The construct is strictly doxycycline-inducible (Figure 53B), does not leak or increase endogenous β-catenin levels (Figure 53A) and produces functional β-catenin as verified by the Wnt-reporter assay (Figure 53C). Ongoing research is focused on generating dox-inducible β-catenin ΔN45 organoid lines. Alongside the Wnt-reporter organoids, the pair will be utilised in bulk RNA-seq analysis to generate a robust signature of Wnt-activation in the human Fallopian tube.

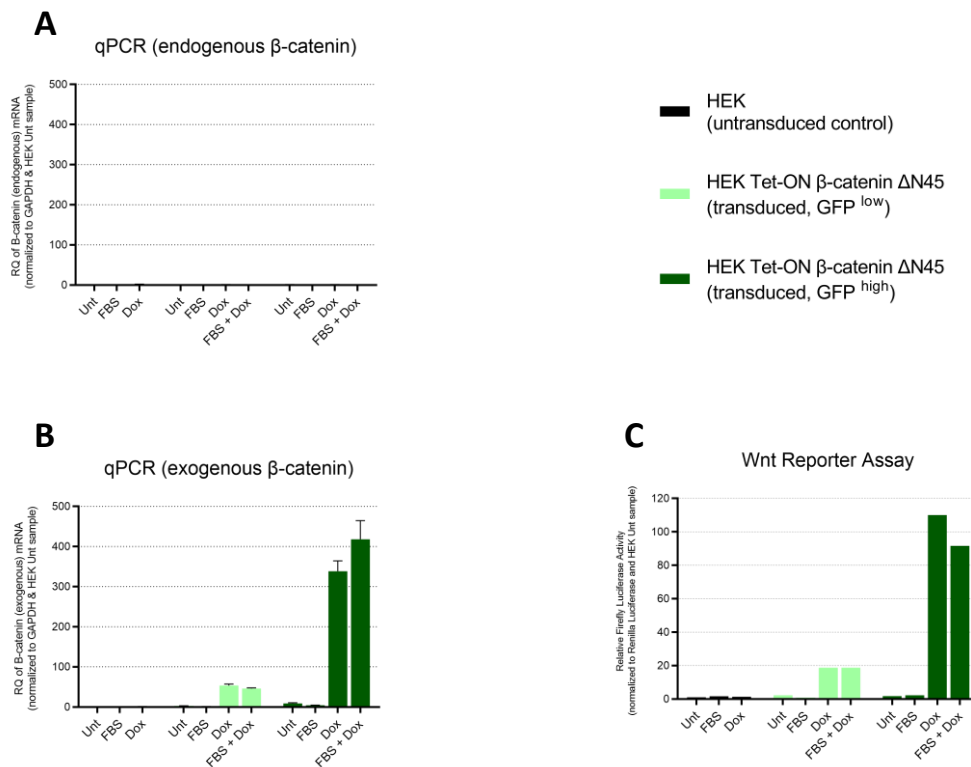


Figure 53: Validating the doxycycline-inducible β -catenin Δ N45 lenti-construct. Analysis of the generated cell lines in comparison to the parent, untransduced, control cell line. **(A)** Endogenous β -catenin is not detected upon dox induction of transduced cell lines. **(B)** Dox-treated samples upregulate β -catenin Δ N45 expression. This is highest in GFP^{high} samples, which contain a higher number of viral integration sites per cell. **(C)** Inducible β -catenin Δ N45 is functional as confirmed by the Wnt Reporter Assay. Doxycycline used at 1 μ g/mL. Endogenous β -catenin levels were detected using qPCR primers targeting the 5' UTR of β -catenin. Exogenous β -catenin was detected using a flag tag primer.

7.4 Chapter Summary

FT stem cells have not been identified to date. *In vivo* studies, on oviducts and other tissues with a shared embryonic origin to the oviduct, hint at a role for Wnt signaling in tissue regeneration and homeostasis. In this chapter, I employed patient-derived and murine organoid cultures as a model of FT regeneration. To this end, RSPO1 is critical for FT organoid regeneration, suggesting Wnt signaling is essential for self-renewal of FT stem cells in organoids. In the presence of RSPO1, PORCN inhibitors abolish organoid regeneration, suggesting that RSPO1 cooperates with an endogenous Wnt ligand to drive self-renewal. Renewal of mOV organoids is affected by inhibition of Wnt signaling at more downstream nodes. However, mOV organoids are not affected by blocking endogenous Wnts, which are shown here to drive Wnt signaling. Furthermore, mouse Wnt-active cells in organoids have no monopoly on organoid regeneration. This suggests that Wnt signaling may be dispensable to mouse organoid formation, albeit XAV-939 and PRI-724 data contradict this conclusion.

Wnt3a CM, widely used in organoid culture systems of various tissues including the hFT, shows no activity and fails to rescue WNT-blocked organoids. To characterise organoid-forming cells, Wnt-reporter organoids were generated and show specific fluorescent-based reporting of Wnt signaling. Wnt-active cells in hFT organoids are PAX8+ secretory cells and are distinct from KRT5+ cells. Single cell transcriptomics shows that Wnt-active cells form a distinct cluster of cells marked by CA125, a clinical marker of HGSOC progression and response to therapy. However, Wnt-active cells do not feature robust Wnt activation markers, and it is possible other specific markers are missed due to technical limitations. A reliable tissue-specific Wnt signature for the hFT is lacking. Bulk RNA-seq approaches, as well as biologically-relevant overexpression of Wnt signaling in hFT organoids, should serve as a tool to address these limitations and more effectively characterize Wnt-active cells. Finally, Wnt-reporter mOV organoids confirm that organoid regeneration in mice is not restricted to Wnt-active cells.

In the next chapter, we explore the nature of the molecular signals that maintain Wnt-active cells in hFT organoids.

CHAPTER 8

WNT7A IS THE DRIVER OF WNT
SIGNALING IN THE HUMAN FALLOPIAN TUBE

WNT7A DRIVES WNT SIGNALING IN FT STEM CELLS

8.1 Chapter Introduction

Thus far, our analysis has focused on characterizing Wnt-active cells as putative hFT stem cells. These are rare cells within organoids and are dependent on Wnt signaling for self-renewal. A hitherto unidentified Wnt ligand cooperates with RSPO1 (Figure 42) to activate Wnt signaling and drive self-renewal of FT stem cells. In line with Objective 3 of the thesis, I sought in this subchapter to identify this Wnt ligand, understanding its basic biochemical nature, identify the receptor through which it signals and characterize the cells that emanate it to support maintenance of FT stem cells.

8.2 WNT7A drives Wnt Signaling in the hFT

1. WNT7A is the only canonical WNT ligand expressed in hFT organoids

Single cell transcriptomic profiling of hFT Wnt-reporter organoids identifies this Wnt ligand as WNT7A (Figure 54A). In fact, WNT7A is the only expressed canonical Wnt ligand in hFT organoids. WNT5A is very lowly expressed and does not participate in canonical Wnt signaling (section 1.1.2-1.1.3). This implicates WNT7A as the endogenously expressed WNT that cooperates with RSPO1 to drive organoid regeneration, and the endogenously expressed Wnt ligand which IWP-2 and LGK-974 block, leading to inhibition of organoid regeneration. WNT3A is not expressed in hFT organoids, explaining why Wnt3a CM does not rescue WNT-blocked organoids, and further weakens the rationale for employing it in FT organoid culture.

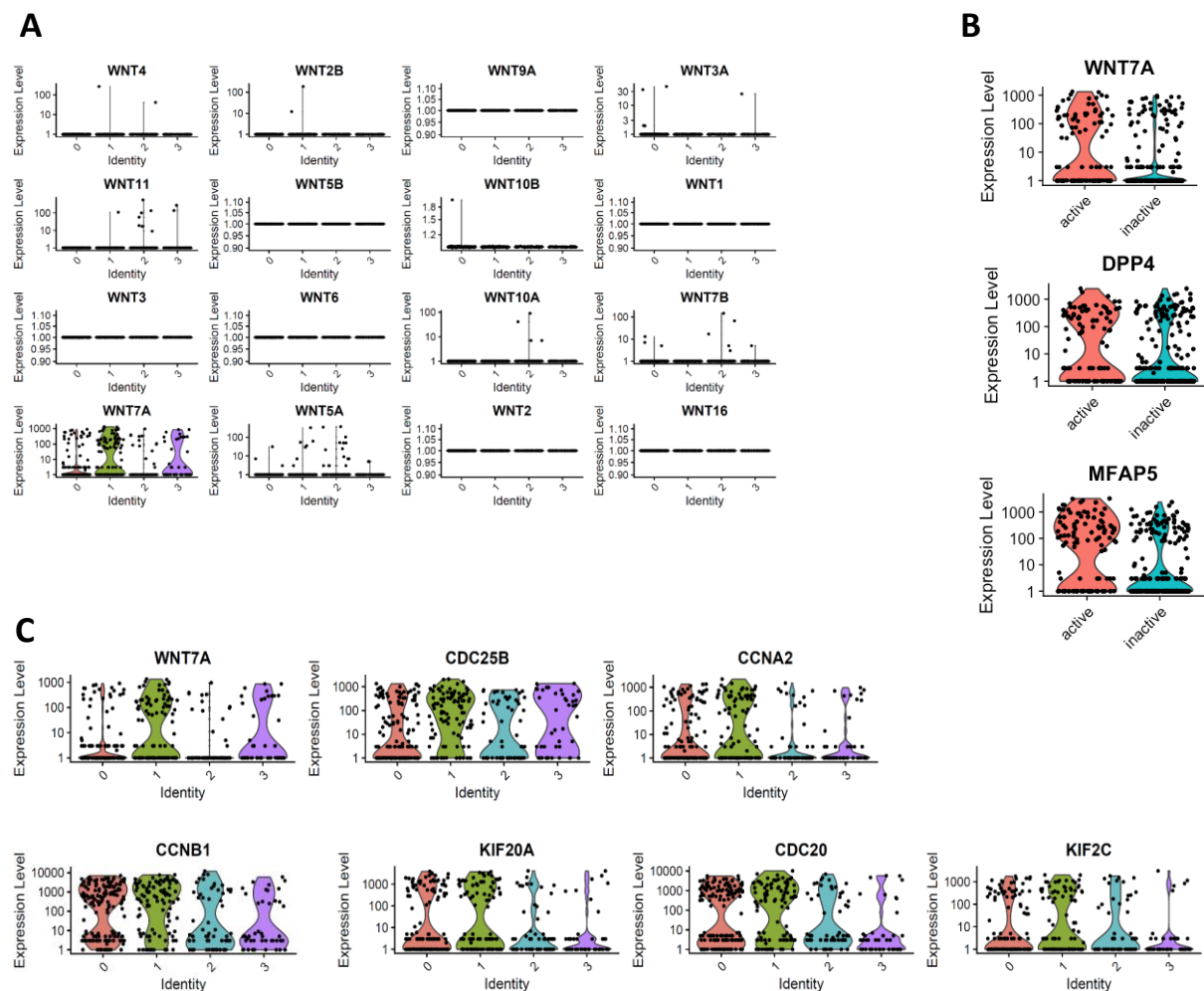


Figure 54: WNT7A is the driver of Wnt signaling and organoid regeneration. (A) Violin plots showing the expression of the WNT ligand family in hFT organoids. WNT7A is the only expressed Wnt. Note that WNT3A is not expressed in hFT organoids. (B) Selected marker genes of Wnt-active cells. WNT7A expression is seen in some but not all Wnt-active cells. (C) WNT7A expression is enriched in cluster 1, which has the highest proportion of Wnt-active cells. This Wnt-active WNT7A+ cluster is proliferative and expresses a number of cyclins and regulators of G2 to M transition of cell cycle. [Bioinformatic analyses conducted by Ahmed Ahmed and Zhiyuan Hu].

The identification of WNT7A as the driver of Wnt signaling in FT organoids is consistent with developmental studies. As discussed in Chapter 2, Wnt7a specifies the oviduct lineage. Wnt7a KO mice have no or severely compromised oviducts, are reproductively sterile and show FRT posteriorization (Parr & Mc Mahon, 1995). Similar to organoids, Wnt7a is the only Wnt ligand expressed in the epithelia of the entire neonatal FRT. As development progresses, Wnt7a expression becomes restricted to oviductal and luminal uterine epithelium. It is unclear how this relates strongly to hFT organoids, but not to mOV organoids.

Interestingly, WNT7A expression in organoids is enriched in Wnt-active cells (Figure 54B). It is clear from Figure 54B that Wnt-active WNT7A- cells also exist. Given organoids are in a constant and

dynamic state of regeneration, Wnt-active stem cells could be yielding progeny that are promptly differentiating to WNT7A+ cells, and what is being captured here is an intermediate cellular state between self-renewal and differentiation.

An alternative explanation could be that WNT7A expression is enriched in Wnt-active cells. This could mean that Wnt-active cells synthesize and secrete their own WNT7A, and receive it to activate Wnt signaling in an autocrine manner. Wnt-active stem cells are generally distinct from Wnt-expressing cells, which constitute the niche for ASCs and secrete multipotency factors required for ASC maintenance. In the intestines, for example, Paneth cells feed LGR5+ Wnt-active stem cells with Wnt3a, Noggin, EGF and protective anti-bacterial factors in a paracrine signaling mode (section 1.4.3). Although the idea that Wnt-active FT cells secrete their own WNT7A (Figure 54B) deviates from the established dogma in ASC biology, it is not without precedent. As we explored in section 1.4.5, Axin2+ hair follicle stem cells behave in this way. *In vivo* studies have demonstrated that ablation of Wnt secretion specifically in Axin2+ cells abolishes hair follicle regeneration (Lim *et al.*, 2016), suggesting these Wnt-active cells secrete and receive their own Wnt ligands in an autocrine manner.

The two hypotheses could be tested by looking at these two cell types in fresh tissue, which is slowly cycling and will probably display a better separation of these cell types. Indeed, in the single cell data of hFT tissue, AXIN2+ or LGR5+ cells do not overlap with WNT7A+ cells (see below, Figure 55C). This suggests that Wnt-active and WNT7A+ cells are different in the hFT. For the organoid single cell data, the absence of a reliable Wnt signaling signature makes characterization of Wnt-active cells this more challenging. The work outlined in Figures 52-53 will help address these issues.

2. Validating WNT7A+ cells

The Wnt-active cluster is proliferative and expresses a number of cell cycle-associated genes, including cyclins A2 and B1 (Figure 54C). Cyclin A2 activates two cyclin-dependent kinases: CDK2 (during S-phase) and CDK1 (at the G2 to M phase). Cyclin B1 binds and activates CDK1 to promote mitosis (Asghar *et al.*, 2015). Likewise, CDC25B and CDC20 promote cell division. These proliferative signals are enriched, but do not exclusively mark Wnt-active cells. The proliferative nature of these cells in hFT organoids is consistent with single cell data from hFT tissue, which shows WNT7A+ cells are proliferative (Hu *et al.*, 2020), although by a subtle margin. WNT7A+ cells have unique transcriptomes relative to other secretory cells (Figure 55B). Upon 2D culture of hFT tissue, cells acquire WNT7A expression (Figure 55C). Note the depletion of LGR5+ and probably Wnt-active cells. This explains why cells cultured for over 2 weeks in 2D culture lose organoid-forming ability, which is

lost in spite of abundant WNT7A+ cells, suggesting that WNT7A+ cells may not be the same as Wnt-active organoid-forming cells.

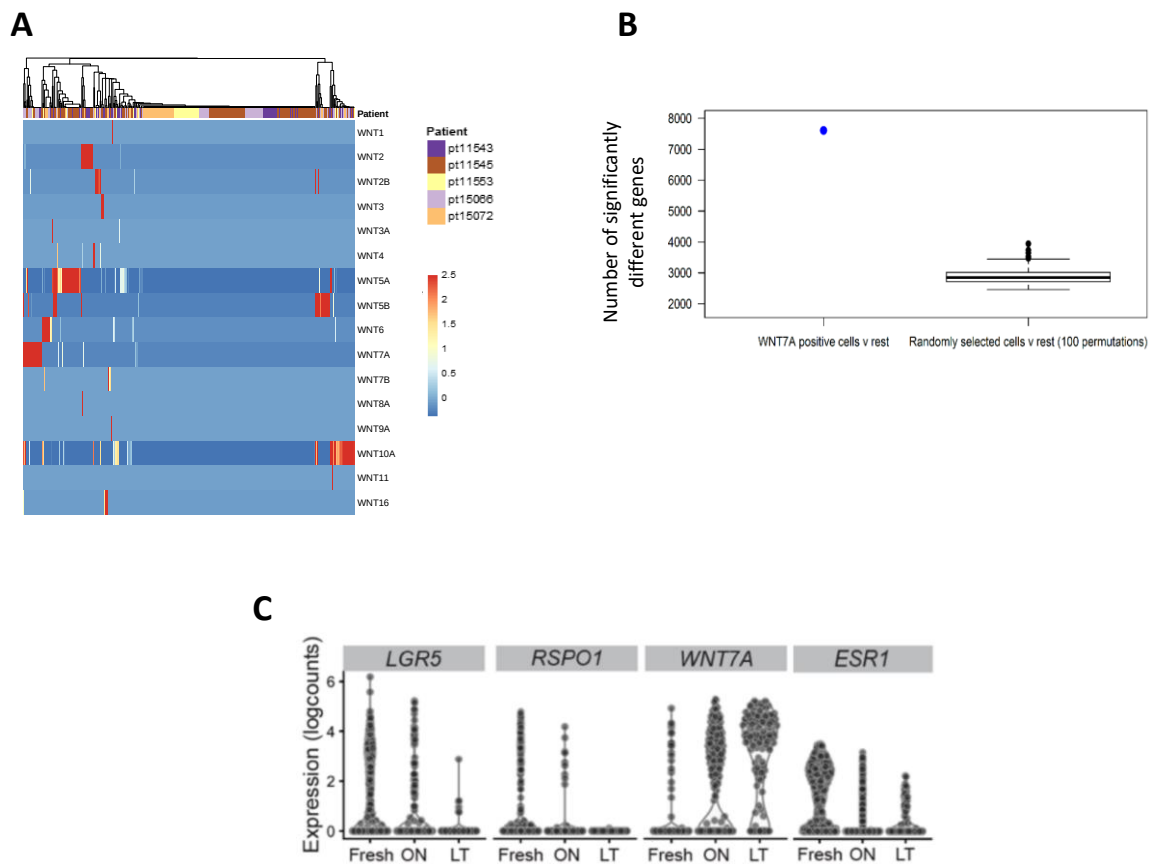


Figure 55: WNT7A cells detected in scRNA-seq of fresh hFT. (A) WNT ligand family expression in fresh hFT tissue. WNT7A is expressed here, but other WNTs are also expressed. **(B)** WNT7A+ cells are unique. Number of differentially expressed genes between WNT7A+ cells and WNT7A- cells. To ensure that the number of DEG's between WNT7A+ and WNT7A- cells is not due to stochastic cell-to-cell differences, permutation analyses were performed to identify the number of DEGs between any two WNT7A- cells selected at random. **(C)** WNT7A+ cells are rare in hFT tissue (around 5%) but make up the majority of cells in 2D-cultured hFT tissue. Note that LGR5+ show the opposite trend, suggesting Wnt-active and WNT7A+ cells are different. ON is overnight culture. LT is long-term culture (2-6 days). [(A) was obtained using dataset generated by Zhiyuan Hu. Analysis in (B) conducted by Ahmed Ahmed. (C) is taken from Hu et al. (2020)].

Next, I set to confirm the presence of WNT7A+ cells by RNA FISH staining. Fixation, hydrogen peroxide treatment and oven hybridization were optimized for hFT and mOV samples, using fluorescent multiplex RNAScope assay (Wang *et al.*, 2012). WNT7A+ cells were detected in hFT organoids (Figure 56B). RNA FISH co-staining of WNT7A with AXIN2 or LGR5, both reliable Wnt activation reports in hFT organoids (shown earlier, Figure 47E) shows that WNT7A+ cells may or may not co-express these Wnt activation markers. These findings were also confirmed in mOV tissue (Figure 57B) and mOV organoids (Figure 57C).

In both hFT organoids and mOV organoids, AXIN2+ and LGR5+ cells are distinct. Our discussion on liver regeneration (section 1.4.8) has shown that Axin2+ cells were homeostatic stem cells while Lgr5 marks facultative stem cells that regenerate the liver during injury repair. Given that both AXIN2 and LGR5 are Wnt target genes that possess WRE elements in their promoters, AXIN2+ and LGR5+ cells in hFT organoids could represent different populations of Wnt-active stem cells. It is unclear which one drives organoid regeneration, but in light of the data in Figures 40 and 42 showing essentiality of RSPO1 to organoid regeneration, as well as data in Figure 64 (shown later), LGR5+ cells are the stronger candidates given their expression of LGR5 as the RSPO1 receptor.

WNT7A+, AXIN2+ and LGR5+ cells were much more common in hFT organoids relative to tissue, validating organoids as a useful tool for expanding and enriching these cell types in a near native format, effectively enabling their characterisation.

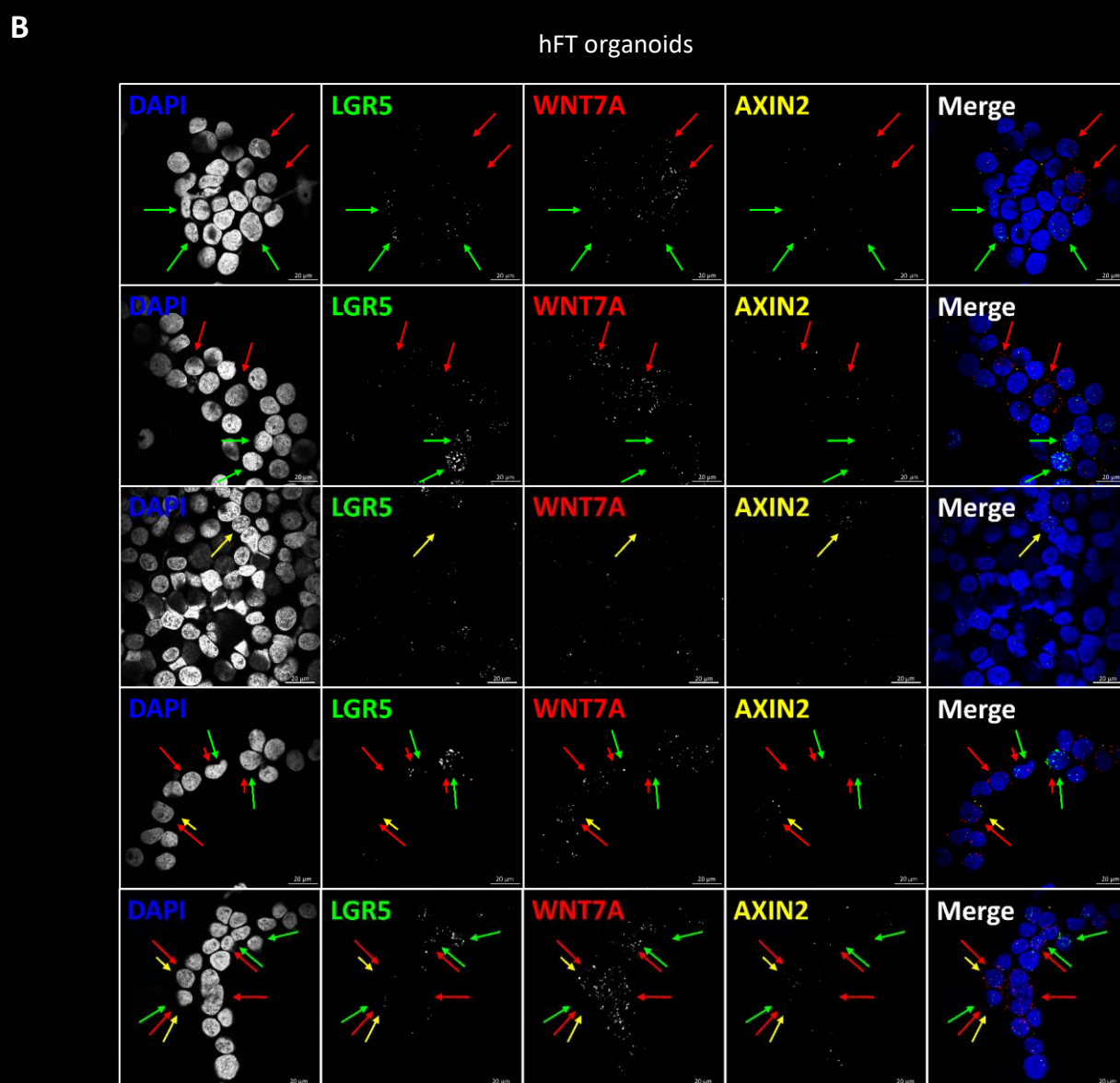
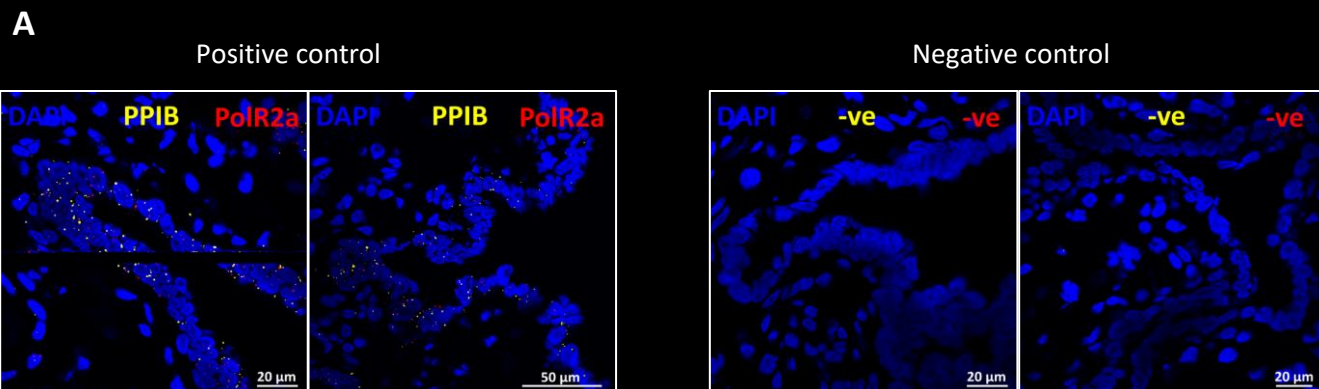


Figure 56: Confirmation of WNT7A+ cells in hFT by RNA FISH. (A) Fluorescent RNAScope technology was optimised for FT tissue sections. Positive control probes target ubiquitously expressed housekeeping genes. Negative control probes target bacterial sequences. **(B)** Confirmation of WNT7A+ cells in hFT organoids. WNT7A+ cells may co-express AXIN2 or LGR5. AXIN2+ cells and LGR5+ cells are largely distinct. Based on single cell data, the frequency of WNT7A+ cells is ~ 5% in hFT tissue and ~20% in hFT organoids. Organoids were dissociated, cytopinned and fixed before RNA FISH staining.

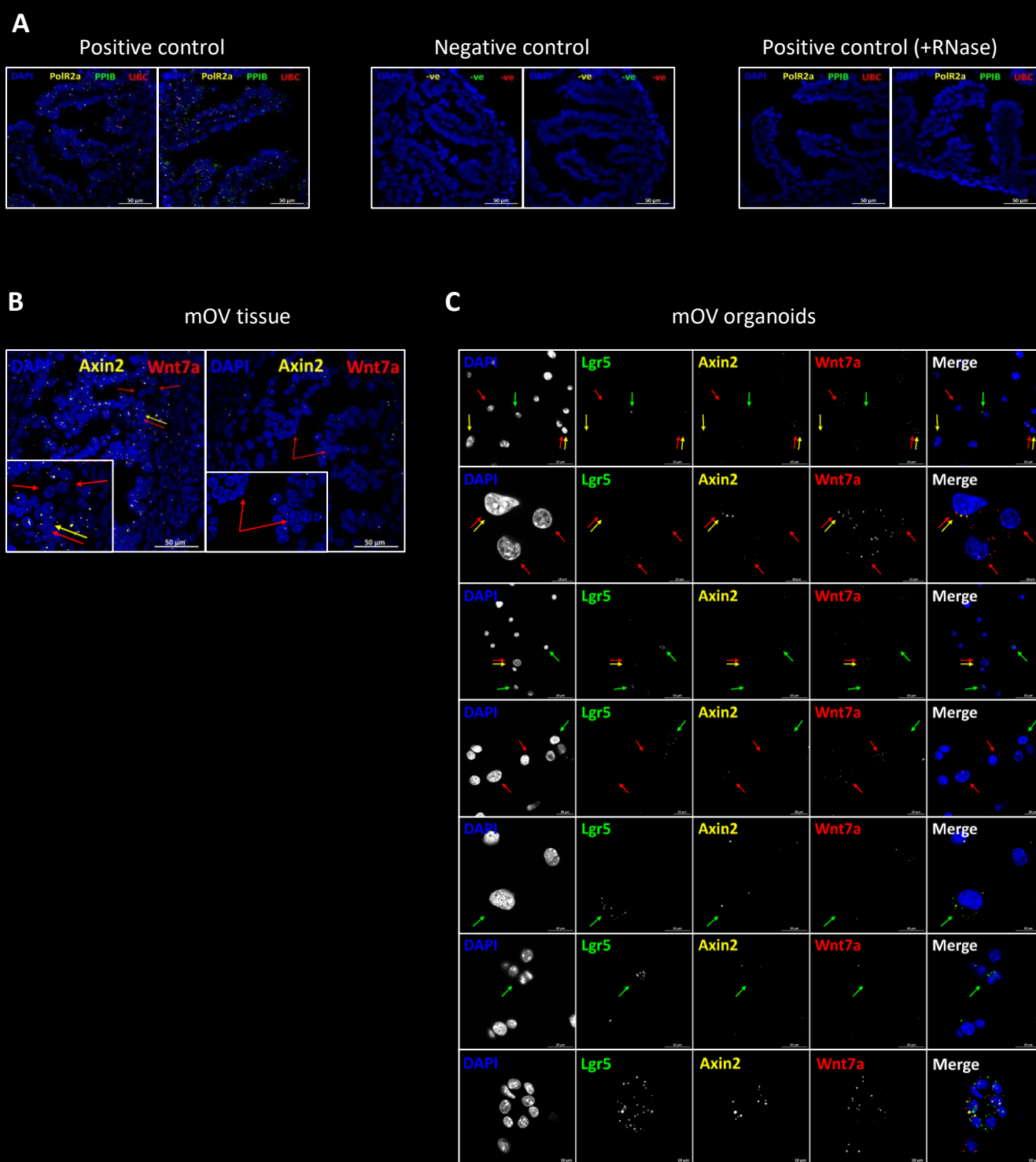


Figure 57: Confirmation of Wnt7a+ cells in mOV by RNA FISH. (A) Control staining on mOV tissue sections. This confirms specificity of RNAScope probes to RNA. RNase treatment sample confirms that probes do not hybridize to genomic DNA. **(B)** Confirmation of Wnt7a+ cells in mOV tissue. As was seen in hFT tissue, Wnt7a+ may or may not co-express Axin2. Insets show enlarged parts of the same image. **(C)** Confirmation of Wnt7a+ cells in mOV organoids. Again, Axin2+ cells are distinct from Lgr5+ cells. Organoids were dissociated, cytopspinned, fixed and stained using RNA FISH.

3. WNT7A protein expression and validation

WNT7A is the only expressed Wnt ligand in hFT organoids. This means it's likely the sole target of IWP-2 and LGK-974, which block endogenous Wnt secretion in organoids and lead to their inhibition. Now that the identity of the driver of Wnt signaling in organoids has been unmasked, I asked the question: can exogenously-supplied WNT7A protein rescue the death of WNT-blocked organoids?

To test this, recombinant WNT7A protein was obtained commercially (R&D). However, due to high hydrophobicity, purified Wnt proteins are generally not active or culture-stable (Tüysüz *et al.*, 2017), and I confirm this for a number of commercially available purified WNT7A proteins (data shown for one of them in Figure 59F). *In vitro*, Wnts such as Wnt3a require presence of FBS during their synthesis, processing and secretion to yield an active and more hydrophilic protein. It is not known why this is the case, but a component within FBS, called Afamin, was shown to complex with synthesized Wnts (Mihara *et al.*, 2016). Active Wnt ligands may be obtained under serum-free conditions in the presence of Afamin (Mihara *et al.*, 2016). Hence, I set out to produce in-house serum-containing active WNT7A CM for use in organoid culture (Figure 58).

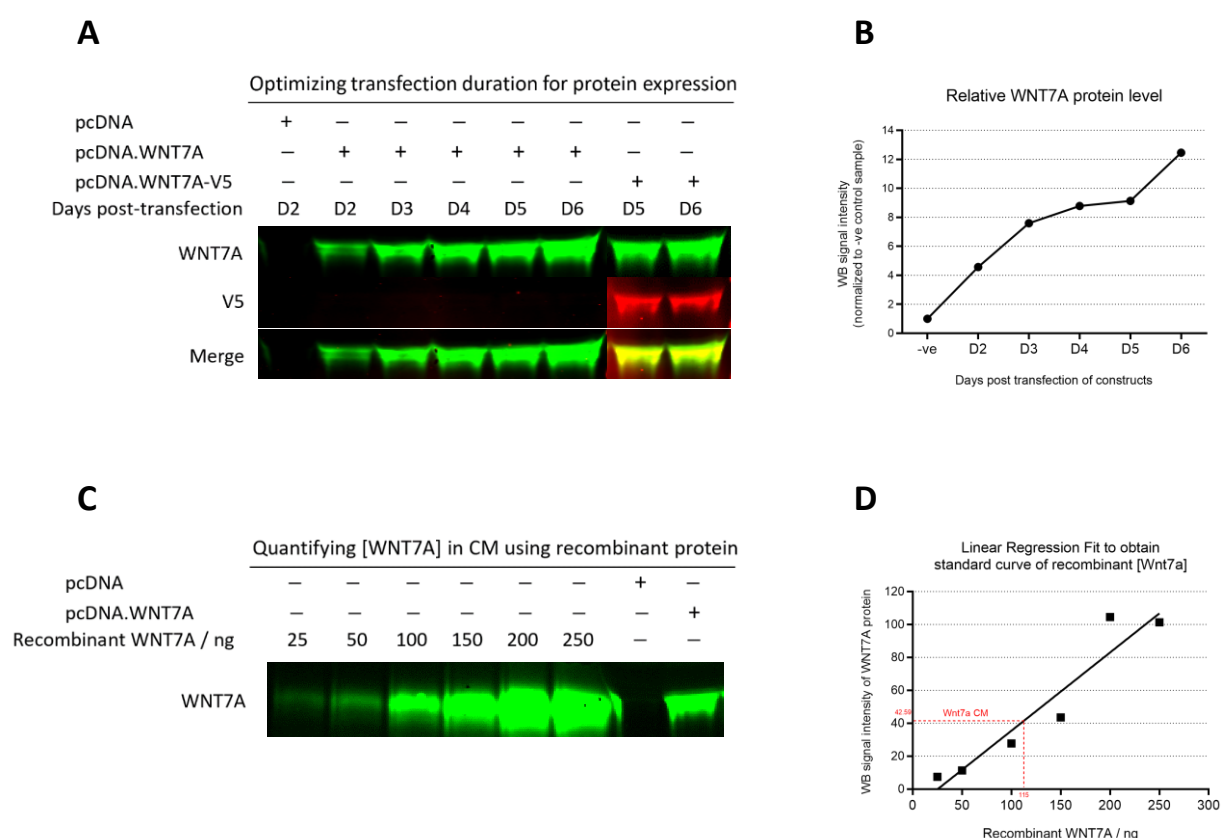


Figure 58: WNT7A protein expression. (A) WNT7A protein was generated using expression constructs, as detailed (section 5.4.1). V5 immunoblotting confirms specificity of WNT7A antibody. (B) Estimation of WNT7A protein amount in (A). (C) Recombinant WNT7A protein, obtained commercially, was used to quantify [WNT7A]. (D) Quantification of data in (C). Recombinant WNT7A was used to generate a standard calibration curve. Linear Regression Fit was used to estimate [WNT7A] in CM, which is 3.4 $\mu\text{g/ml}$. WNT7A CM was used at 25% in downstream assays, so final [WNT7A] used is 850 ng/ml. CM contained DMEM, serum and P/S. 195

After that, WNT7A CM and the construct used to produce it were tested using the Wnt-reporter assay. In addition, a number of other WNT7A reagents were generated and tested. For example, the single cell data showed that most FT cells become WNT7A+ upon 2D culture (Figure 55C). I confirm this by qPCR (Figure 59A) and exploit this as a means of obtaining WNT7A protein (Figure 59B). The lysates of these cells contain abundant WNT7A (Figure 59C), which disappears upon activating Wnt signaling (Figure 59C). As a side point, this points to the notion that WNT7A+ and Wnt-active cells are two cell types / states that are programmed to be mutually exclusive. Finally, native WNT7A protein was also isolated using the same construct (Figure 59E).

All reagents were tested with the Wnt-reporter assay. The WNT7A construct gave a robust and reproducible activation of Wnt signaling. Unfortunately, all other protein-based reagents failed to match this (Figure 59F). This could point to a mechanism that deactivates WNT7A upon secretion, or that intracellular forms of WNT7A may be active. Native WNT7A protein was harvested intracellularly from lysates, and also failed to show activity. Two protein secretion inhibitors were used to test if WNT7A secretion is a prerequisite to Wnt activation. Brefeldin A (Orcl *et al.*, 1991) and Monensin (Tartakoff, 1983) inhibit protein transport from the endoplasmic reticulum to the Golgi complex. Brefeldin A and Monensin abolish the activation of Wnt signaling by the WNT7A construct, confirming that WNT7A secretion is a prerequisite to activation of Wnt/ β -catenin signaling.

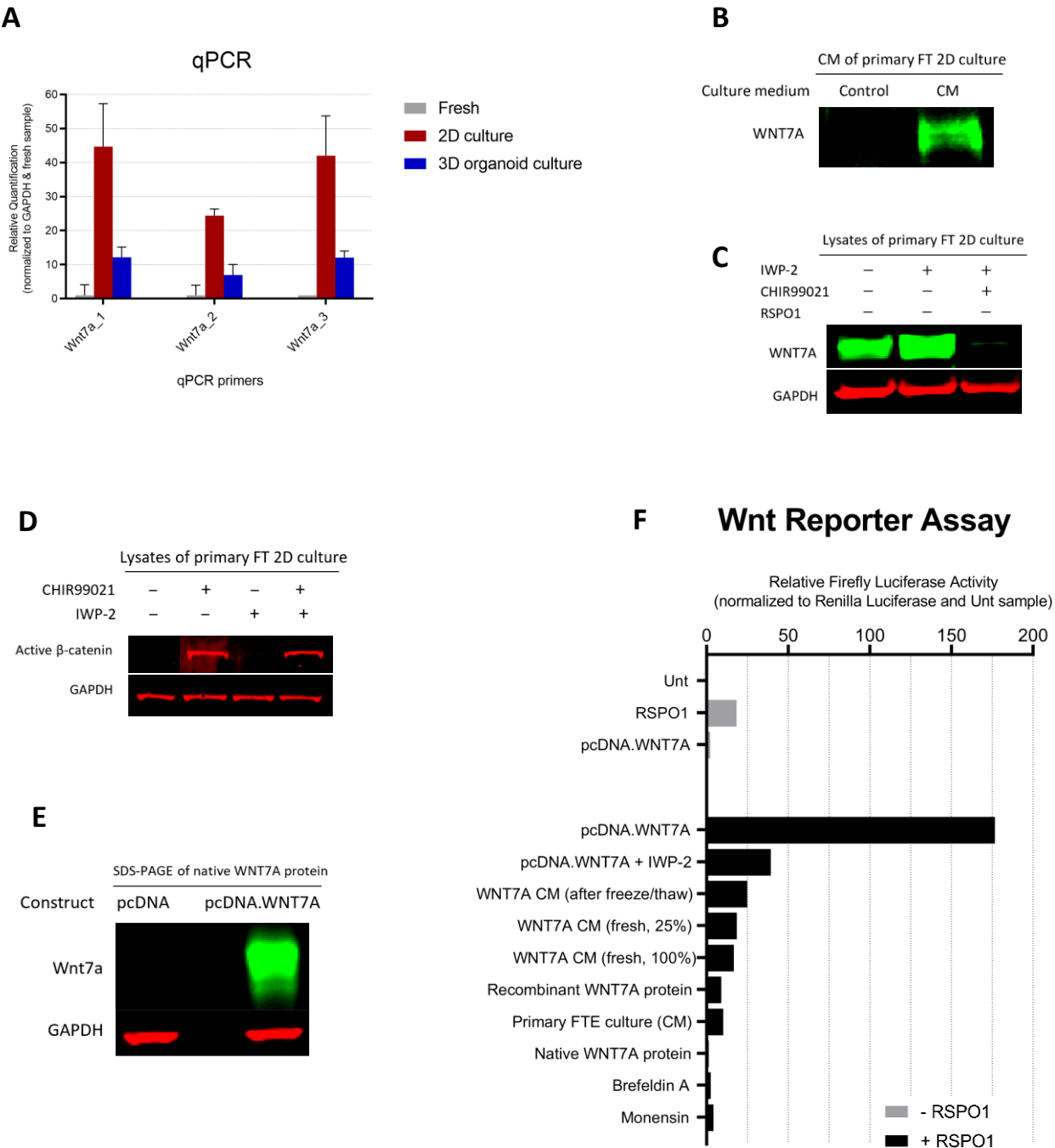


Figure 59: WNT7A protein reagents are not functional. Activity of WNT7A protein in CM was tested using the Wnt-reporter assay. **(A)** WNT7A levels are massively overexpressed in 2D-cultured FT cells. This confirms the single cell data shown in Figure 55C. All samples here were from the same patient. **(B)** WNT7A protein is readily detectable in the CM of 2D-cultured cells. Conditioned medium was harvested after 4-day incubation of 1-week old culture. Control here is the same medium prior to incubation with cells. **(C)** WNT7A protein can be seen in lysates of 2D-cultured FT cells. **(D)** Confirmation that CHIR99021 treatment increases intracellular levels of stabilized β -catenin and Wnt signaling. Active β -catenin antibody specifically recognizes β -catenin unphosphorylated in Ser 33, 37 and 41. Phosphorylation of these serine residues by GSK-3 β and CK1 α targets β -catenin for degradation (section 1.3.4). Unt sample was run concurrently, on a different gel in the same SDS-PAGE gel tank. **(E)** 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. **(F)** Wnt-reporter (TOPFlash) assay confirms activation of Wnt signaling by pcDNA.WNT7A construct. All other protein-based WNT7A reagents failed to activate Wnt signaling. Some samples were tested on different days, and are normalized to their own Unt controls.

4. WNT7A signals through a short-range gradient

Wnt ligands are known to function as gradient morphogens during development. Signaling range of Wnt ligands may be increased by carrier proteins or chaperones. However, recent biochemical and *in vivo* evidence suggests that most Wnts function through a very short-range gradient (Farin *et al.*, 2016), possibly 1-3 cells long. With this in mind, the signaling range for WNT7A protein was tested using a co-culture assay (Figure 60A). Indeed, WNT7A is active and functional when producing and receiving cells are co-cultured and in very close range, possibly through direct physical interaction (Figure 60B). Therefore, deeper biochemical and mass spectrometry analysis are needed to identify the mechanisms that prohibit WNT7A signaling in longer range. This may not be due to WNT7A structure or post-translational modifications. Alternatively, Wnt ligand hydrophobicity could be to blame. It is a barrier to *in vitro* use of Wnt ligands, and unlike Wnt3a, WNT7A may require other factors not present in FBS that can promote its activity in an aqueous and hydrophilic *in vitro* environment.

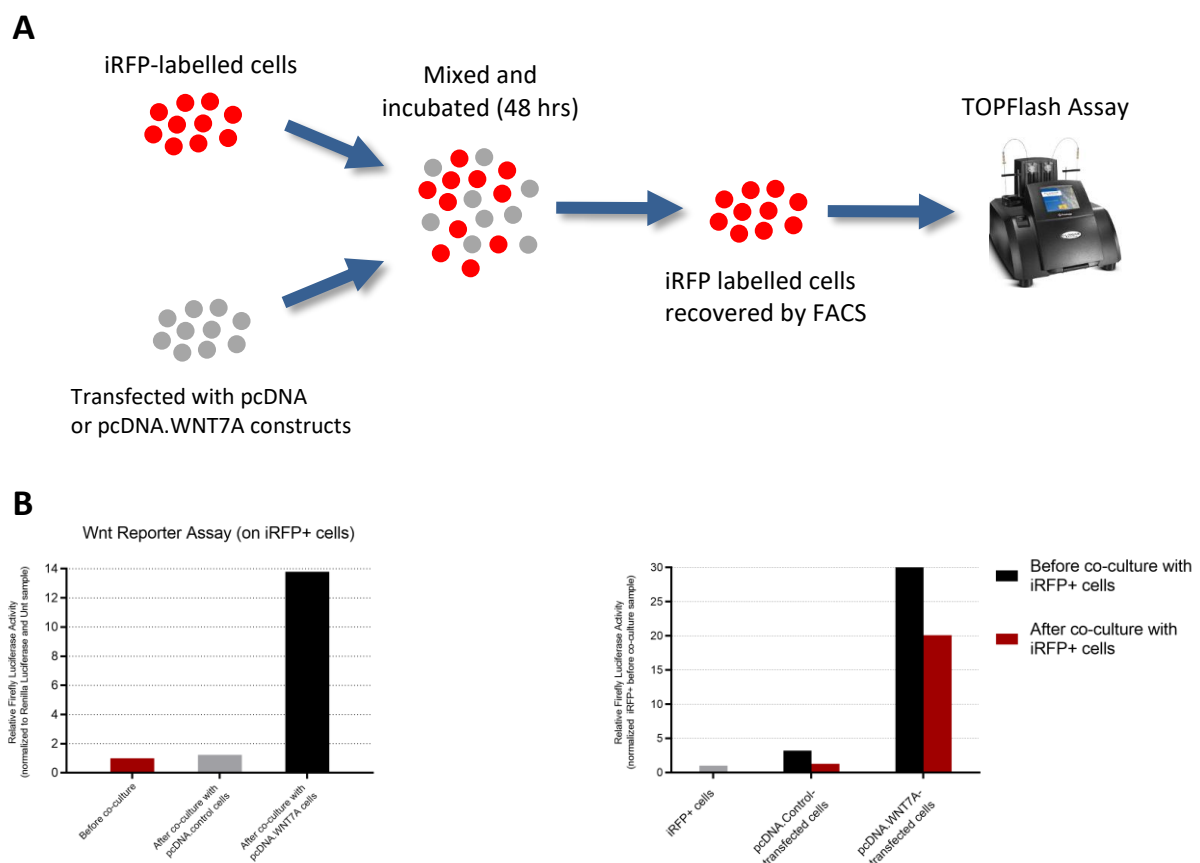


Figure 60: WNT7A signals through a short-range gradient. (A) Co-culture assay. HEK cells were labelled with TOPFlash plasmids and iRFP. They were then co-cultured for 48 hrs with HEK cells transfected with TOPFlash plasmids and either pcDNA or pcDNA.WNT7A constructs. iRFP+ and iRFP- cells were then isolated by FACS and subjected to the Wnt-reporter (TOPFlash) assay. **(B)** Wnt signaling level increases in iRFP+ WNT7A- cells upon co-culture with WNT7A-expressing cells, but not controls. **(C)** Wnt signaling levels drop in pcDNA.WNT7A transfected samples after co-culture with iRFP+ cells, suggesting that a fraction of produced WNT7A was utilized by iRFP+ cells containing no WNT7A. All samples were treated with RSPO1. Data representative of two biological replicates.

5. Structural analysis of WNT7A protein

In addition to signaling in close range, WNT7A protein is also targeted for glycosylation. PNGase F is an amidase that cleaves N-linked oligosaccharides from glycoproteins (Maley *et al.*, 1989). Treatment of WNT7A with PNGase F leads to a mobility shift on SDS-PAGE (Figure 61), in both intracellular and secreted forms of the protein. As discussed (section 1.2.2), Wnt glycosylation is essential for secretion. Absence of glycosylation inhibits Wnt3a secretion, but removal of glycosylation from already secreted Wnt protein does not affect its activity (Nusse & Varmus, 1992). Extrapolating from what is known about mouse Wnt3a (Komekado *et al.*, 2007), and upon pairwise protein sequence alignment, I predict that Asparagines 83 and 295 are the sites of glycosylation in human WNT7A. These residues are conserved in mouse Wnt7a protein, implying functional importance.

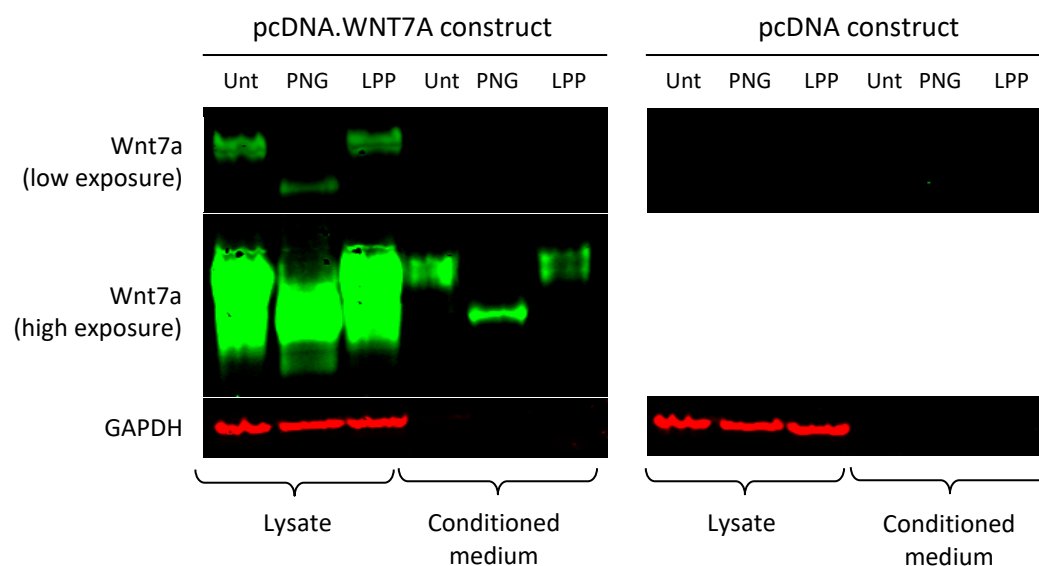


Figure 61: WNT7A protein is glycosylated. HEK cells were transfected with pcDNA.WNT7A construct or pcDNA (control) construct. Harvested lysate and CM were subjected to treatment by PNGase F (PNG) and Lambda Phosphatase (LPP). For technical details, please refer to section 5.13.

Although much progress has been made in delineating components of the Wnt signaling pathway, little is known about the structural basis of Wnt activation at the cell surface. This is due to lack of knowledge about Wnt protein structure. It has been technically challenging to solve Wnt structure, due to Wnt hydrophobicity and difficulty in crystallizing the protein. Thus far, the crystal structures of only two Wnts have been solved in their active form containing important post-translational modifications: *Xenopus* Wnt8 (Janda *et al.*, 2012) and human WNT3 (Hirai *et al.*, 2019). Both were characterized in a complex with a FZD receptor, and modified from the original protein to aid crystallization.

Using *Xenopus* Wnt8 as a template, I employ the Phyre2 bioinformatic tool to build a predicted structure for human WNT7A (Figure 62). The predicted structure shows a diminished linker domain in WNT7A. Other major domains are largely conserved, particularly the Hairpin 2 (thumb) and Hairpin 3 (index finger) domains. These create protruding 'thumb and finger' β -strands that are critical for engaging FZD receptors through their FZD-CRD domains. As with other Wnt ligands, the 24 conserved cysteine residues are present in human WNT7A. These create 11 pairs of inter-cysteine disulphide bonds that are crucial for establishing the 3D structure of Wnt ligands (Figure 63). Mouse Wnt7a contains the same distribution of Cysteine residues at the exact same amino acid locations. Mutations in these cysteines were shown, in other Wnts, to block Wnt secretion or inhibit Wnt activity (section 1.2.3). C-to-A mutagenesis in Hairpin 1 are well-tolerated in Wnts. Cysteine mutations in the index finger domain affect Wnt activity, while mutations in the thumb domain abolish Wnt secretion. In the hFT, it is not currently known which FZD receptor WNT7A engages using its predicted thumb and finger domains. This is the subject of discussion in the next section.

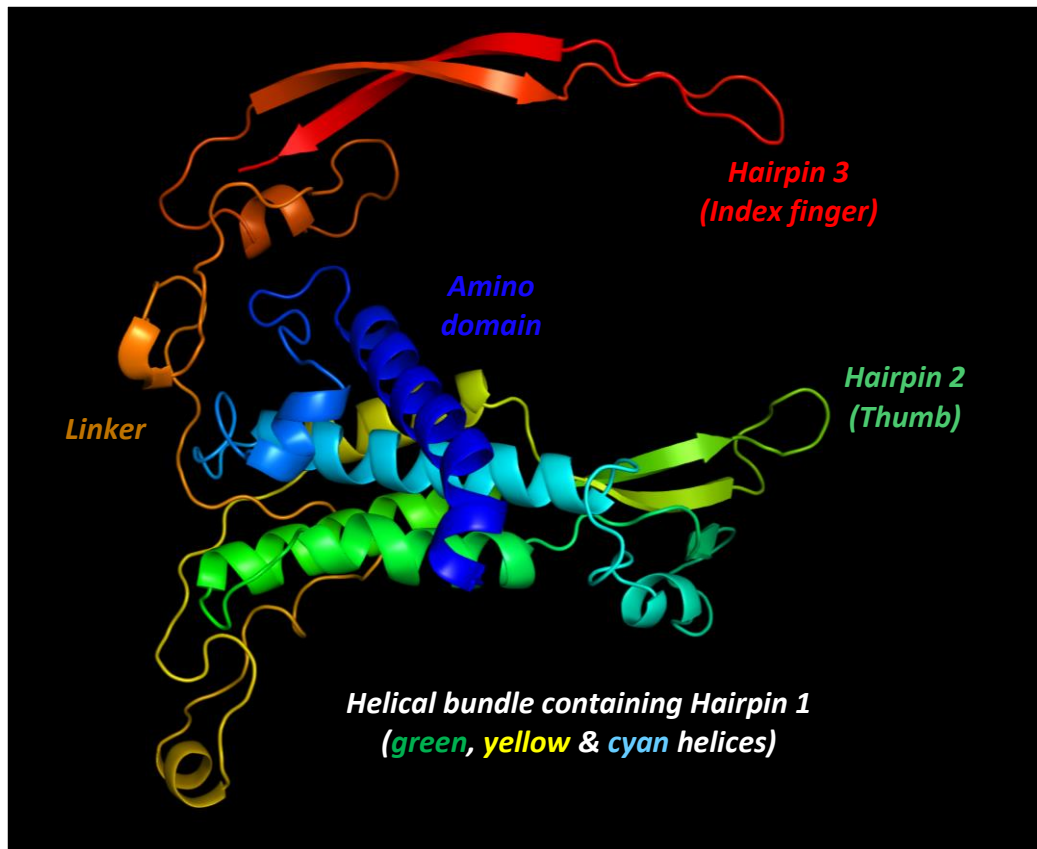
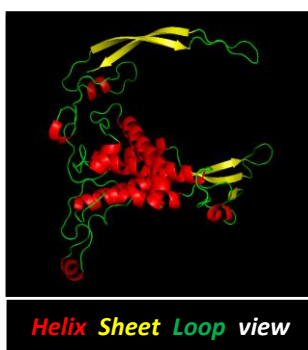
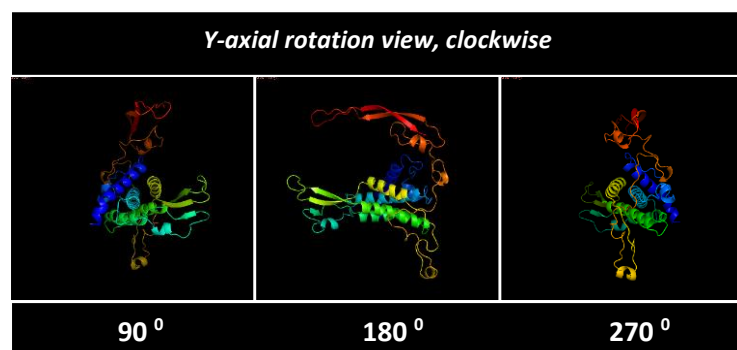
A**B****C**

Figure 62: Bioinformatic prediction of WNT7A protein structure. (A) Prediction of human WNT7A 3D structure, based on its primary sequence (RefSeq, NCBI gene), using the Phyre2 bioinformatic tool. Phyre2 employs advanced remote homology detection methods to infer 3D structure (Kelley et al., 2015). Crystal structure of *Xenopus* Wnt8 (Janda et al., 2012) was used as domain homology template. Domains are rainbow colour-coded, with blue to red representing N to C terminals, respectively. Domains are labelled with matching colour insets. (B) Secondary structure view showing location of helices, sheets and loops. (C) Still images of Y-axis rotations shown in 90° increments. For 3D movie, please refer to accompanying file, which can be played with Windows Media Player.

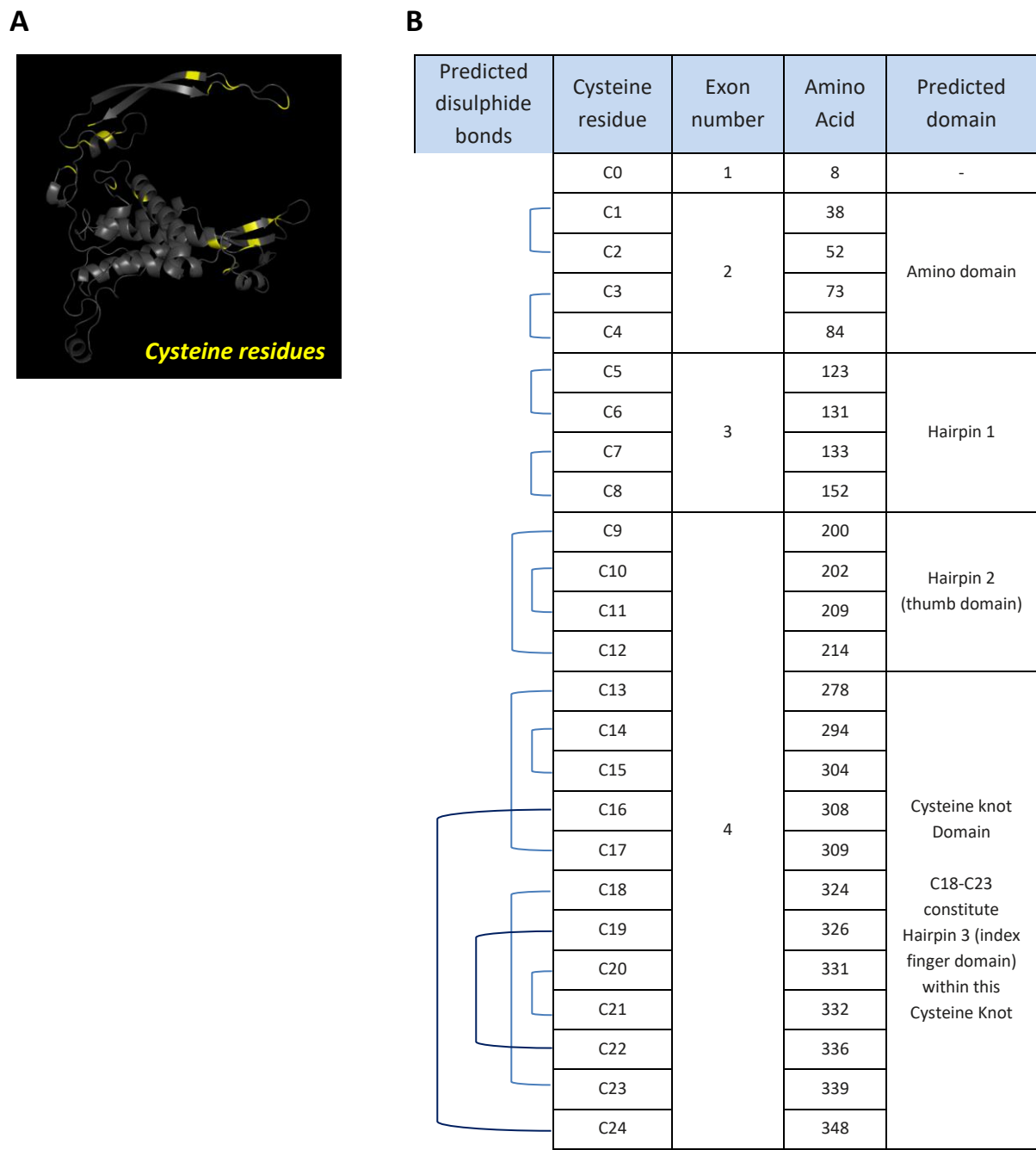


Figure 63: Distribution of Cysteine residues in the predicted structure of human WNT7A.
(A) Location of cysteine residues in WNT7A’s structure. Cysteine residues are shown in yellow.
(B) Distribution of Cysteine residues in WNT7A protein. Disulphide bonding pairs are shown with blue connecting lines. Disulphide bridge map predicted based on the crystal structure of *Xenopus* Wnt8 (Janda et al., 2012). Note that Cysteine 0 lies N-terminal to the signal peptidase cleavage site, so it is probably cleaved away and does not contribute to the overall structure of mature WNT7A. Cysteine 73 is well-characterized in other Wnts and was initially thought to be the PORCN palmitoylation site, which is now known to be Serine 209. Cysteine 24 is the penultimate amino acid in Wnt7a. C-to-W mutation of this amino acid in Wnt1 compromises protein activity (Nusse & Varmus, 1992).

8.3 FZD5 & LGR5 mediate Wnt/ β -catenin Signaling in the hFT

1. FZD5 is the WNT7A receptor

Frizzleds (FZDs) are the natural receptors of Wnt ligands. Wnts bind FZD receptors and LRP5/6 co-receptors to initiate the recruitment of Dishevelled, which sequesters some components of the β DC complex, effectively disrupting its assembly. Unregulated (and now stabilized) β -catenin translocates to the nucleus, where it activates canonical Wnt signaling. Although FZDs are G-protein coupled receptors, FZD-mediated G-protein activity is only seen in noncanonical Wnt signaling (Katanaev *et al.*, 2005).

Single cell transcriptomic profiling identifies three FZD receptors expressed in hFT organoids. These are FZD3, FZD5 and FZD6 (Figure 64A). This is also the case for hFT tissue (Figure 64B). FZD receptor family categorizes into 4 clusters: FZD5/8 cluster, FZD4/9/10 cluster, FZD1/2/7 cluster and FZD3/6 cluster (section 1.2.4, Figure 3C). The FZD3/6 cluster is the divergent cluster from this family, and participates in non-canonical Wnt signaling (Dong *et al.*, 2018). Therefore, FZD5 is the likely receptor for WNT7A and transducer of canonical Wnt signaling. Sequence similarity of FZD5 to FZD3/6 receptor cluster is low (Figure 64C). Given their divergent sequences and therefore structures, it is unlikely that WNT7A can activate Wnt signaling through both sets of FZD receptors.

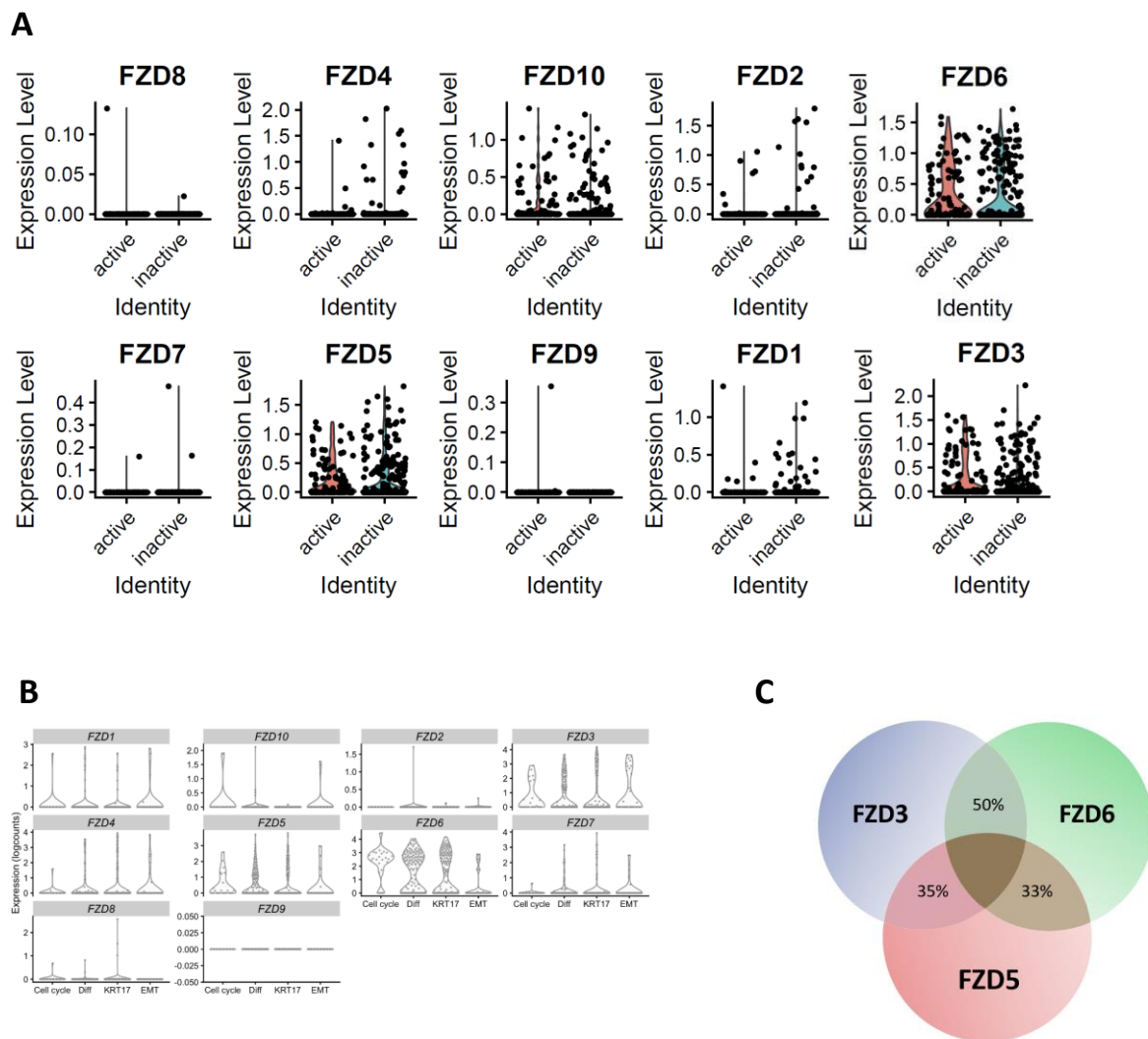


Figure 64: Expression of FZD family of receptors in hFT. (A) FZD expression in hFT organoids. FZD3, FZD5 and FZD6 are the only expressed FZDs, with some FZD10 expression seen. **(B)** FZD receptor expression in hFT tissue. FZD3, FZD5 and FZD6 are also the most highly expressed here. **(C)** Similarities between the protein sequences of FZD's expressed in the hFT, obtained by pairwise sequence alignments of RefSeq sequences. [Image (B) obtained from dataset generated by Zhiyuan Hu].

RSPO1 is also required for organoid regeneration, through its LGR receptors. As such, one of these two receptors, the WNT7A receptor (FZDs) or the RSPO1 receptor (LGRs), is the limiting factor in dictating multipotency. From Figure 64A, it can be surmised that expression of FZDs is not specific to Wnt-active cells, and is expressed in a higher % of cells compared % of cells with organoid formation capacity. I hypothesize that the RSPO1 receptor is the limiting factor in endowing cells with multipotency, but thus far, limited cell numbers might be masking its expression in the single cell data of hFT organoids.

To test if WNT7A can signal through these FZD receptors, the Wnt-reporter assay was used, as HEK cells endogenously express FZD3/5/6 (Figure 65A). FZD3/5/6 were knocked down using siRNAs (Figure 65B). WNT7A-induced TOPFlash signal is reduced only upon FZD5 siRNA knockdown, not FZD3 or FZD6 (Figure 65C). Furthermore, WNT7A construct induces the highest TOPFlash signal in a background of FZD5 overexpression (Figure 65D). This is consistent with a report that demonstrated, using ELISA-based protein-protein binding assays, that WNT7A binds FZD5 at its extracellular Cysteine-Rich Domain (CRD) in the nanomolar range (Yu *et al.*, 2012).

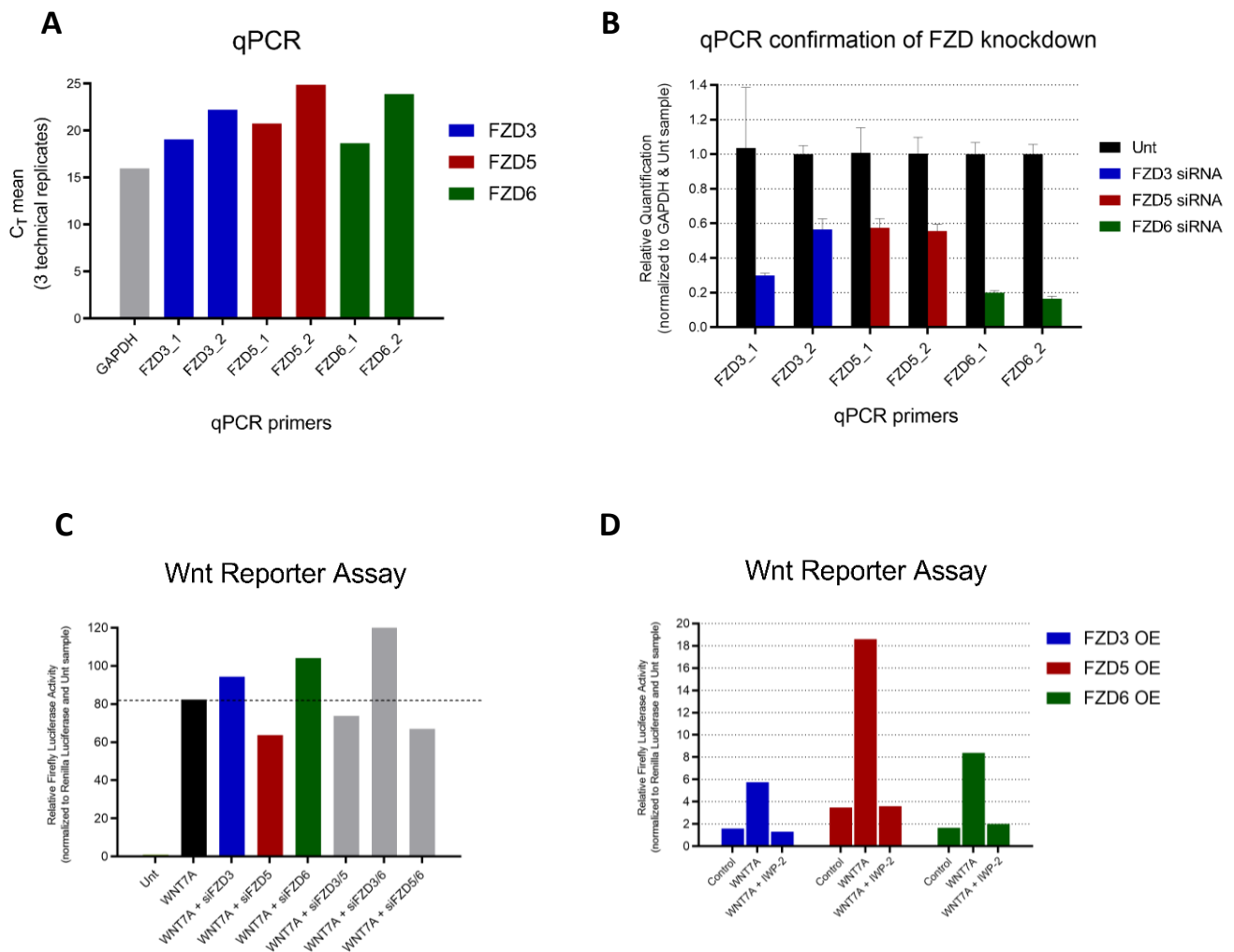


Figure 65: FZD5 can act as a receptor for WNT7A. (A) FZD3, FZD5 and FZD6 are expressed in HEK cells. C_T values shown for an untreated sample. GAPDH C_T value is shown for comparison. (B) qPCR confirmation of FZD siRNA knockdown. (C) WNT7A-induced TOPFlash signal reduces upon knockdown of FZD5, but not FZD3 or FZD6. All samples contained RSPO1, except Unt. WNT7A denotes pcDNA.WNT7A construct. Data from (A), (B) and (C) are from the same experiment. (D) WNT7A induces highest TOPFlash in a background of FZD5 overexpression. IWP-2 treatment confirms that this signal is due to WNT7A overexpression. Control is pcDNA construct. OE is overexpression. Data is representative of two biological replicates.

Although the data in Figure 65 is strongly suggestive of FZD5 as a WNT7A receptor, direct evidence from organoids is lacking. No reliable protocols exist for siRNA knockdown in organoids, while stable gene knockdown using viral techniques does not guarantee effective knockdown (data not shown). To circumvent these technical limitations, I utilize a novel, selective, antibody-based inhibitor of FZD5, generated using an antibody phage-display system. The anti-FZD5 antibodies, IgG-2919 and IgG-2921, were isolated in a screen for binders to FZD5 CRD domain, to which endogenous Wnt ligands bind using their thumb and finger motifs. IgG-2919 and IgG-2921 exhibited high-affinity binding to human FZD5 (Steinhart *et al.*, 2017). IgG-2919 and IgG-2921 have a higher affinity to FZD5 than OMP-18R5, a FZD-inhibiting antibody that has activity against a range of other FZDs (including FZD5), and is currently in clinical trials (Gurney *et al.*, 2012).

The anti-FZD5 antibodies reduce FZD5-mediated Wnt-activation in TOPFlash assay (Figure 66A). As predicted, FZD5 inhibition abolishes regeneration of hFT organoids (Figure 66 C-D). This phenocopies blocking endogenous WNT secretion. Of note, FZD8 is the only FZD with high sequence similarity to FZD5 (around 70%), and categorizes with FZD5 in the FZD5/8 cluster of this family. IgG-2919 and IgG2921 have some activity against FZD8. However, FZD8 is not expressed in hFT organoids (shown earlier, Figure 64A). As such, it can be inferred with certainty that FZD5 is the cognate receptor for WNT7A, and that a WNT7A-FZD5 signaling axis drives Wnt activation and self-renewal of FT stem cells in hFT organoids.

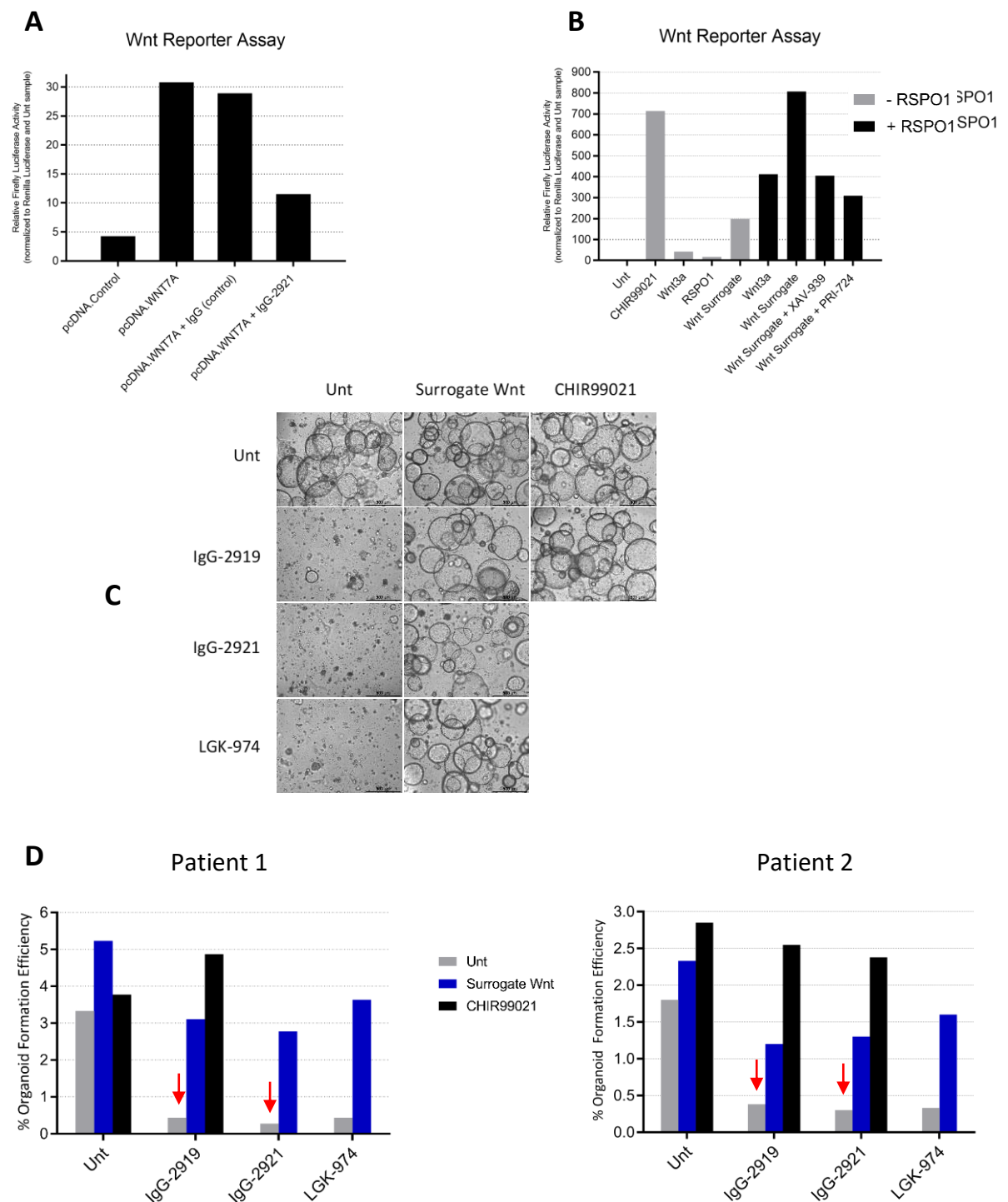


Figure 66: FZD5 is the WNT7A receptor in hFT organoids. (A) Anti-FZD5 antibodies show activity against WNT7A-induced FZD5-mediated Wnt activation in TOPFlash assay. FZD5 OE construct encoded mouse Fzd5, suggesting anti-FZD5 IgG's are active against mouse Fzd5 as well. (B) Surrogate Wnt shows potent activation of Wnt signaling in Wnt-reporter assay. Surrogate Wnt is as potent as CHIR99021 and superior to Wnt3a. Wnt activation by Surrogate Wnt reduces with XAV-939 and PRI-724 treatment, further confirming that this activation happens through the canonical Wnt machinery. Data shown is representative of two biological replicates. (C) Anti-FZD5 antibodies abolish organoid regeneration, phenocopying WNT7A inhibition by LGK-974. Both are rescued by Surrogate Wnt or CHIR99021 treatment. Surrogate Wnt was used at 0.5 nM. FZD5 inhibitors IgG-2919 and IgG-2921 were used at 1 μ M. RSPO1 was included in all samples. Representative images on day 10 shown. (D) Quantification shown for two patients. Each experiment was conducted on separate days, constituting biological replicates. 3-4K cells were plated per well on 96-well plates.

Blocking of WNT7A (and other Wnts), as well as FZD5 inhibition, are rescued by Surrogate Wnt; a synthetic peptide containing an antibody epitope that binds the extracellular FZD CRD domain, 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). In essence, Surrogate Wnt selectively heterodimerizes FZDs with LRP6 co-receptor extracellularly, mimicking the action of endogenous Wnt ligands in receptor binding and activation.

Surrogate Wnt is superior to Wnt3a in activating Wnt signaling (Figure 66B), as well as in expansion of Wnt3a-dependent organoids (Clevers H., personal communication). Surrogate Wnt is highly potent, selective and water-soluble, overcoming the challenges of expressing and isolating active Wnt proteins for functional studies (Figures 56 and 57). It is thus a welcomed resource and a useful addition to Wnt signaling and organoid-based research.

By tuning the FZD CRD epitopes, Surrogate Wnts were customized to activate specific FZD receptors (Miao *et al.*, 2020). These so-called Next Generation Surrogate Wnts (or NGS Wnts) can be very useful in identifying functional requirements of Wnt-active cells *in vitro* and *in vivo*. However, only FZD4 NGS Wnt and FZD8 NGS Wnt are monospecific, with others showing activity against 2 or more FZDs. In this regard, it is worth mentioning that the Surrogate Wnt used in Figure 66 has broad-spectrum activity against FZDs 1/2/5/7/8. Nonetheless, it has no activity against FZDs 3/6. This is further evidence that FZD5 is the FZD receptor associated with canonical Wnt signaling and regeneration of hFT organoids.

2. mOV organoids are unaffected by FZD5 inhibition

Interestingly, FZD5 inhibition has no effect on mouse oviduct organoids (Figure 67). By pairwise protein sequence alignment, mouse Fzd5 is over 97% similar to human FZD5. Anti-FZD5 antibodies were designed to have cross-species activity (Steinhart *et al.*, 2017). I confirm that anti-FZD5 IgG's possess activity against mouse Fzd5 (Figure 66A). The lack of inhibition of mOV organoids by anti-FZD5 antibodies is consistent with lack of inhibition seen in Wnt-blocked mOV organoids (treated with LGK-974). This suggests that, unlike hFT organoids, the WNT7A-FZD5 signaling axis is not essential for regeneration of mOV organoids.

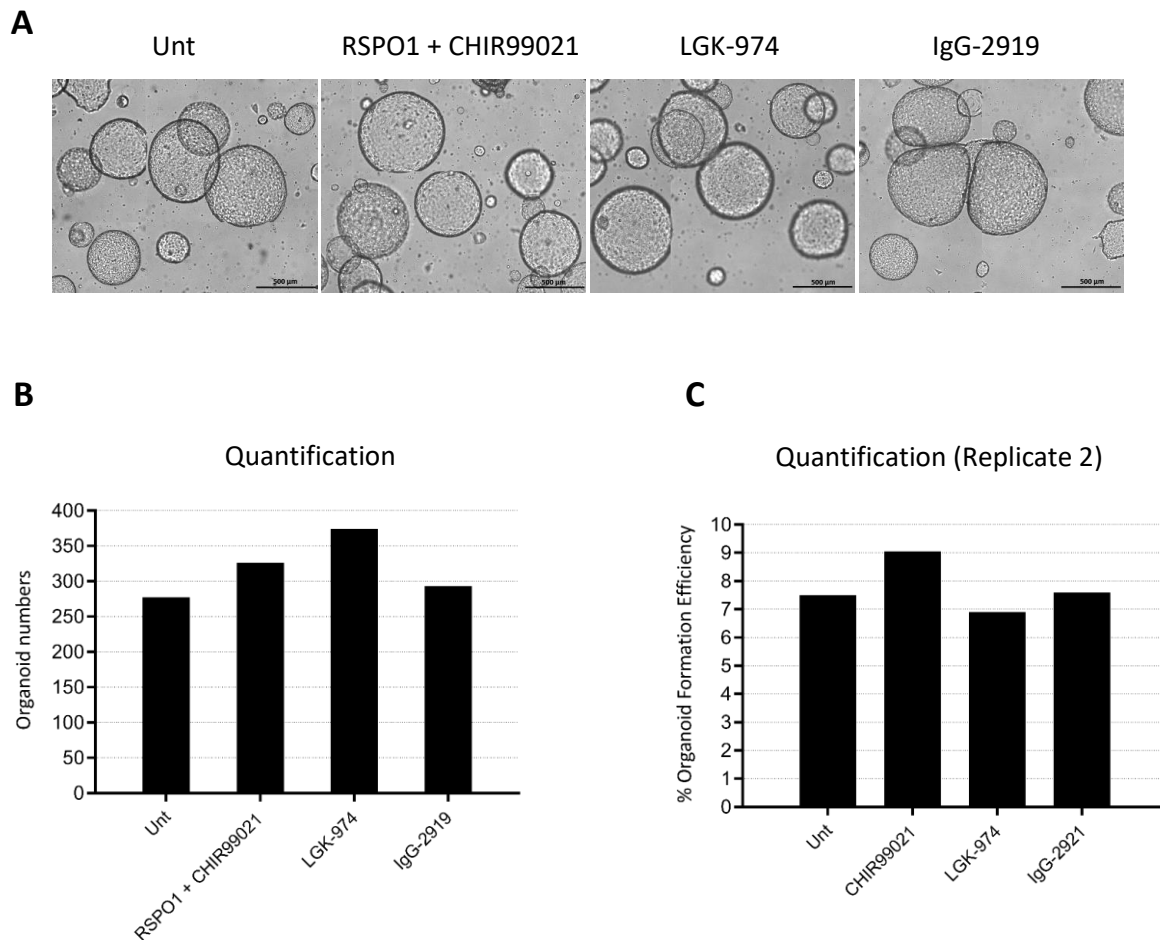


Figure 67: mOV organoids are unaffected by Fzd5 inhibition. (A) Representative images on day 10. **(B)** Quantification of images shown in (A). **(C)** Quantification of a replicate, using a different anti-FZD5 antibody.

3. LGR5 is the RSPO receptor in hFT organoids

Thus far, we have observed the importance of WNT7A-FZD5 signaling for regeneration of hFT organoids. Single cell profiling shows that FZD5 expression is diffuse and not restricted to a minority of cells. It is also not enriched in Wnt-active cells. In the presence of WNT7A, withdrawal of RSPO1 compromises organoid renewal, suggesting that the RSPO1 receptor is an obligate requirement, and probably the limiting factor, in endowing cells with multipotency. Its identification might hold the key to specific biomarking of ASCs in the hFT.

Leucine-rich repeat-containing G protein-coupled Receptors (or LGRs) are the cognate receptors for RSPOs. RSPO-LGR signaling potentiates Wnt signaling, by degrading RNF43/ZNRF3, an E3 ubiquitin ligase that targets FZD receptors for degradation (section 1.2.5). The LGR family of receptors are characterized by a large ectodomain and seven transmembrane domains (shown

earlier, Figure 5, section 1.2.6). Three subfamilies exist, and direct biochemical and *in vivo* evidence shows that LGRs 4-6 bind all RSPOs (RSPO1-4) with no evidence of specific ligand-receptor preferences (de Lau *et al.*, 2011; Carmon *et al.*, 2011; Glinka *et al.*, 2011).

Single cell transcriptomic profiling has ruled out LGR4 as the RSPO1 receptor in hFT organoids, as its expression is not restricted to a minority of cells, and not enriched in Wnt-active cells (Figure 68A). LGR5 and LGR6 expressions are not well-captured, including within Wnt-active cells (Figure 68A) for reasons outlined earlier. However, a number of studies have reported detection of LGR5/6 in hFT and mOV organoids (Table 15).

	AXIN2	LGR5		LGR6
hFT tissue	✓ (this thesis)	✓ (this thesis) (Hu <i>et al.</i> , 2020)	✗ (Kessler <i>et al.</i> , 2015)	✓ (Kessler <i>et al.</i> , 2015) (Lee <i>et al.</i> , 2020)
hFT organoids	✓ (this thesis) (Kessler <i>et al.</i> , 2015)	✓ (this thesis) (Chang <i>et al.</i> , 2020) – spheroids	✗ (Kessler <i>et al.</i> , 2015)	✓ (Kessler <i>et al.</i> , 2015) (Y. Yamamoto <i>et al.</i> , 2016) – ALI culture
mOV tissue	✓ (Ghosh <i>et al.</i> , 2017) (this thesis)	✗ (Seishima <i>et al.</i> , 2019) (Ng <i>et al.</i> , 2014)		?
mOV organoid	✓ (this thesis)	✓ (this thesis)		?

Table 15: Summary of reported Wnt / stem cell markers in the hFT and mOV. Green tick denotes confirmed expression, red cross denotes confirmed lack of expression. AXIN2/Axin2 is the only documented Wnt-activation marker in all contexts. *In vivo* lineage tracing of Lgr5-EGFP in mice conclusively ruled out LGR5 expression in mouse oviducts. LGR6 expression was documented in hFT tissue and organoids, using IHC techniques. ALI culture is Air-Liquid Interface culture.

Using microarray-based analyses, a study detected strong expression of LGR6 in hFT tissue and organoids, and reported lack of LGR5 expression in both (Kessler *et al.*, 2015). I confirm LGR6 expression in hFT organoids, and show that LGR5 is expressed at equivalent or higher levels (Figure 68B). Furthermore, I confirm presence of LGR5+ cells in hFT tissue and organoids by RNA FISH (shown earlier, Figures 56). The host lab also documents this in single cell data from hFT tissue (Figure 68C). RNA FISH and scRNA-seq are essentially single cell approaches, and are superior to microarray-based approaches, in which RNA amplification and sample distribution across array introduce biases in sample hybridization and transcript detection.

I predict one of these, LGR5 or LGR6, is the RSPO1 receptor that marks putative FT stem cells. To test this, I conduct functional analyses in organoids by direct inhibition of these LGRs using IgGs. Anti-LGR5 IgG was validated to bind the extracellular ectodomain of LGR5 (Kemper *et al.*, 2012; de Lau *et al.*, 2011). Anti-LGR6 IgG also binds LGR6 at the extracellular N-terminal ectodomain (Kessler *et al.*, 2015). In the presence of RSPO1, inhibition of LGR5 (and not LGR6) abolishes organoid regeneration (Figure 68 D-E). This phenocopies withdrawal of RSPO1 (shown earlier, Figures 40, 42 and 44).

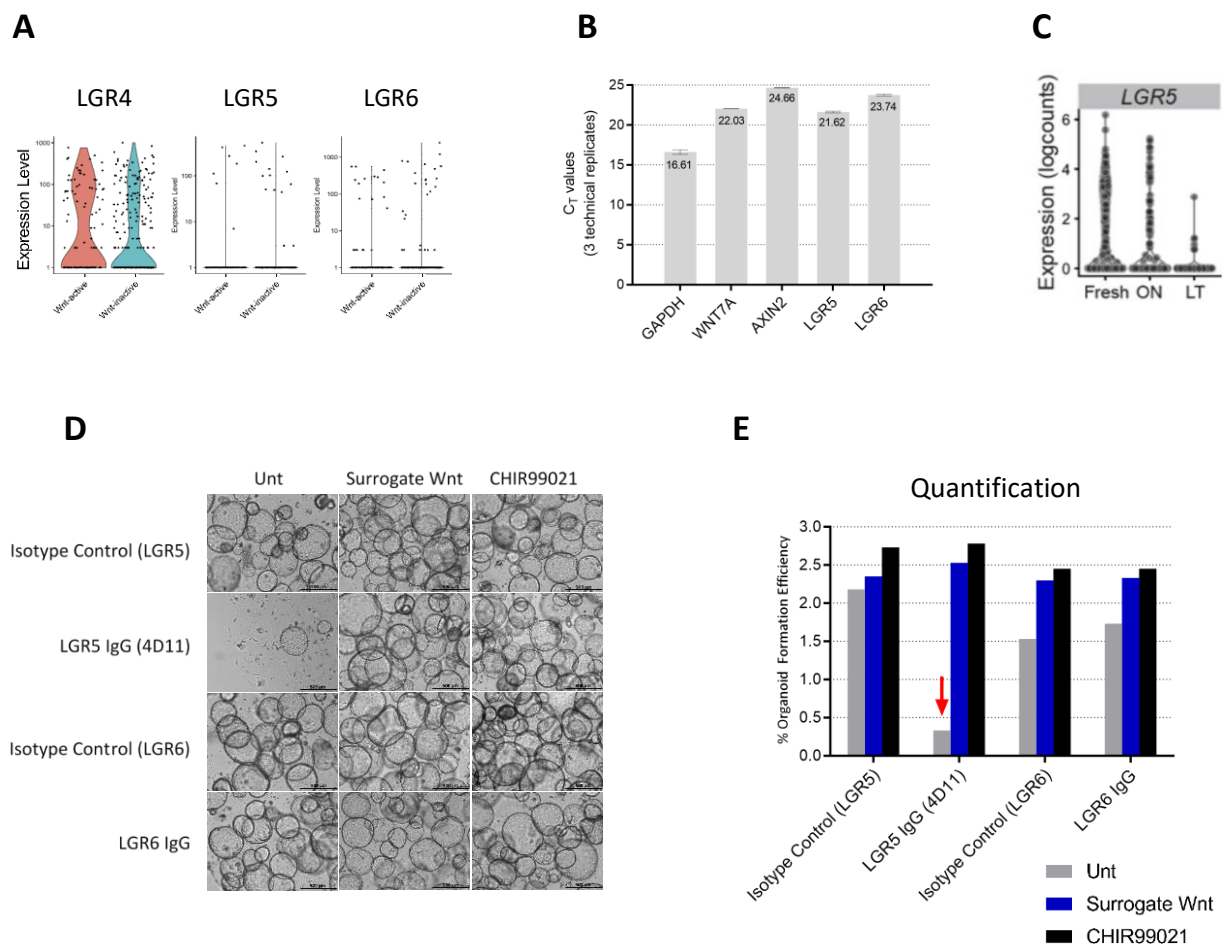


Figure 68: LGR5 is the RSPO1 receptor in hFT organoids. (A) Expression of the RSPO receptors LGR4-6 in the single cell data of hFT organoids. (B) Quantification of the relative expression of LGR5/6 in untreated hFT organoids using qPCR. Both are expressed in hFT organoids. Data shown is representative of 3 organoid lines from 3 different patients. (C) LGR5 expression in the single cell data of hFT tissue. ON is overnight-cultured, LT is long-term cultured (2-6 days). Both were 2D-cultured. Reproduction of Figure 55C. (D) LGR5 inhibition by anti-LGR5 antibody abolishes organoid regeneration, phenocopying RSPO1 withdrawal. This is not rescued by LGR6, indicating LGR6 does not act as an RSPO1 receptor. However, it is rescued by Surrogate Wnt or CHIR99021 treatment, indicating this is a Wnt signaling effect and not a non-specific IgG effect, which was also controlled for by an isotype control. Representative images shown on Day 10. (E) Quantification of the images in (D). [(C) is taken from Hu *et al.* (2020)].

The anti-LGR5 IgG is better validated than the anti-LGR6 IgG. The LGR6 inhibition result may not be reliable and LGR6 may have the potential to act as an RSPO1 receptor. However, if this was the case, LGR6 would have substituted for LGR5 upon its inhibition, and rescue inhibition of LGR5-blocked organoids. In the absence of this rescue, it can be surmised that LGR5 is the cognate receptor for RSPO1 in hFT organoids. LGR5 may therefore mark hFT stem cells in tissue and organoids.

The anti-LGR5 IgG was confirmed to lack reactivity against mouse Fzd5. This prohibited conducting the same functional analysis on mOV organoids. However, absence of the LGR5 agonist (RSPO1) does not abolish regeneration of mOV organoids (shown earlier, Figures 43 A-D and 50 B-F). This indicates that, although Lgr5+ cells were detected in mOV organoids (shown earlier, Figure 57C), Lgr5+ cells in mOV organoids do not mark organoid-forming stem cells. *In vivo*, Lgr5 is not detectable in mOV tissue (Table 15). Therefore, Lgr5+ cells do not play a significant part in tissue and organoid regeneration in mice, in a stark difference to humans.

4. Estrogen regulates WNT7A and Wnt signaling

As outlined in section 2.3, Estrogen acts as a ligand that homo- or hetero-dimerizes ER nuclear receptors. Liganded ER α promotes Wnt signaling and proliferation in the female reproductive tract. It is not completely understood how this is achieved, but Wnt ligand-dependent and independent mechanisms have been described. For instance, Estrogen treatment of endometrial primary culture induces β -catenin stabilization and hyperproliferation. This Estrogen-induced proliferation is abolished upon treatment with soluble frizzled-related protein 2 (Sfrp2) (Hou *et al.*, 2004), suggesting this is dependent on a Wnt ligand.

On the other hand, liganded ER α was reported to interact with Akt and p85, and together the complex inhibits GSK-3 β , promoting canonical Wnt signaling (Wang *et al.*, 2010) downstream of Wnt ligands. Liganded ER α may also bind β -catenin directly (Kouzmenko *et al.*, 2004) and stabilize it, suggesting that Estrogen-induced activation of Wnt signaling is independent of a Wnt ligand. Not much is known about whether Estrogen regulates Wnt signaling in the adult FT/oviduct, and whether this process is Wnt ligand-dependent or independent.

Progesterone antagonizes Estrogen and Estrogen-induced Wnt activation. As mentioned (section 2.3.2), this was seen in endometrial biopsies of patients treated with Estrogen or Estrogen + Progesterone (Hanifi-Moghaddam *et al.*, 2007; Wang *et al.*, 2010). In endometrial cell lines, Progesterone was shown to inhibit Estrogen-induced Wnt activation. This is abolished upon siRNA

knockdown of either Dickkopf-1 or FOXO1, suggesting the pair mediate progesterone's inhibition of Estrogen-induced Wnt activation (shown earlier, Figure 13C). It is also possible that Progesterone inhibits Wnt signaling by directly antagonizing Estrogen or its ER receptors.

Thus far, I have documented a synonymous relationship between WNT7A and Wnt signaling in hFT organoids. When WNT7A secretion (and other WNTs) is blocked using LGK-974, or its receptor (FZD5) is inhibited using antibodies, Wnt signaling is abolished and hFT organoids fail to regenerate. However, from our discussion in section 2.3.3 on the Estrogen analogue DES, and how neonatal mice exposed to DES phenocopy Wnt7a KO mice, Estrogen seems to uncouple this synonymous relationship, promoting Wnt signaling on the one hand while simultaneously inhibiting WNT7A on the other hand.

To address this apparent contradiction, Estrogen and Progesterone were tested for their ability to induce Wnt-reporter signal. Estrogen robustly activates WRE elements in TOPFlash assay (Figure 69A). This happens independently of Wnt ligands (Figure 69B), and is unaffected by inhibitors of Wnt signaling at midstream (XAV-939) and downstream (PRI-724) levels (Figure 69B). This could mean that liganded ER α binds to and stabilizes β -catenin, giving it independence from its transcriptional co-activators. Alternatively, liganded ER α may bind WRE elements directly and independently of β -catenin.

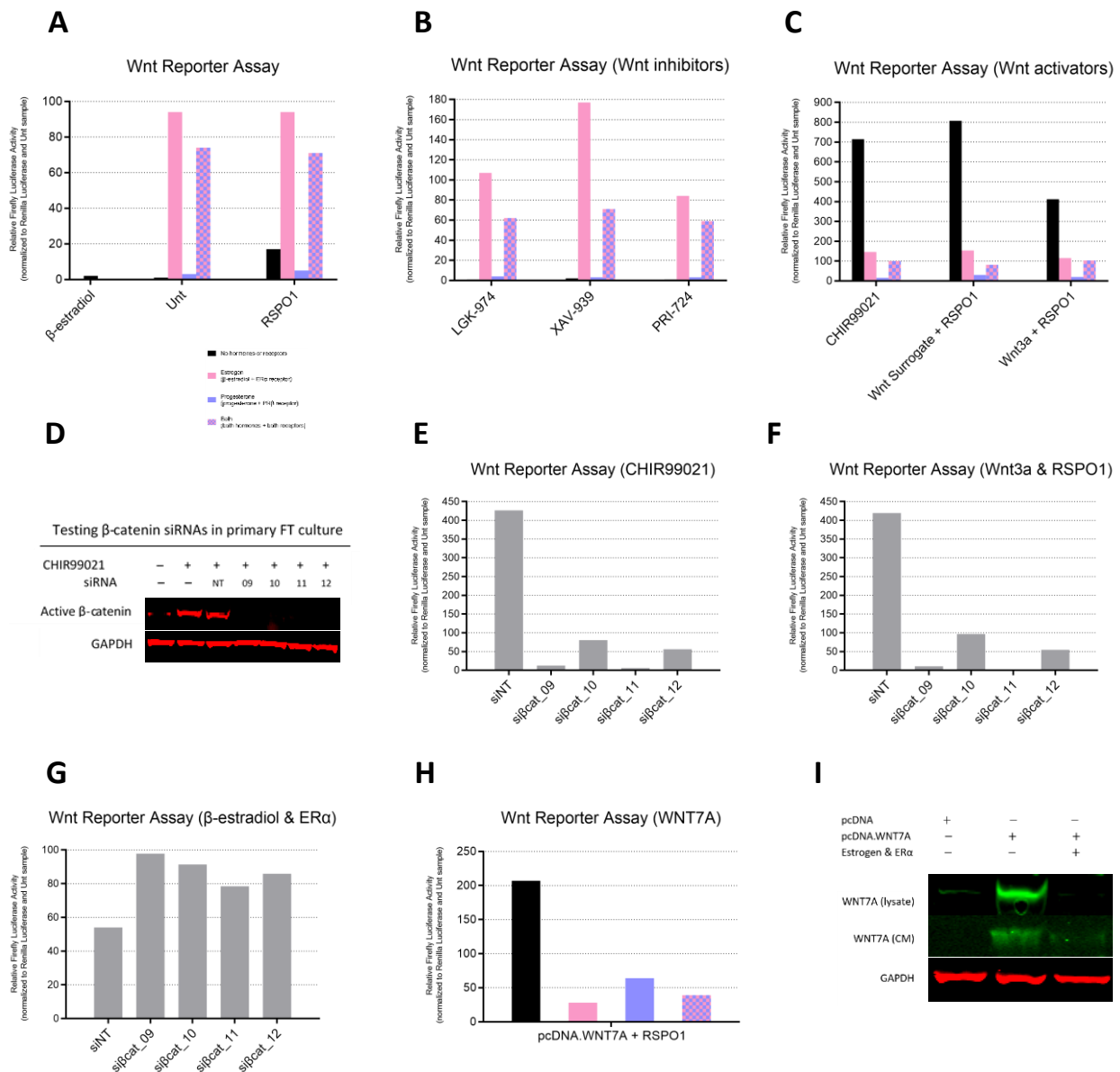


Figure 69: Estrogen activates transcription at WRE elements independently of the Wnt/ β -catenin pathway. (A) Estrogen robustly activates WRE elements. Progesterone antagonizes this. (B) Estrogen activates WRE elements even in the presence of LGK-974, suggesting this activation is independent of Wnt ligands. Estrogen-induced transcription of Wnt-target genes is unaffected by midstream (XAV-939) and downstream (PRI-724) inhibition of the Wnt pathway. (C) Estrogen counteracts elevated activation of Wnt signaling, reducing it to levels comparable with Estrogen-only treated samples. (D-F) β -catenin siRNAs effectively reduce protein level (D) and activity (E-F) of β -catenin. (G) Estrogen activates WRE elements independently of β -catenin. This activation is higher in the absence of β -catenin, suggesting competition between the pair. (H) Estrogen reduces WNT7A-induced Wnt signaling, to levels well-below Estrogen-treated samples. This hints that liganded ER may downregulate WNT7A protein directly. (I) Western Blot confirmation that liganded ER α downregulates WNT7A protein. CM is conditioned medium.

Estrogen (β -estradiol) was used at 100 nM. Progesterone at 1 μ M. Figures representative of two biological replicates. ER α and PR β were used at 200 ng per well for Wnt-reporter assays, and 1 μ g per well of a 6-well plate for Western Blotting.

To investigate this, Estrogen's ability to activate WRE elements was tested in the presence of β -catenin siRNA. These siRNAs were validated to effectively abolish β -catenin's protein level (Figure 69D) and activity (Figure 69 E-F). Interestingly, Estrogen's induction of transcription at WRE elements is independent even of β -catenin (Figure 69G). In fact, it's higher in the absence of β -catenin, suggesting the pair compete for binding to WRE elements.

The above data confirms that Estrogen can activate transcription of Wnt-target genes independently of Wnt signaling. *In vivo* studies also document that Estrogen suppresses Wnt7a, which counter-intuitively, is the activator of Wnt signaling. To test the relationship between Estrogen and WNT7A, the Wnt-reporter assay was used. Interestingly, Estrogen abolishes WNT7A-induced Wnt activation (Figure 69H), this time without independently activating Wnt signaling as seen in Figure 69C. It can be speculated that Estrogen inhibits transcription of WNT7A, but in the setting described above, WNT7A expression is driven by a general promoter (pcDNA.WNT7A) in a transfected construct. Hence, liganded ER α probably downregulates WNT7A at the protein level, and this is confirmed in Figure 69I. It is possible that Estrogen promotes expression of a factor that destabilizes WNT7A and leads to its degradation.

Estrogen's differential effects on WNT7A and Wnt signaling are interesting. They were confirmed here using the Wnt-reporter assay (Figure 69), which although useful, is not biologically relevant to the FT. Organoids offer a superior model to understand hormonal control of Wnt signaling and stem cell self-renewal. As confirmed by single cell transcriptomics, hFT organoids express ER α (Figure 70A) as seen in hFT tissue (Figure 70B). Estrogen treatment of organoids reveals an interesting phenotype of crippled, deeply staining organoids with extensive internal foldings and invaginations (Figure 70 C-D). This is reminiscent of differentiation signs that appear in FT organoids cultured for over a month (shown earlier, Figure 31), as well as in differentiated organoids of other tissues (Yin *et al.*, 2013).

The difference here is that Estrogen triggers these changes within 72 hrs. Characterisation of these organoids shows that Estrogen downregulates WNT7A at the transcriptional level, in addition to its suppression of WNT7A at the protein level as shown above (Figure 69 H-I). Moreover, downregulation of WNT7A is accompanied by suppression of Wnt-related markers (Figure 70E) and induction of ciliated cell markers (Figure 70F). From this data, we can conclude that Estrogen's activation of Wnt signaling (Figure 69 A-G) does not happen in organoids, but its suppression of WNT7A does take place (Figure 69 H-I) and triggers differentiation.

Although Estrogen's induction of differentiation in the oviduct has been documented (Chang *et al.*, 2019; Okada *et al.*, 2004), this is the first report identifying the molecular mechanism of differentiation, via suppression of WNT7A. Of note, these changes were observed upon 72 hr treatment of organoids with Estrogen. The proliferative phase of the menstrual cycle in humans, in which Estrogen levels peak, lasts for 7 days. Treatment of organoids for an equivalent period of time may precipitate even more pronounced differentiation. This is confirmed upon long-term Estrogen treatment of organoids (Figure 70G), which become completely differentiated and undergo major morphological changes in comparison to normal organoids. Ongoing work is focusing on characterising these completely differentiated organoids. I predict that WNT7A suppression alone (in matured organoids) is sufficient to trigger Estrogen-induced differentiation in the absence of Estrogen. Ongoing work is also testing this, by comparing differentiation in Estrogen-induced versus WNT-blocked organoids.

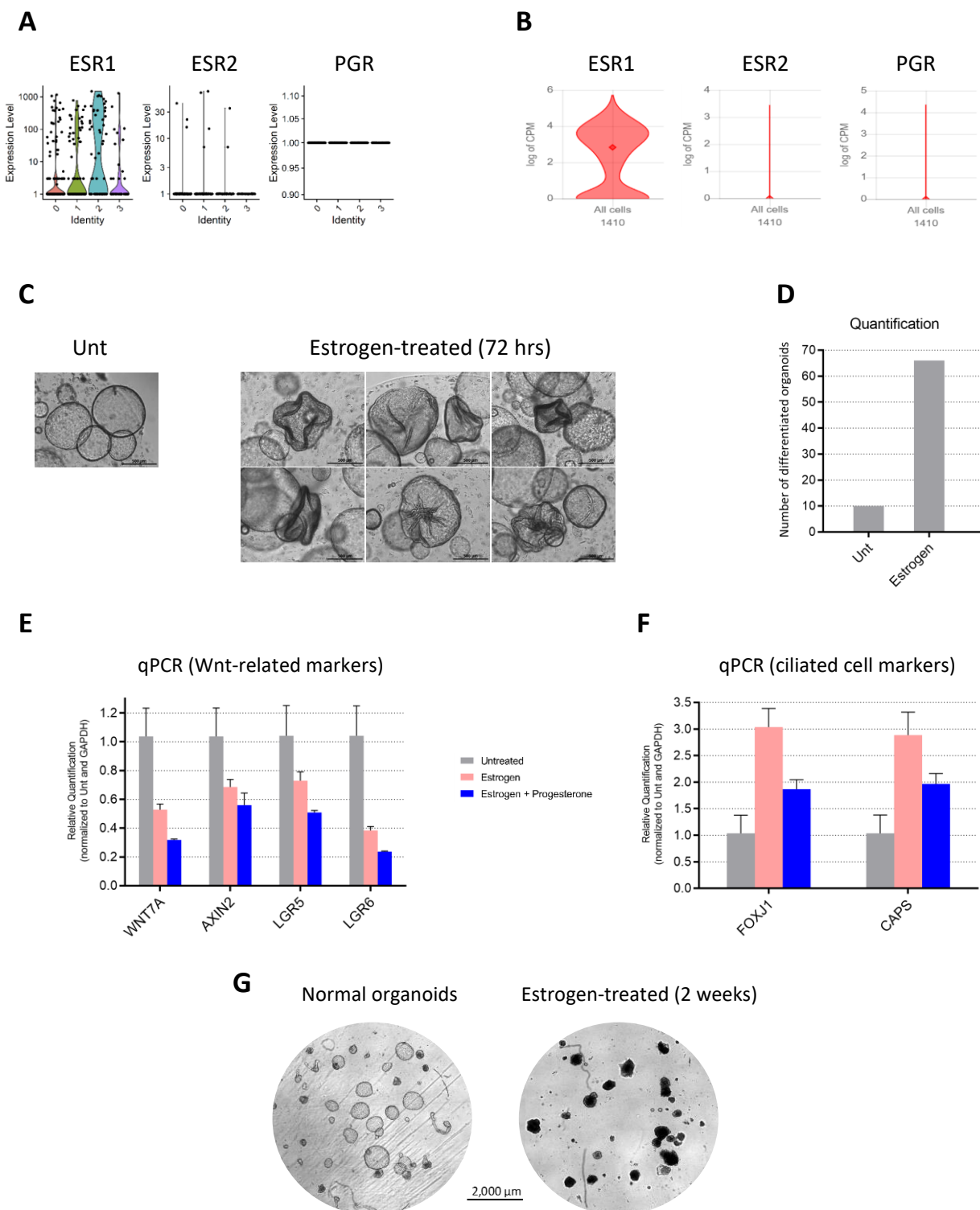


Figure 70: Estrogen suppresses WNT7A transcription and promotes organoid differentiation. (A) $ER\alpha$ (ESR1) is expressed in organoids. As expected, PR (also called PGR) is not expressed, as its expression is promoted by Estrogen. Organoids were not treated with Estrogen prior to scRNA-seq. (B) Expression of the 3 hormone receptors in hFT tissue. This mirrors expression seen in organoids. (C) Estrogen treatment triggers morphological changes in organoids, associated with differentiation. (D) Quantification of images in (C). (E) Estrogen treatment suppresses WNT7A transcription. This is accompanied by a reduction in markers of Wnt-activation. (F) Estrogen promotes differentiation of FT stem cells towards the ciliated cell fate. qPCRs in (E) and (F) are for samples shown in (C) and (D). (G) Estrogen treatment of organoids for 2 weeks produces fully differentiated organoids with major morphological changes.

8.4 Chapter Summary

The previous chapter identified an obligate requirement of putative FT stem cells for a Wnt ligand and RSPO1. In this chapter, single cell approaches identify WNT7A as the Wnt ligand cooperating with RSPO1 to drive organoid regeneration. WNT7A is the only expressed ligand in hFT organoids. This is consistent with *in vivo* developmental studies. Wnt-active cells are enriched for WNT7A expression, but it is not definitive yet whether the mode of signaling is autocrine or paracrine. The Wnt-active cluster is proliferative and expresses a number of cell cycle-associated genes. Presence of WNT7A⁺ cells, AXIN2⁺ and LGR5⁺ cells was confirmed in hFT tissue and organoids, as well as mOV tissue and organoids.

WNT7A protein signals in very close range, possibly through direct physical interaction between donor and recipient cells. This might explain why protein-based reagents of WNT7A are not functional *in vitro*. With few exceptions, WNT7A is a classic Wnt ligand, harbouring protruding thumb and finger domains for FZD engagement and featuring sites for glycosylation. FZD5 is the cognate receptor of WNT7A in hFT organoids, and its inhibition, much like WNT blocking, abolishes organoid regeneration. This is rescued by Surrogate Wnt, which has activity against a broad range of FZDs including FZD5, but not FZDs 3 and 6, suggesting FZD5 is the mediator of WNT7A-induced canonical Wnt signaling. LGR5 is the likely receptor for RSPO1 in hFT organoids. Its inhibition abolishes organoid regeneration, phenocopying withdrawal of RSPO1 from organoids. Like FZD5 inhibition, LGR5 inhibition is rescued by Surrogate Wnt and CHIR99021 treatment. Mouse oviduct organoids show a deviation from human organoid behaviour, exhibiting resistance to WNT ligand blocking, FZD5 inhibition and RSPO1 withdrawal.

Given the broad expression of FZD5 in Wnt-active as well as Wnt-inactive cells, it is conceivable to propose that FT stem cells and their obligate requirement for WNT7A and RSPO1, will express FZD5 and LGR5 receptors. The latter is the limiting factor in dictating multipotency under these conditions. Finally, this thesis confirms Estrogen's opposing effects on WNT7A (suppression) and Wnt signaling (activation). The latter does not happen in organoids. Estrogen downregulates WNT7A at the transcriptional and protein levels, triggering a remarkable phenotype of differentiation within few days. The implications of this regulatory loop are further explored in the Discussion chapter.

CHAPTER 9

SUMMARY & DISCUSSION

SUMMARY & DISCUSSION

Ovarian Cancer is the fifth most common cancer in women. Its dismal survival rate, which remained persistently low over decades, is driven by its most malignant histotype: High Grade Serous Ovarian Cancer. Also called HGSOC, it features ubiquitous TP53 mutations, low somatic mutational load, extensive CNAs and dysregulation in BRCA and HR repair pathways. HGSOC shares many features with basal-like breast cancer, and aside from early TP53 mutation, not much is known about tumour initiation. This, in part, is due to previous research efforts focusing on the ovarian surface epithelium as the presumptive origin of HGSOC, which is now accepted to be the FT.

The current working hypothesis of HGSOC initiation is that secretory cell outgrowths in the FT, called SCOUTs, acquire TP53 mutation and DNA damage, not necessarily in that order. This gives rise to non-invasive p53 signature lesions. These lesions progress to STICs and seed to the ovary or peritoneum by a yet unknown mechanism, leading to HGSOC. Upon advanced progression, HGSOC establishes metastatic colonies in more distant anatomical locations.

Major studies extensively profiled advanced HGSOC at the genomic, transcriptomic, epigenetic and CNA levels, but not early HGSOC. This is in part due to limitations in early detection and lack of availability of early stage samples. Hence, there is currently a blackhole in our understanding of the FT-specific processes between initiating TP53 mutations and advanced HGSOC. A major unaddressed question is the cell-of-origin of HGSOC, which has remained elusive.

STIC lesions display an unusual localization to the distal (fimbrial) end of the FT. This is a region enriched in stem-like cells. As discussed, SCOUTs have been described as outgrowths of stem-like cells blocked in differentiation capacity. Furthermore, tumourigenesis is an event where genetic instability meets hyperproliferation, and the pair provide a platform for an iterative process of mutagenesis and clonal selection. It is unclear how the TP53-mutant tumour-initiating cell acquires a growth advantage relative to other cells. Such growth advantage may not need to be acquired ectopically, if TP53 mutation can target an already actively and constantly cycling cell. In many tissues, longevity and constant cell division are characteristics unique to tissue-resident stem cells. Coupled with the unusually specific localization of STICs to stem cell-enriched regions of the FT, this provides a rationale for interrogating the role of stem cells in HGSOC. Alternatively, growth advantage could be acquired by early CNAs. Loss of heterozygosity at chromosomes 6p, 17p and 17q were associated with HGSOC (Dodson *et al.*, 1993), and may precede TP53 mutation. Chromosome 17p LOH was shown to associate with growth advantage and proliferative capacity in primary human breast cancer (Chen *et al.*, 1991).

The two hypotheses may not be mutually exclusive. For instance, chromosome 17q harbours the AXIN2 locus. LOH at this locus can ectopically activate Wnt signaling and stemness into an otherwise differentiated cell. Further accumulation of TP53 mutation may represent the loss of caretaker function and promote tumourigenesis in this ectopic stem-like cell. Irrespective of the two hypotheses, whether initiating TP53 mutations are targeting endogenous stem cells or differentiated cells that acquire stemness through early CNAs, this warrants further interrogation of these cells in HGSOC initiation.

Although identification of FT stem cells will be of benefit to several fields including regenerative medicine, FT pathologies and sexual and reproductive health, the ultimate aim in this thesis was to shed light on HGSOC initiation. Better understanding of HGSOC initiation may pave the way towards developing effective screening tools. Current figures show that 90% of HGSOC patients diagnosed at stage I survive the disease for over 5 years, compared with less than 10% by stage IV. Unfortunately, over two-thirds of patients are diagnosed at late stages, making the disease deadly. An effective screening tool is much needed in tilting the balance in favour of early detection. This will lead to better survival outcomes for many patients, even with current treatment regimens.

9.1 Fallopian Tube Organoid Culture

To address the lack of reliable models of the FT and its stem cells, I optimized the organoid culture technique for hFT tissue and made it amenable to cell isolation and characterisation. I confirmed its structural and molecular relevance using direct side-by-side comparisons with hFT tissue. Organoid regeneration is used throughout the thesis as a measure of self-renewal of FT stem cells. I used this optimized technique to conduct functional analyses on the requirements of stem cell renewal, the characteristics of FT stem cells and the nature of molecular signals that maintain them.

Work in this thesis builds up and optimises the hFT organoid culture method reported by Kessler *et al* (2015). Generally speaking, addressing stem cell requirements within organoid culture allows its regeneration from single cells. In this respect, FT stem cells are dependent on Wnt activation and TGF- β inhibition.

1. FT organoids as in vitro models of FT biology

In this thesis, I validated FT organoids and their relevance to FT biology. Organoids emerge from single cells. They do not mix when bulk cultured (Kessler *et al.*, 2015), although this does not rule out niche cells of one organoid supporting stem cells of another organoid in close proximity. Organoids form structures that have a hollow interior, are monolayered and exhibit internal foldings and invaginations, characteristic of hFT tissue. Organoids contain all the major differentiated cell types expected in hFT tissue, including PAX8+ secretory cells and TUBB4+ FOXJ1+ ciliated cells.

Using scRNA-seq of fresh hFT tissue, work in the host laboratory uncovered marked heterogeneity within secretory cells (Hu *et al.*, 2020). In this thesis, I validate some of the identified markers in hFT tissue. KRT7 and CAPS were seen to mark secretory and ciliated cells, respectively. I sought to extend this validation to organoids, which confirmed these observations. Rare cells expressing markers of both secretory and ciliated cells (KRT7+ CAPS+) were detected in hFT tissue, and were enriched in organoids. It was shown that ciliated cells were shown to emerge from a subpopulation of secretory cells (Gosh *et al.*, 2017). This, coupled with the observation that ciliated cells emerge a few days after establishment of organoid culture (this thesis; Kessler *et al.*, 2015), suggests FT organoids can serve as a tool for cell lineage and specification studies, as reported for other ASC-based organoids (Yin *et al.*, 2013).

For global comparisons, this thesis has generated the first dataset of transcriptomic profiling of hFT organoids at the single cell level. Ongoing work is focused on comparative transcriptome-wide analyses between hFT tissue and organoids.

2. Immuno-epithelial cells

In addition to secretory and ciliated cells, a basal cell type was documented in hFT tissue. The nature of these cells remains a bone of contention. A report has shown that these are CD44+ ITG6A+ epithelial cells that possess higher spheroid-formation efficiency, albeit only by 5-fold. In this thesis, I show that these cells are epithelial (EpCAM+) and secretory (KRT7+) in nature. They are proliferative (phospho-Histone H3+), basally-located and express immune-specific marker CD45. Further work in the host lab has shown these cells to be lymphocyte-like, expressing CD3, CD69 and CD103 (Hu *et al.*, 2020).

Free-floating EpCAM+ CD45+ cells were detected in HGSOC ascites, co-migrating with TP53-mutant tumour cells. As described in this thesis, one study finds these cells to be proliferative, marked by CD44 and other myeloid and lymphoid markers, but stain negatively for epithelial keratins

(Sukhbaatar *et al.*, 2017). It is not known how these cells arise, but a hypothesis postulates that they are the product of an unsuccessful phagocytic process, leading to the fusion of a CD45+ macrophage with an EpCAM+ tumour or non-tumour cell. This was shown in ovarian carcinoma, where the immune-epithelial cells show increased migratory phenotype (Ramakrishnan *et al.*, 2013), drug resistance (due to expression of SIRT1, ABCA1 and BCL2) and an EMT phenotype (Akhter *et al.*, 2018). It is also suggested that the tumour cell acquires a number of haematopoietic receptors due to this fusion event, such as CXCR4/CXCL12, which contribute to the acquisition of the migratory phenotype *in vivo* (Ramakrishnan *et al.*, 2013).

It is important to note, although this fusion event can lead to reprogramming and stemness of an epithelial or tumour cell (Cowan *et al.*, 2005), this is thought to happen in response to inflammation, elicited by already formed carcinogenesis. As such, it hypothesizes a mechanism for HGSOC progression (Akhter *et al.*, 2018), not HGSOC initiation. However, ovulation is known to induce damage and inflammation in distal FT (fimbrial) cells (Bahar-Shany *et al.*, 2014; King *et al.*, 2011). By extrapolation, if for unknown reasons this fusion event takes place in a fimbrial cell and promotes CNAs in it, before or after acquiring TP53 mutation, this may represent the acquisition of growth advantage required to promote tumourigenesis. As mentioned, basal CD44+ FT cells were shown to have a 5-fold increase in forming epithelial spheroids containing differentiated FT cells (Paik *et al.*, 2012). This thesis and the work of the host lab (Hu *et al.*, 2020) has shown these basal CD44+ cells to be the immune-epithelial cells, suggesting they do acquire a growth advantage. Thus, unless proven otherwise, an open mind should be kept about a possible role for immune-epithelial cells in HGSOC initiation.

Finally, it is intriguing that these cells are also present in hFT and mOV organoids. By FACS quantification, I see that these cells are exceedingly rare (0.1%) and as such, do not represent organoid-forming stem cells. Their disappearance in later passages suggests lack of maintenance. Future work may focus on identifying chemokine or immune-supporting factors that can promote survival and expansion of these cells for functional studies.

3. KRT17+ cells

Single cell work has identified a novel cluster of cells in hFT tissue, marked by KRT17. In this thesis, I confirm the robustness of this observation in hFT tissue and organoids. Other marker genes of this cluster, including MHC class II genes, suggest its involvement in FT immune biology and perhaps HGSOC tumour immunology. Unlike immune-epithelial cells, KRT17+ cells are not rare, and are persistent upon organoid passage. Thus, the organoid protocol described in this thesis can be harnessed in genomic engineering of organoids, to facilitate marking, isolation and characterisation

of this cell type. This thesis did not explore whether KRT17+ cells associate with Wnt-active cells. Ongoing work, particularly the one described in Figures 52-53, should address this relationship.

4. KRT5+ cells

KRT14 and KRT5 mark basal cells, stem cells or both in various tissues. They are generally co-expressed, but their exact function remains unknown. The hFT is largely luminal in nature, and I confirm most cells express luminal marker KRT8. However, rare KRT5+ and KRT14+ cells were detected, which are interestingly distinct. The thesis validates KRT5+ cells in hFT tissue and organoids, and nullifies the presence of KRT14+ cells, at least at the protein level. It is possible KRT14 mRNA is present in these cells, but unidentified endogenous mechanisms may suppress its translation.

Of interest is the fact that the pair are not co-expressed as seen in other tissues. Furthermore, KRT5 is a basal cell marker, but here it is detectable in the largely-luminal FT tissue. This was documented in mammary tissue (Nguyen *et al.*, 2018).

Since KRT5+ cells and the transcriptional KRT14+ cells are enriched in KRT17+ cluster, the above described future work should address their role in this context. In this thesis, I attempted to introduce virally-integrated fluorescence-based reporters of KRT5 expression in hFT organoids, using a published construct (Axlund *et al.*, 2013). These set of experiments were unsuccessful. However, for the purposes of this thesis, KRT5+ cells did not overlap with Wnt-active cells. Since functional work identified Wnt signaling as essential for self-renewal of stem cells in organoids (section 9.2), it can be deduced that KRT5+ cells are not stem cells under these conditions. However, Progesterone treatment induces KRT5+ cells in luminal breast cancer (Axlund *et al.*, 2013), and as such it cannot be ruled out that these cells may have a role to play as facultative ASCs in hormone-induced regeneration.

9.2 Fallopian Tube Stem Cells

As discussed in section 3.3.3, there is reliable evidence from *in vivo* studies in the oviduct, as well as other FRT tissues, that distally located FT stem-like cells may be Wnt-active. Interrogation of the role of FT stem cells in HGSOE initiation has not been possible, for two reasons. Firstly, reliable models of the FT, the organ from which HGSOE initiates, are largely lacking. In this thesis, I improve the reported method for hFT organoid culture and show its relevance to hFT tissue. Transcriptomic analysis of tissue and organoid at the single cell level is pending. Secondly, FT stem cells have not been identified to date, precluding investigations into their potential role in HGSOE initiation.

To identify FT stem cells, I use FT organoids as a means to reconstruct the FT stem cell niche *in vitro*. This was necessary, as work in section 6.2.1 identified major limitations in traditional 2D culture, even when derived from patient material. 2D culture is not tractable and cannot propagate epithelial cells beyond 6 weeks. Inherent biases in dimensionality, as well as poor medium formulation, prohibit activation of pathways necessary for stem cell maintenance. Single cell work from our lab has shown that, upon 2D culture of patient-derived cells, most cells transition within 24-48 hrs to become WNT7A+ cells (Hu *et al.*, 2020). There is loss of ciliated cells, and the heterogeneity seen in the secretory cell compartment is abolished. I report in this work that putative stem cells (Wnt-active cells) are also lost, consistent with loss of organoid-forming capacity. Taken together, this thesis argues that 2D culture methods are not a suitable model of FT biology or FT stem cells.

1. WNT7A-FZD5 signaling axis maintains FT stem cells

The central hypothesis of this thesis is that Wnt-active cells are the stem cells of the FT. Functional work has shown that Wnt signaling is essential for self-renewal of FT stem cells in organoids. Inhibition of Wnt signaling, either by withdrawal of RSPO1 or blocking endogenous Wnt secretion abolishes stem cell renewal. Functional experiments suggest Wnt-active cells drive organoid regeneration. To fully characterise these, stable fluorescent-based Wnt-reporter organoids were generated and characterized. Testing whether organoid formation is restricted to Wnt-active cells in hFT organoids has not been shown in this thesis, and is currently ongoing. However, single cell analysis of Wnt-reporter organoids identifies WNT7A as the only expressed canonical Wnt in hFT organoids, and thus the only canonical target of Wnt blocking agents which abolish organoid regeneration. I propose that RSPO1 and WNT7A are essential niche signals for FT stem cells. It is evident that WNT7A is derived from dedicated WNT7A+ cells in the FT epithelium. This is consistent with developmental studies. The source of RSPO proteins *in vivo* is less clear, and future work should investigate this source, which could possibly be mesenchymal.

By extrapolation, FT stem cells should express the WNT7A and RSPO1 receptors. Single cell data point to FZD5 as the WNT7A receptor in organoids, and functional work confirms this. Like WNT blocking, FZD5 inhibition using antibodies targeting FZD5-CRD domain abolishes organoid regeneration. These antibodies have activity against FZD8, which is not expressed in organoids, suggesting the effect seen is due to FZD5 inhibition. This effect can be rescued by Surrogate Wnt, which activates canonical Wnt signaling through FZD5 and other FZDs, but has no activity against FZD3 and FZD6; the other two FZDs expressed in hFT organoids. This further substantiates FZD5 receptor as the transducer of the WNT7A signal.

2. RSPO-LGR5 signaling sustains the WNT7A-FZD5 signaling axis in FT stem cells

RSPOs potentiate Wnt signaling by inhibiting RNF43/ZNRF3, which are ubiquitin ligases that target FZD receptors for destruction. Although RSPOs are, in theory, potentiators of Wnt signaling, their absence causes high turnover of FZD receptors, effectively shutting down Wnt signaling. I document this in hFT stem cells, which are inhibited by RSPO1 withdrawal, even in the presence of endogenous WNT7A and FZD5. It can be speculated that RSPO and its receptor prevent the quick turnover of FZD5 on the cell surface of FT stem cells, and allow persistent activation of Wnt signaling.

Functional testing attributes organoid-forming ability to 3-5% of cells. Single cell work shows that FZD5 expression is broad spectrum and not restricted to a minority of cells. In addition, FZD5 expression is not specifically enriched in Wnt-active cells. Therefore, although FZD5 is essential for renewal of stem cells, I hypothesize that FZD5+ LGR- cells do not possess stem cell character, and that expression of the RSPO1 receptor within the FZD5+ compartment marks multipotent cells, at least in the setting described.

Biochemical evidence has shown that RSPO's are ligands to LGRs 4, 5 and 6 (de Lau *et al.*, 2011). Like FZD5, LGR4 expression in hFT organoids is diffuse, not restricted to Wnt-active cells and expressed in many cells as opposed to the minority of cells with organoid-forming capacity. A number of studies have investigated the expression of LGR5 and LGR6 in the FT context (Chapter 8, Table 15). Few studies reported lack of LGR5 expression in hFT tissue and organoids. Here, I confirm LGR5 expression using RNA FISH, and scRNA-seq also reliably captures LGR5+ cells in hFT tissue (Hu *et al.*, 2020). Both of these are single cell approaches, and are superior to microarray-based approaches, which were employed to negate LGR5 expression in the hFT (Kessler *et al.*, 2015). Using functional analyses, I also show that anti-LGR5 IgG that was shown to block RSPO1-LGR5 interaction (de Lau *et al.*, 2011) abolishes organoid regeneration, phenocopying RSPO1 withdrawal from

organoids. This is rescued by downstream activation of Wnt signaling, but not by LGR6 which the antibodies do not bind to.

A limitation of this preliminary result is that the anti-LGR5 antibody was not validated in-house. A number of studies have conducted these validations in a background of LGR5 overexpression in cell lines (Kemper *et al.*, 2012; de Lau *et al.*, 2011). The anti-LGR5 antibody (clone 4D11) has been employed for isolation of LGR5+ cells from cell lines, but is not effective in isolating LGR5+ cells from FT organoids (this thesis, data not shown). However, when compared to its isotype control, the anti-LGR5 IgG acts as an effective inhibitor of RSPO1-LGR5 binding in live organoids. To my knowledge, this is the first report that documents such an inhibitory effect, which might qualify this reagent as a useful anti-LGR5 inhibitor in organoid and *in vivo* research. Ongoing work is validating this reagent further, to ascertain the specificity of its binding to RSPO1 and confirm its downregulation of Wnt signaling in organoids.

To fully characterize LGR5+ cells, ongoing work is focused on conducting single cell analysis of more Wnt-reporter organoid lines established from multiple patients. Furthermore, bulk RNA-seq of Wnt-active cells, as well as of organoids overexpressing β -catenin, should enable identification and further characterisation of LGR5+ Wnt-active cells, which may hold the key to biomarking of FT stem cells. Overall, this thesis proposes the following model for FT regeneration, shown in Figure 71.

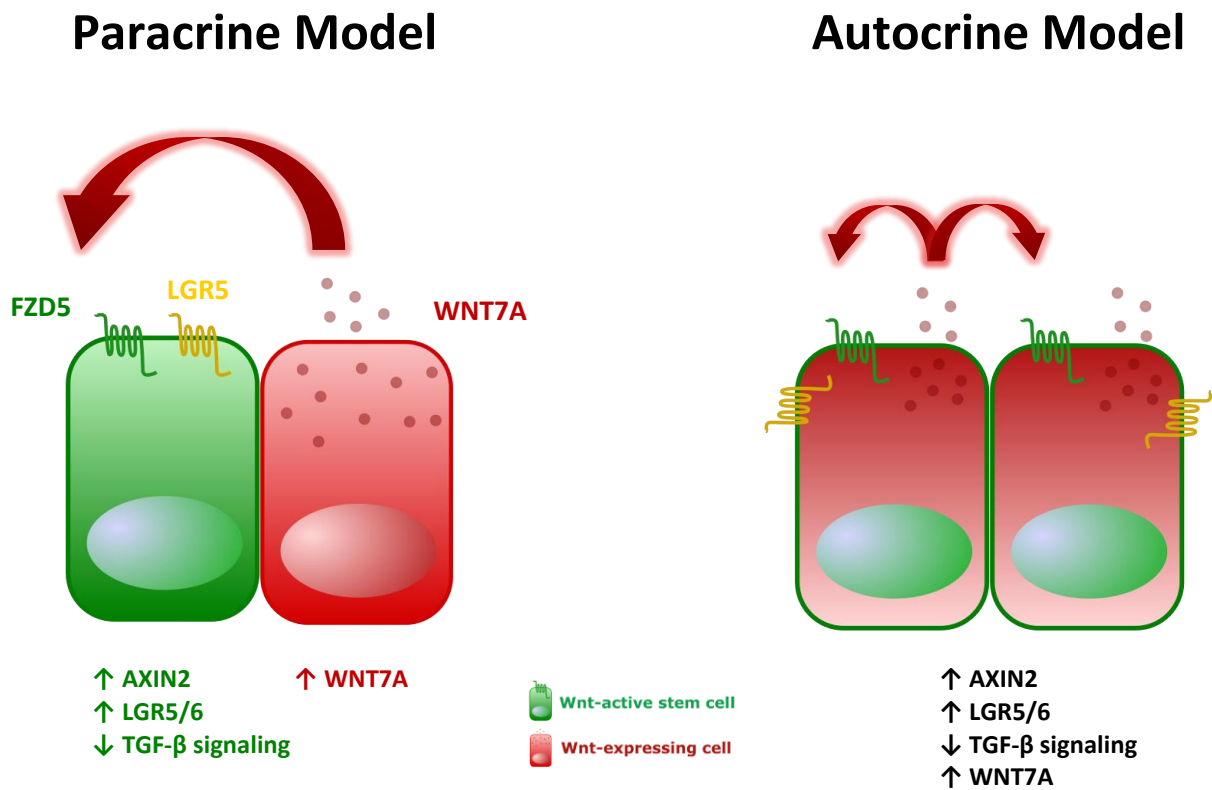


Figure 71: Proposed model of maintenance of FT stem cells. In the Paracrine model, WNT7A+ cells constitute the niche for Wnt-active FT stem cells. WNT7A ligand is transferred between niche cells and stem cells in very short range, possibly through direct physical contact. The ASC niche also signals RSPO and secreted antagonists of TGF- β signaling, but the sources of these in the in vivo setting is unclear. In the Autocrine model, FT stem cells secrete and utilize their own WNT7A, as seen in hair follicle stem cells (section 1.4.5).

Data in this thesis lend more support to a paracrine mode of stem cell maintenance, as RNA FISH points to WNT7A+ and AXIN2+ cells being distinct but juxta-posed in hFT tissue. This is also the case in organoids, although these two cell types may overlap. 2D-cultured WNT7A+ cells are unable to generate organoids after 2 weeks in 2D culture. Enforcing Wnt activation on 2D-cultured FT cells abolishes WNT7A expression. scRNA-seq of fresh vs 2D-cultured hFT cells shows loss of Wnt activation markers but gain of WNT7A expression upon 2D-culture (Figure 55D). These and other observations suggest that the two cell types are distinct.

The Autocrine model is supported by scRNA-seq of Wnt-reporter organoids, in which Wnt-active cells show enrichment of WNT7A expression. As discussed, this may represent a transitional cell state between Wnt-active stem cells and differentiated WNT7A+ cells.

3. *In vitro* activation of Wnt signaling (Wnt3a CM)

Wnt signaling is critical for ASC maintenance in various organs (section 1.4). In this respect, *in vitro* activation of Wnt signaling is necessary for stem cell maintenance in organoids from many tissues. Although few studies employ the GSK-3 β inhibitor CHIR99021, this is not ideal as the inhibitor targets other cellular kinases. GSK-3 β itself is one of the most promiscuous kinases, and its artificial inhibition deviates organoids from the character of the tissue they derive from. It is best to rely on biological activators of the pathway for physiological relevance of tissue modelling, as well as for stem cell-based research.

Wnt3a protein is widely used, often in organoids derived from tissues where Wnt3a plays no role *in vivo*. Wnt3a is used in the format of serum-containing medium conditioned from L cells overexpressing Wnt3a. This was originally used in the first report on organoid culture method (Sato *et al.*, 2009), and was a reasonable supplement as Wnt3a was identified as the endogenously-secreted Wnt ligand in the intestinal epithelium, *in vivo* and in organoids. However, serum-containing mediums are undefined and confound investigations into the functional requirements of stem cell self-renewal. Studies document serum to promote differentiation and have negative effects on organoid culture (Huch *et al.*, 2015; Seino *et al.*, 2018).

In this thesis, I note that serum encourages growth of mOV organoids in a stem cell-independent manner. Furthermore, I document that serum negatively impacts self-renewal of hFT stem cells. Single cell transcriptomic analysis shows Wnt3a is not expressed in fresh (Hu *et al.*, 2020) or organoid (this thesis) FT cells. Unlike intestinal organoids, growth inhibition of FT organoids is not rescued by Wnt3a upon blocking endogenous WNTs, suggesting Wnt3a has no activity. Hence, although widely used in almost all published studies on FT/oviduct organoid culture (Löhmußaar *et al.*, 2020; Zhang *et al.* 2019; Kessler *et al.*, 2015; Hoffmann *et al.*, 2020; Kessler *et al.*, 2019), there is no basis for use of Wnt3a conditioned medium in organoids from this tissue.

In reported studies, the two main sources of serum in FT organoids are RSPO1 and Wnt3a. To avoid these, purified and highly stable RSPO1 is available commercially or can be expressed and purified in-house, as shown in this thesis. As for a Wnt source, a recent and potent alternative is Surrogate Wnt (Janda *et al.*, 2017) or its next generation derivative, NGS Wnt (Miao *et al.*, 2020). Both were shown to support growth of organoids from a number of tissues, including kidney, colon, hepatocyte and breast. This thesis appends the FT to this growing list of organoid systems.

Although currently available Surrogate Wnts are broad spectrum, they are sufficient for organoid expansion in serum-free settings. Research is underway to generate NGS Wnts targeting

specific FZD receptors, and some have already been described (Tao *et al.*, 2019). Overall, in the absence of a Wnt source, it is perhaps most physiologically relevant to rely on endogenously-expressed Wnts for organoid expansion. I document in this thesis that hFT organoids robustly express endogenous WNT7A and require no further Wnt sources for maintenance of stem cells *in vitro*.

4. Human Fallopian Tube versus Mouse Oviduct

Mouse oviduct organoids were employed in this thesis to scrutinize cross-species differences. Currently, *in vivo* lineage tracing in mice is the gold standard for evidencing stem cell character of a cell population, and generally correlates well with human biology. *In vivo* studies driving TP53 and other mutations in oviduct epithelium are currently in use as murine-based models of HGSOC (Kim *et al.*, 2018). Therefore, close biological resemblance between the hFT and mOV is essential for accurate modelling of FT biology and HGSOC initiation, progression, *in vivo* drug screening and other aspects of tumour biology.

This thesis documents major differences in renewal of human and mouse stem cells. As above-mentioned, regeneration of mOV organoids is unaffected by TGF- β signaling. Oviduct stem cells persist in self-renewal and organoid formation despite withdrawal of RSPO or FZD5 inhibition. Blocking endogenous Wnts does not inhibit organoid regeneration here, despite depleting Wnt-active cells to 0%, as confirmed by Wnt-reporter organoids. Ectopic Wnt activation does not increase organoid formation, despite increasing proportion of Wnt-active cells by 10-fold, as confirmed in Wnt-reporter mOV organoids. Isolated Wnt-active cells in mOV organoids do not display enhanced organoid regeneration under a range of tested conditions. I conclude from these observations that Wnt signaling is dispensable for mOV stem cells, and that Wnt activation markers do not biomark oviduct stem cells. This lack of Wnt requirement may explain why *in vivo* studies blocking Wnt signaling in the oviduct have not shown a striking phenotype (Gosh *et al.*, 2017) similar to the severe phenotypes observed upon Wnt inhibition in Wnt-dependent organs (Korinek *et al.*, 1998; Kuhnert *et al.*, 2004).

However, mOV organoids are inhibited by XAV-939 and PRI-724, inhibitors of Wnt signaling at the β DC complex and β -catenin levels, respectively. It is unclear how this observation can be reconciled with the above functional data from organoid regeneration and Wnt-reporter organoids. Finally, Notch inhibition significantly reduces but does not abolish regeneration of mOV organoids. This is consistent with reports showing increased differentiation of stem cells to ciliated cells upon Notch inhibition (Xie *et al.*, 2018; Kessler *et al.*, 2015).

It is important to point out that organoid formation capacity in mOV organoids, although slightly higher than human, is restricted to 5-10% of cells. Mouse oviduct organoids can still be employed for effective characterisation of these putative stem cells. However, in a stark contrast to hFT organoids, Wnt signaling does not play a major role here.

9.3 Hormonal Regulation of WNT7A & Wnt signaling

In Chapter 2, we explored the role of Estrogen in regulating WNT7A and Wnt signaling during development. A number of studies suggest that Estrogen activates Wnt signaling and proliferation (section 2.3.2), while other reports note a negative relationship between Estrogen and WNT7A (Wagner & Lehmann, 2006; Li *et al.*, 2001). Work in this thesis has confirmed both observations in Wnt-reporter assays. However, in FT organoids, only Estrogen's suppression of WNT7A is confirmed, both at the transcriptional and protein levels, and leads to differentiation. Estrogen's activation of transcription of Wnt-target genes does not happen in organoids.

Given WNT7A is the driver of Wnt signaling, Estrogen's opposing effect on Wnt signaling on the one hand, and WNT7A on the other hand, appear contradictory. To reconcile these observations, it is important to note that these two are different processes. With some exceptions (eg. hair follicles), these processes also happen in distinct cells that are generally juxtaposed to each other (Sato *et al.*, 2011; Reyfman *et al.*, 2018). Activation of the Wnt pathway may suppress WNT7A expression, independently of Estrogen (shown earlier, Figure 59C). In this case, Estrogen's downregulation of WNT7A mRNA and protein may be mediated by activation of Wnt signaling. However, data from hFT organoids argues against this, and proposes Estrogen to suppress WNT7A transcription and protein function by a yet unidentified factors.

On Estrogen's opposing effects on WNT7A and Wnt signaling, another school of thought argues that Wnt ligands themselves are Wnt target genes (Wang *et al.*, 2010). This is seen in the intestine, where the source of epithelial Wnts, Paneth cells, are Wnt-active (Yin *et al.*, 2013). Another example is upregulation of Wnt4 expression during Estrogen-dominant phase of the menstrual cycle, in the rat pituitary gland. This is reduced in ovariectomized rats (lacking Estrogen), but rescued upon Estradiol supplementation (Miyakoshi *et al.*, 2009). In hippocampus neurons cultured from rat brains, Estradiol supplementation activates Wnt5a and Wnt7a expression (Quintanilla *et al.*, 2005). Therefore, Estrogen may upregulate both Wnt7a expression and Wnt signaling. However, data in this

thesis does not support this model for the FT, and argues for Estrogen as a WNT7A suppressor. A graphical summary of the proposed model is shown in Figure 72.

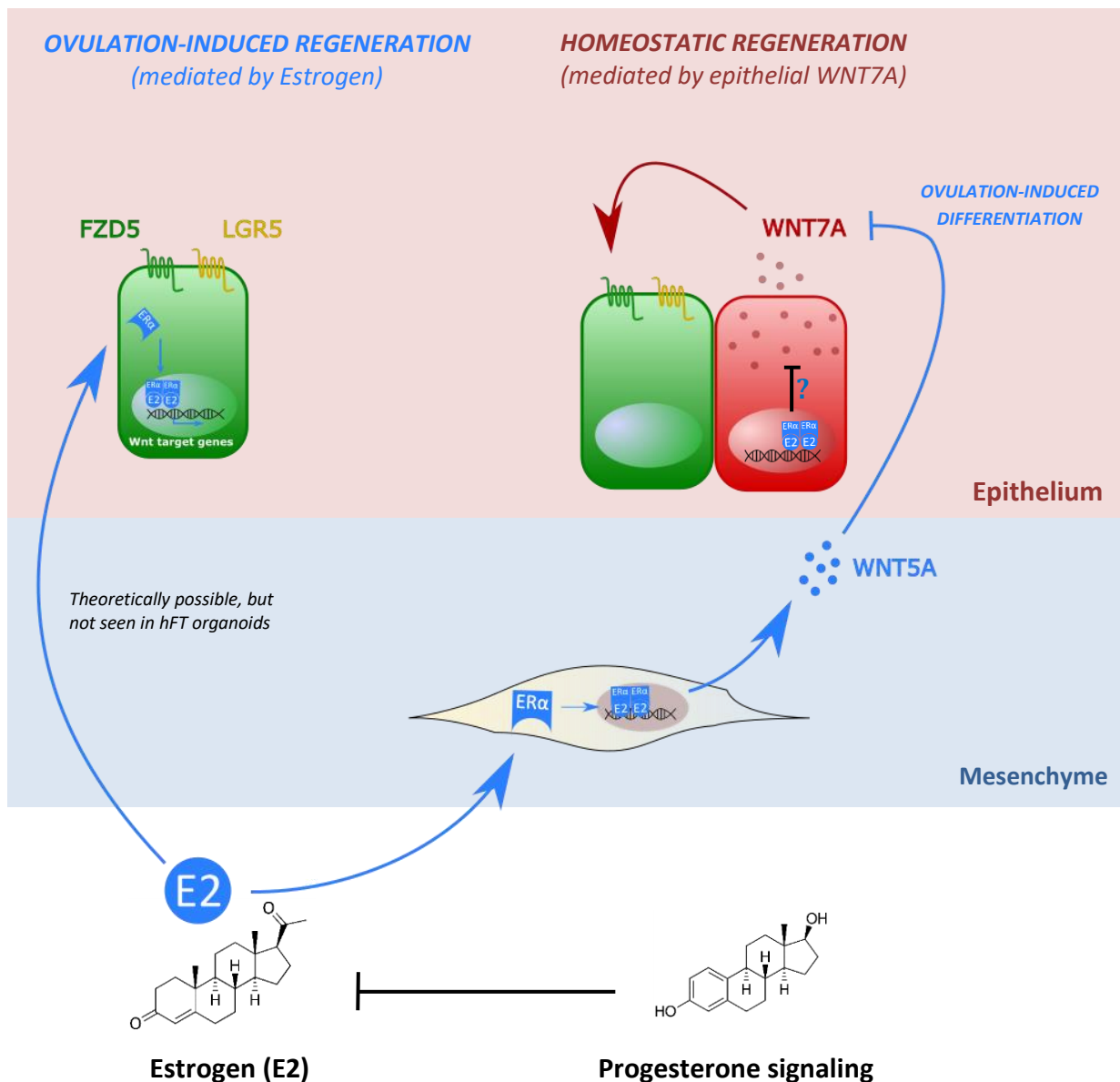


Figure 72: Proposed model of Estrogen's regulation of FT stem cells. The default route of FT regeneration is through epithelial WNT7A, which activates Wnt signaling and stem cells. On the one hand, Estrogen disrupts this loop by suppressing WNT7A. This could be through a yet unknown factor (shown as a question mark) in ER+ WNT7A+ cells, or via WNT5A secreted from ER+ fibroblasts (see section 2.3.4). Estrogen's suppression of WNT7A is seen in hFT organoids, and triggers differentiation.

On the other hand, Estrogen can promote regeneration by activating Wnt-target transcription independently of Wnt signaling and Wnt ligands. This regeneration is ovulation-linked, as Estrogen levels peak around ovulation during the proliferative phase of the menstrual cycle. This was documented in this thesis in Wnt-reporter assays, but is not seen in hFT organoids. This may indicate that Wnt-active cells in hFT organoids do not express ERα.

1. Limitations of the proposed model

Of note, *in vivo* studies have confirmed the presence of ER+ Wnt5a+ fibroblasts that regulate epithelial Wnt7a (section 2.3.4). This was shown in the endometrium, which shares similar embryonic origins and expresses epithelial Wnt7a as seen in the oviduct. scRNA-seq of hFT tissue (Hu *et al.*, 2020) was performed on post-menopausal patients (in which ovaries cease to secrete Estrogen). Thus, validating the presence of ER+ WNT5A+ mesenchymal cells was not possible. This might require samples from patients with specific characteristics, including premenopausal age and lack of use of contraceptive pills, or at least use of contraceptive pills not involving progesterone, so that transcriptomic characteristics of ER+ fibroblasts can be seen.

Another inherent limitation is the notion of modelling Estrogen's complex interactions *in vitro*. Cells are known to elicit different responses to Estrogen depending on its local concentration, which varies significantly and dynamically during the menstrual cycle (shown earlier, Figure 13A). An example of this was discussed in section 2.3.3, whereby dynamic maternal Estrogen levels do not affect fetal FRT development. However, upon maternal consumption of DES (an Estrogen analogue), FRT malformations are seen in new-born mice, as well as in adult female humans exposed to DES *in utero*. The cyclic nature and physiological doses of Estrogen *in vivo* are difficult to recapitulate *in vitro*, and the phenotypes observed in Estrogen-supplemented organoids, 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*.

9.4 Unanswered Questions & Future Directions

Work in this thesis has progressed our understanding of the molecular nature of FT regeneration. Findings were shown in FT organoids, a superior model of hFT tissue. Nonetheless, *in vivo* lineage tracing and cell / gene ablation studies are the gold standard in conclusively evidencing the findings reported here. However, if mouse oviducts *in vivo* behave as predicted by mOV organoids, the inter-species differences between hFT and mOV present a challenge in modelling hFT biology. The below discussion explores future directions in mouse studies by assuming such differences are not profound. Addressing the inter-species difference is explored in section 9.4.6.

1. Lineage tracing of FT stem cells

I propose future direction should focus on a number of studies. Firstly, it is crucial to confirm that Wnt-active cells are the FT/oviduct stem cells. Lgr5 lineage tracing has negated a role for Lgr5+ cells (Ng *et al.*, 2014; Seishima *et al.*, 2019). However, the oviduct is a low turnover tissue. As discussed (section 3.3.3), low turn-over tissues such as the liver, pancreas and kidney express Lgr5 only upon injury-mediated repair. In fact, lineage tracing in the liver has shown that Axin2 marks the homeostatic multipotent ASCs, where Lgr5 failed to do so (Wang *et al.*, 2015). In light of this, as well as studies showing that Axin2 marks homeostatic ASCs in the endometrial (Syed *et al.*, 2020) and vaginal (Ali *et al.*, 2020) epithelium, I propose Axin2 lineage tracing as an alternative strategy for marking Wnt-active cells.

Conditional on success of the above strategies, it is important to be cautious in extrapolating findings to hFT biology. As noted, LGR5 expression in hFT tissue and organoids is robust, and LGR5 plays a critical role in renewal of FT stem cells in organoids. LGR5 lineage tracing of hFT organoids in an *in vivo* setting should be envisaged. Alternative strategies that can enable this are explored in section 9.4.6.

2. Molecular maintenance of FT stem cells

Furthermore, essentiality of WNT7A to oviduct homeostasis could be tested by conditional and inducible deletion of WNT7A *in vivo*. This may not produce a phenotype, as extra-epithelial sources of Wnt ligand could be involved, as was shown for other tissues (Degirmenci *et al.*, 2018; Shoshkes-Carmel *et al.*, 2018). A likely candidate here is WNT4, which is expressed in a subset of fibroblasts as seen in hFT tissue single cell data. In this thesis, WNT4 constructs were used to express and validate WNT4 protein (data not shown). Ongoing work is testing whether WNT4 can rescue regeneration of WNT-blocked organoids.

An alternative strategy for evidencing the WNT7A-FZD5 signaling axis *in vivo* is via FZD5 deletion, as FZD5 is the only expressed canonical Wnt receptor (in tissue and organoids) and will be critical for Wnt activation. Importantly, such *in vivo* ablation studies should be conducted in the absence of Estrogen signaling, which can promote FT regeneration independently of WNT7A-FZD5 signaling. I suggest these studies to be undertaken in ovariectomized mice or ER α KO mice.

3. Estrogenic regulation of FT stem cells

A number of unanswered questions surround the role of Estrogen in FT stem cell biology. The observation that liganded ER α downregulates WNT7A protein is intriguing. Using co-

immunoprecipitation approaches, future work should address whether ER α binds to WNT7A directly and, if not, whether WNT7A binds an Estrogen-induced factor. The latter can be conducted using tagged-WNT7A constructs, one of which was validated in this thesis (WNT7A-V5) but seen to be weaker at Wnt signaling activation compared to the non-tagged construct (data not shown).

Furthermore, Estrogen exerts its functions through genomic and non-genomic interactions (Deroo & Korach, 2006). A big part of the latter is performed by liganded ER α that is tethered to the plasma membrane, modifying and activating a number of intracellular targets (Revankar et al., 2005). A palmitoylated C451 residue is important for this tethering. As such, Estrogenic inhibition of WNT7A can be investigated using C451A mutant ER α , in which membrane-associated ER α functions are abolished (Gagniac et al., 2020). This may be conducted *in vitro*. Alternatively, WNT7A levels were observed to decrease in mice during the Estrogen-dominated phase of the estrous cycle. Comparing normal female mice with C451A- ER α mice may be a good first step in establishing the nature of the relationship between Estrogen and WNT7A.

4. Towards complex FT organoid cultures

The organoid model presented in this thesis is purely epithelial. Optimisation of complex organoid co-culture methods involving fibroblasts or/and other microenvironmental components could help increase the relevance of the culture system to the FT stem cell niche. As explored (section 3.4.1), proof-of-principle investigations have shown that mesenchymal, immune, stromal and other microenvironmental components can be individually or collectively co-cultured with epithelial organoids, and yield complex and macro-sized tissue structures that are self-organizing and reflective of tissue-of-origin (Well, 2020, unpublished data; Koehler, 2020, unpublished data).

An early benefit of this complex organoid culture would be to enable investigating the crosstalk between ER $^{+}$ fibroblasts and Wnt-active/WNT7 $^{+}$ epithelial cells (Figure 72). Such a culture system can also investigate mesenchymal sources of WNT ligands, RSPOs and TGF- β regulators *in vitro*, and the nature of extra-epithelial signals that regulate FT regeneration. Given the major differences identified between human and mouse organoids, complex human-based organoid culture systems can be an innovative solution to dissect hFT biology in a near *in vivo* format without relying on mouse studies.

5. Role of FT stem cells in HGSOc initiation

The identification of FT stem cells will find applications in various fields. As emphasized, the motivation in this thesis is to shed light on HGSOc initiation. Findings in this thesis require further *in vivo* confirmation before one can begin to look at the role of FT stem cells in HGSOc initiation.

Studies have shown that targeting HGSOc's initiating mutation (in TP53 gene) to the oviduct secretory cell compartment is not sufficient for instigating STIC formation and subsequent HGSOc (Perets *et al.*, 2013). If FT stem cells are indeed Wnt-active, their role in HGSOc initiation can be tested by targeting initiating TP53 mutation specifically to rare Wnt-active cells. Will this lead to tumour formation? Upon addition of further mutations, does this lead to higher efficiencies of tumour formation relative to bulk targeting of the oviductal secretory compartment?

Previous studies have utilized genomic engineering approaches to insert HGSOc mutations into oviduct organoids. These were then xenografted into mice to test tumour-formation capabilities (Zhang *et al.*, 2019; Löhmußaar *et al.* 2020). This approach could be undertaken in Wnt-reporter organoids, which will enable separation of Wnt-active and Wnt-inactive mutated organoids, that can be separately xenografted into mice to test their tumour formation efficiencies *in vivo*.

Another useful approach would be to use existing mouse models to induce STIC and HGSOc, concomitantly with or without genetic ablation of Wnt-active oviduct cells. Does ablation of Wnt-active cells abolish STIC formation? Previous reports have utilized CRISPR/Cas9 genetic engineering approaches to introduce inducible Caspase 9 (Shimokawa *et al.*, 2017) or Diphtheria toxin (De Sousa E Melo *et al.*, 2017) specifically in Wnt-active cells. In this setting, mutated hFT organoids can be transplanted into mice, and their STIC formation ability can be observed upon selective ablation of Wnt-active cells.

For effective modelling of HGSOc initiation, *in vivo* targeting of initiating TP53 mutation to a relevant cell type may not be sufficient. Other microenvironmental factors need to be considered. For instance, Wnt-active cells are enriched in the fimbrial (distal) end of the FT, which is subject to constant ovulation-induced inflammation and damage at periodic intervals. Epidemiological studies have shown that natural (pregnancy or lactation) or induced (contraceptive methods) pauses to ovulation have a protective effect against HGSOc, quantified as a 50% reduction in risk (Kurman & Shih, 2011). A study reported higher death rates from ovarian cancer among 31,000 Catholic nuns (Fraumeni *et al.*, 1969; Bast *et al.*, 2009), who generally lead celibate lives with uninterrupted ovulatory cycles. Data from the Million Women Study confirmed the reduced ovarian cancer risk

amongst parous women, with further risk reductions with additional births (Gaitskell et al., 2018). This supports a role for ovulation in HGSOc initiation.

Indeed, it was shown that cultured hFT cells exposed to ovulation-released Follicular Fluid (FF) develop DSB breaks, upregulate DNA repair pathways, accumulate TP53 protein and undergo apoptosis (Bahar-Shany et al., 2014; King et al., 2011). The FF component responsible for this effect remains unknown, but intra-patient comparison of FTs shows that fimbrial cells, which are closer to the ovulation site, contain higher levels of endogenous reactive oxygen species (ROS) than proximal FT cells. *In vitro* treatment of cultured fimbrial cells with ROS-inducing factors triggers the same changes as FF treatment, including DSBs breaks and apoptosis of normal fimbrial cells, but not TP53-mutant cells (Huang et al., 2015). Interestingly, the same study showed that TP53-mutant mice developed tumorigenesis upon repeated exposure to patient-derived ROS^{high} FF, not ROS^{low} FF (Huang et al., 2015). This ROS^{high} induced tumorigenesis in TP53^{-/-} mice was ameliorated by melatonin or N-acetyl L-cysteine, both potent anti-oxidants (Huang et al., 2015). Therefore, the role of Wnt-active cells in HGSOc initiation should be contextualized within the ovulatory, FF, ROS and the generally inflammatory environment surrounding fimbrial cells *in vivo*.

6. Differences between human FT and mouse oviduct

Differences were identified in this thesis between hFT and mOV organoids (section 9.2.4). It is important to emphasize that the future directions involving *in vivo* studies are conditional on resolving these differences. Deeper characterisation of mouse oviduct tissue is necessary, and is currently ongoing in the host lab, which is employing scRNA-seq approaches to profile mOV tissue. This should enable comparative profiling between hFT tissue, hFT organoids and mOV tissue at the single cell level, and address biological similarities and differences.

If indeed mouse oviducts are dramatically different to hFTs, innovative solutions are required to model hFT biology. Organoids will be part of the answer. Complex hFT organoids can be optimized as a near physiological complex culture system to model hFT. Alternatively, a hybrid human-mouse system might partially address these differences. In this setting, patient-derived hFT organoids can be genomically engineered and manipulated *ex vivo*, and *in vivo* lineage tracing or cell ablation studies can be conducted upon transplantation into mice. As mentioned, this was shown to be feasible (Shimokawa et al., 2017; De Sousa E Melo et al., 2017).

Finally, to address inter-species differences, it might be a daring approach to model hFT tissue using another animal species altogether. The hen is emerging as a strong contender in this

respect. Hens are the only species that develop ovarian cancer spontaneously, at a very high rate. Hen's ovarian cancer remarkably resembles its human counterpart, decreases upon blocking ovulation by contraceptive pills and expresses oviductal proteins, indicating tubal origin (Johnson & Giles, 2013). Resemblance of aetiology and progression of ovarian cancer between hens and humans could be an indication of shared FT/oviduct biology. This awaits further confirmation by molecular and genome-wide approaches. If this holds true, it may be more appropriate to conduct lineage tracing and cell ablation studies in hens. Given that transgenic technologies have been optimised for hens (Bednarczyk *et al.*, 2018; McGrew *et al.*, 2004; Sid & Schusser, 2018), and that the hen genome has been sequenced (Hillier *et al.*, 2004), this daring approach should not be impossible.

9.5 Summary and Overall Contributions

This thesis has contributed to our knowledge of FT biology in the following ways:

- ✓ Showed that 2D culture is not suitable for modelling hFT tissue
- ✓ Optimised the hFT organoid culture and made it amenable to isolation and characterisation of specific cell population
- ✓ Validated presence of intriguing cell types in hFT organoids, including KRT17+ and immune-epithelial cells, opening the door to their functional characterisation
- ✓ Identified Wnt signalling and TGF- β signaling as essential drivers of self-renewal of FT stem cells
- ✓ Identified WNT7A as the driver of Wnt signaling in FT stem cells, via the FZD5 receptor
- ✓ Showed that WNT7A signals in very close range, possibly through direct cell-to-cell contact
- ✓ Showed essentiality of RSPO signaling to stem cell maintenance
- ✓ Showed that FZD5 and LGR5 mark hFT stem cells, with the latter likely to be a more specific marker
- ✓ Showed that widely used Wnt3a protein has no utility in hFT organoids
- ✓ Generated the first single cell atlas of hFT organoids
- ✓ Showed that Estrogen regulates WNT7A to trigger differentiation of hFT organoids
- ✓ Described major differences between human and mouse oviducts, which should be accounted for when using mouse organoids or *in vivo* studies to model hFT biology and HGSOC

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