Characterisation of paracrine Wnt regulators in colorectal cancer microenvironment

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Declaration

I Sang Eun Lee confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Aberrant Wnt activation is commonly found in colorectal cancer (CRC), especially in cells adjacent to the tumour microenvironment (TME). This indicates the crucial interaction between tumour cells and their niche. Comprehensive transcriptomic analyses by the Cancer Genome Atlas project show genes that are differentially upregulated in tumour stromal population. Of these, a cluster of genes includes markers of cancer-associated fibroblasts (CAFs) that are associated with poor survival. I hypothesise that a subset of CRCs is dependent on paracrine Wnt activation from the surrounding CAFs for tumour progression.

PERIOSTIN (POSTN) is a matricellular protein that is often upregulated in CAFs and is associated with poor CRC prognosis. Human primary fibroblasts established from CRC tissues are found to retain transcriptomic and functional profiles that are distinct between normal and cancer-associated populations *in vitro*, including POSTN upregulation in CAFs. *In vitro* gain-of-function studies show that POSTN potentiates Wnt activation in Wnt ligand-dependent manner, and promotes Wnt receptor stabilisation via yet unclear mechanism(s) interacting with Wnt components on cell membrane. To better study paracrine Wnt activation, a 3D model is developed in which intestinal epithelial cells reside on top of fibroblasts. Co-culture of normal small intestinal epithelial cells with normal fibroblasts show an organised, differentiated crypt/villi-like structure of the epithelial layer, whereas disorganised multi-layered epithelial cells with altered polarity are observed with CAFs. Moreover, the contribution of CAF-derived POSTN in tumour progression is assessed utilising the 3D model system and *in vivo* xenograft formation approaches.

This project demonstrates POSTN is upregulated in CRC and acts as a potential CAF-derived Wnt regulator, providing insight into a novel Wnt regulation that may be at play in CRC tumour progression. The tissue-engineered 3D co-culture system will be a useful tool to study different cell type interactions without sacrificing biomimetic organisation by providing a simple and malleable system.

Impact Statement

Colorectal cancer has one of the highest cancer-related morality rates. Numerous studies have demonstrated the role of the Wnt pathway in CRC, and growing evidence now highlight the contribution of the tumour microenvironment (TME) in the progression of the disease. Wnt hyperactivation is observed regardless of mutations in the Wnt pathway components, further underscoring paracrine regulation of Wnt activity. High-throughput sequencing of a large cohort of CRC patients have identified not only Wnt mutations, but also altered transcriptomic profiles of the TME. In particular, the role of cancer-associated fibroblasts (CAFs) is beginning to be emphasised in tumour progression as they provide both biomechanical and biochemical cues to modulate epithelial cell behaviour.

Based on the available transcriptomic data and what is known of the Wnt pathway, we investigated a putative paracrine factor PERIOSTIN (POSTN). Our data demonstrate that POSTN upregulated in CAFs can potentiate Wnt activity in a ligand-dependent manner, suggestive of its role as a Wnt potentiator. In addition, we identified novel interactions between POSTN and transmembrane Wnt regulators, LGR and RNF43, as well as Wnt receptor LRP6, which potentially lead to stabilisation of FZD receptor observed in the presence of POSTN. The findings offer insights into the complex tableau of Wnt regulation at cell surface by stromal factors, which can be exploited for therapeutic application.

Moreover, we have generated a simple and flexible collagen I 3D co-culture model where human primary epithelial cells and fibroblasts can be cultured long-term in a biomimetic system. Similar to *in vivo*, normal epithelial cells show differential fate decisions and behaviour depending on the fibroblast population cultured together. In addition to the fibroblast-derived secretory factors, the model is capable of recapitulating the fibroblast-mediated ECM remodelling. The *ex vivo* 3D co-culture system allows investigation of heterotypic communication of human primary epithelial cells and fibroblasts preserving the *in vivo* architecture, and can be further employed to study the role of additional stromal cell population or ECM components.

In addition, I have consistently communicated with other scientists in the field. During my PhD, I co-authored a book chapter titled "*Extracellular Matrix Remodelling in Intestinal Homeostasis and Disease*" in 2018, and presented a poster at European Wnt Meeting in 2018 and at EMBO workshop on Mesenchymal cells in cancer in 2019. Finally, we intend to publish a research article to share our findings with the broader scientific community.

Acknowledgement

Doing a PhD was more comprehensive than I initially imagined. I am certainly not the same person I was 4 years ago when I first started this. There were blue days, and I was able to endure with the invaluable support from those around me.

First of all, I would like to thank my supervisor, Vivian, for taking me on for this journey, and for her enduring support and advice throughout my PhD. I was lucky enough to have a supervisor who prioritised my concerns and problems whenever I was stuck, and who generously shared their knowledge and ideas. My thanks to my thesis committee Dr Erik Sahai, Dr Ilaria Malanchi, and Professor Paolo De Coppi, for their advice and suggestions. Last but not least, I am eternally grateful to the Li lab members. You kept me sane until I reached this finish line. I will always remember our long discussions, random chats, and board game and movie nights.

Hard to find friends in a foreign city, but still I was lucky enough to be surrounded by compassionate people. Some were fellow PhD students, some were already earning their bread, or some were way ahead. Thank you for being there when all I could think and talk about was experiments. Thank you for being a voice of reason whenever I needed one and letting me be a part of your journey as well.

Finally, a big shout-out to my family in Korea. I would not be here without your tremendous support and understanding. Saranghae.

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Abbreviations

2D	Two-dimensional
3D	Three-dimensional
AB-PAS	Alcian blue-periodic acid-Schiff
ACTA2	α-smooth muscle actin
AFM	Atomic force microscopy
AFM	Atomic force microscopy
ALPi	Intestinal alkaline phosphatase
APC	Adenomatous polyposis coli
ATF2	Activating transcription factor 2
BMP	Bone morphogenetic protein
CAF	Cancer-associated fibroblast
CamKII	Calmodulin-dependent kinase II
CBC	Crypt base columnar
CID	β-catenin inhibitory domain
CK1	Casein kinase 1
CMS	Consensus molecular subtypes
CRC	Colorectal cancer
CRD	Cysteine-rich domain
CSC	Cancer stem cell
CXCL12	C-X-C motif chemokine 12
DAB	Diaminobenzidine
DKK	Dickkopf
DLL	Delta-like
DMEM	Dulbecco's modified eagle medium
DTT	Dithiothreitol
DVL	Dishevelled
ECM	Extracellular matrix
EdU	5-ethynyl-2’deoxyuridine
EF-1α	Elongation factor-I alpha
EGF	Epidermal growth factor
EGFR	EGF receptor

EMI	Emilin
EMT	Epithelial-to-mesenchymal transition
ER	Endoplasmic reticulum
EV	Empty vector
Evi	Evenness interrupted
FAP	Fibroblast-activation protein
FAS1	Fasciclin-like domain
FBS	Foetal bovine serum
FGF	Fibroblast growth factor
FN	Fibronectin
FSP1	Fibroblast-specific protein
FZD	Frizzled
gRNA	Guide RNA
Gro	Groucho
GSK3	Glycogen synthase kinase 3
GTPase	Hydrolyse guanosine trisphosphate
H&E	Eosin-haematoxylin
Hes	Hairy and enhancer of split
HGF	Hepatocyte growth factor
HTLV	Human T-cell Leukaemia Virus
IF	Immunofluorescence
IFABP	Intestinal fatty acid-binding protein
IL	Interleukin
IP	Immunoprecipitation
IP	Immunoprecipitation
ISC	Intestinal stem cell
JAG	Jagged
JNK	c-Jun N-terminal kinase
KO	Knock-out
LEF	Lymphoid enhancer-binding factor
LGR	Leucine-rich repeat-containing G protein-containing receptor
LOX	Lysyl oxidase
LOXL	Lysyl oxidase-like

LRP	Low-density lipoprotein receptor-related protein
MAPK	Mitogen-activated protein kinase
MEM	Minimum essential media
MIF	Macrophage migration inhibitory factor
MMP	Matrix metalloproteinase
MSI	Microsatellite instability
MSS	Microsatellite stable
NF	Normal fibroblast
NICD	Notch intracellular domain
NTR	Netrin-related motif
OPN	Osteopontin
PAGE	Polyacrylamide gel
PCNA	Proliferating cell nuclear antigen
PCP	Planar cell polarity
PDX	Patient-derived xenograft
PEI	Polyethylenimine
PFA	Paraformaldehyde
PGE	Prostaglandin E
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
POSTN	Periostin
PPIB	Peptidylpropyl isomerase B
PTK	Protein tyrosine kinase
PTM	Post-translational modification
PVDF	Polyvinylidene difluoride
RBPJ	Recombination Signal Binding Protein For Immunoglobulin Kappa J Region
RNF43	RING finger 43
ROCK	Rho-associated protein kinase
ROR	Receptor tyrosine kinase-like orphan receptor
RSPO	R-spondin
RT-qPCR	Quantitative real-time PCR
RTK	Receptor tyrosine kinase

SCNA	Somatic copy number alteration
SDS	Sodium dodecyl sulfate
SFRP	Secreted frizzled-related protein
SOST	Sclerostin
TA	Transit-amplifying
TCF	T cell-specific factor
TCGA	The Cancer Genome Atlas
TGF-β	Transforming growth factor-β
TLE	Transducin-like enhancer of slipt
TME	Tumour microenvironment
TNC	Tenascin-C
TP53	Tumour protein 53
TRIB3	Tribbles pseudo-kinase 3
VEGFA	Vascular endothelial growth factor A
WIF-1	Wnt-inhibitory factor 1
Wls	Wntless
ZNRF3	Zinc and RING finger 3

Chapter 1. Introduction

The intestinal tract is responsible for absorption of nutrients and provision of a barrier against a hostile environment, necessitating a constant renewal of the intestinal epithelium facing the lumen. The turnover is carried out by intestinal stem cells (ISCs) whose proliferation and differentiation are rigorously controlled by multiple signalling pathways. Wnt signalling is central in regulating ISC proliferation and homeostasis, and is counterbalanced by Notch signalling that promotes differentiation (Korinek et al., 1998, Fre et al., 2005). Similarly, EGFR signalling is involved in proliferation of early progenitors whereas the BMP pathway drives derivation of differentiated cell types (Haramis et al., 2004, Wong et al., 2012). Intestinal tissue homeostasis is maintained by tight regulation of these pathways, including Hippo and Hedgehog pathways, with varied degrees of defined functional impacts (Beumer and Clevers, 2016).

The importance of the Wnt signalling pathway in particular has been emphasised since aberrant activation of the Wnt pathway is commonly found to be one of the key initiating events in colorectal cancer (CRC) (Fearon, 2011). Moreover, it is becoming increasingly clear that the interplay between epithelial cells and surrounding cells can contribute to abnormal behaviour of transformed cells in cancer. This chapter will review our current understanding of the ISC biology with a detailed focus on the Wnt signalling pathway, and studies on CRC and the microenvironment will also be summarised.

1.1 Intestinal stem cell biology

The intestinal tract in mammals is comprised of the small and large intestine. The small intestine consists of duodenum, jejunum, and ileum, while the large intestine includes the colon and rectum. The surface facing the lumen is a single layer of epithelial cells, termed the mucosa, which performs food processing and nutrient absorption in addition to stool compaction (Sancho et al., 2003). In the small intestine, villi protrude into the lumen maximising the surface area of the absorptive epithelium. Inferior to villi are the crypts of Lieberkühn which are organised as invaginations of

the single layer of epithelial cells into deeper mesenchyme, the submucosa, whereas only the crypts are present in the colon (Figure 1.1).

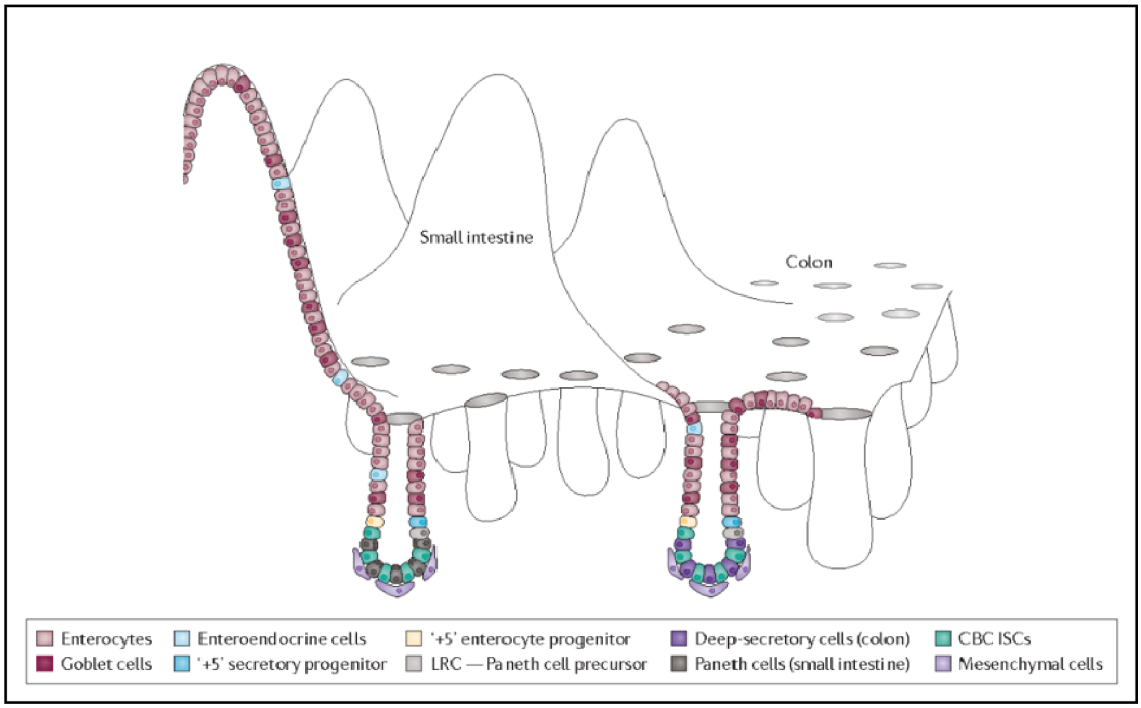

Figure 1.1 The intestinal epithelium.

The epithelial layer of the small intestine consists of crypts and villi, and only crypts in the colon. The crypt base columnar (CBC) intestinal stem cells (ISCs) reside at the bottom of the crypt along with early progenitors. Along the crypt-villus axis, differentiated cell types are found. Figure modified from Vermeulen & Snippert, 2014.

Reflective of its everyday exposure to foreign compounds and pathogens in the lumen, the intestinal epithelium possesses a surprising regenerative capacity (Potten and Morris, 1988, Heath, 1996). The epithelial layer in the gut is renewed every 4-5 days, repopulated by ISCs that reside at the bottom of the crypts and can give rise to all differentiated epithelial cells (Beumer and Clevers, 2016). Early progenitors derived from stem cells, termed transit-amplifying (TA) cells, move upwards out of the crypt as cells proliferate and give rise to either secretory or absorptive lineages

(Beumer and Clevers, 2016). Secretory cells produce hormones, mucus, and anti-microbial peptides against challenges in the intestinal lumen, whilst absorptive cells are responsible for uptake of nutrients (Santos et al., 2018). At the tip of the villus, epithelial shedding into the lumen takes place whereby differentiated cells undergo anoikis-mediated cell death triggered by detachment from the basement membrane (Hall et al., 1994, Grossmann et al., 2001) (Figure 1.2).

Differentiated cell types performing secretory and absorptive functions are found in the intestine but distribution varies throughout the length (Barker et al., 2008). In the small intestine, cells of both secretory and absorptive lineages are found. Enterocytes are the most common cell type in the small intestine, and absorb nutrients and secrete hydrolytic enzymes into the lumen (Barker et al., 2008). Cells of the secretory lineage include goblet cells, enteroendocrine cells, and Paneth cells that produce mucous, hormones, and digestive lysozymes as well as anti-microbial peptides respectively (Noah et al., 2011, Clevers, 2013). Enteroendocrine cells are a rare cell type in the intestine, representing less than 1% of the total epithelium, and have multiple subtypes depending on the hormone they secrete (Barker et al., 2008, Schonhoff et al., 2004). Unlike other differentiated epithelial cells, Paneth cells migrate back down to the crypt guided by EphB3/EphrinB interaction to reside next to the stem cells (Batlle et al., 2002). Other differentiated cells such as Tuft cells are found but their specific functionality is less well defined (Gebert et al., 1996, Nabeyama and Leblond, 1974, Barker et al., 2008).

The proliferation and differentiation of ISCs is regulated by the ISC niche in which other cell types and extracellular matrix (ECM) collectively provide appropriate signals (Barker et al., 2008). The following section will describe the ISC niche and key underlying signalling pathways involved in ISC homeostasis.

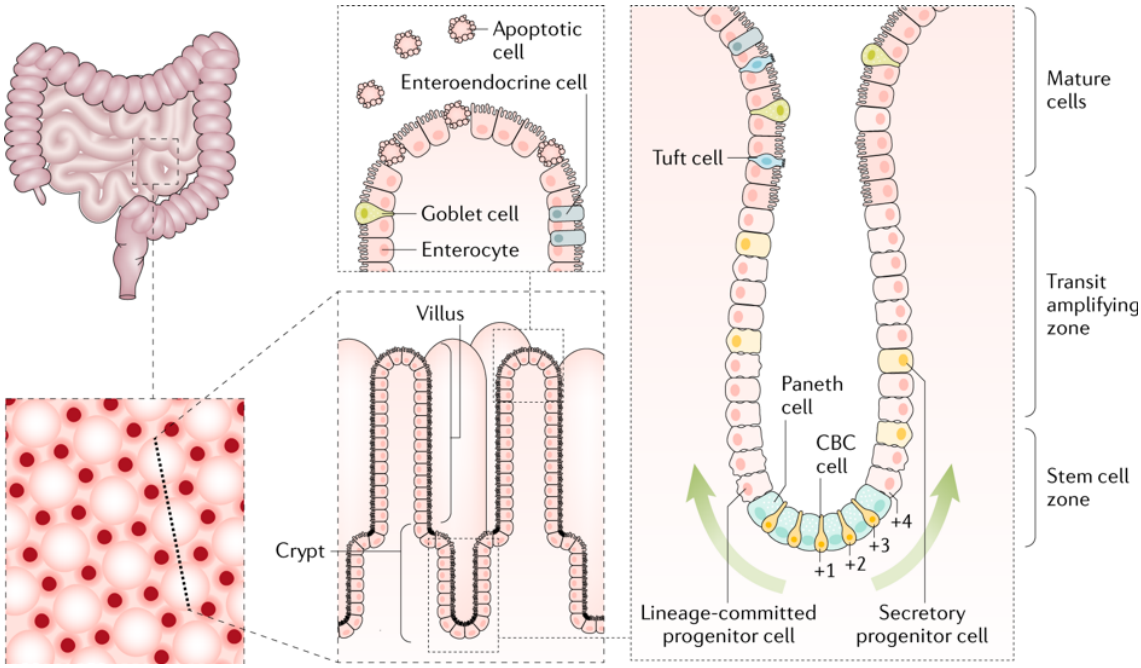

Figure 1.2 The intestinal epithelial cells.

The ISCs (or CBCs as labelled here) allow regular turnover of cells at the tip of the villus after a series of cell migration upwards along the crypt-villus axis and differentiation. Cells in transit amplifying zone pushed out by cells generated in the stem cell zone rapidly divide and give rise to differentiated cells found in the intestinal epithelium, which then apoptose at the villus tip. Figure adapted from Gehart & Clevers, 2019.

1.1.1 Intestinal stem cells and their niche

Two early models proposed the ISC identity and location at the bottom of the crypt but the ISC population is now recognised to be a heterogeneous pool with differential proliferation dynamics. In the first model, cells at the +4 position (by counting cell nuclei from the crypt bottom) were termed +4 cells with the ISC-indicative property of DNA-label retention (Potten et al., 1974). These Bmi1-positive cells have been characterised to be quiescent stem cells that become active upon injury (Sangiorgi and Capecchi, 2008). In the second model, crypt base columnar (CBC) cells residing between Paneth cells were first suggested to be stem cells in 1974 and have been shown to give rise to all differentiated cell types (Cheng and Leblond, 1974, Bjerknes and Cheng, 1999). Identification of the CBC-specific marker leucine-rich repeat-

containing G protein-containing receptor 5 (Lgr5) has revealed nigh daily proliferation of CBCs by lineage tracing analyses, indicating that homeostatic turnover of intestinal epithelium depends on CBC cells, whilst Bmi1-positive cells function as reserve ISCs and replace the pool of CBC cells when needed (Barker et al., 2007, Tian et al., 2011). In addition, Lgr5-positive cells with high expression of RNA binding protein Mex3a have been suggested to represent another slow-cycling reserve $Lgr5^+$ ISC pool that is resistant to radiation and chemotherapy (Barriga et al., 2017). Following asymmetric cell division of ISCs, stochastic cell fate decisions of resultant daughter cells are driven by a range of signals supplied by the niche (Lopez-Garcia et al., 2010, Schepers et al., 2011, Snippert et al., 2010b).

The ISC niche is comprised of both physical and cellular components that are required to support ISC maintenance in part by providing morphogen gradients (Santos et al., 2018) (Figure 1.3A). The intestinal organoids, an *ex vivo* expansion of ISCs, are enabled by *in vitro* supply of ISC niche components including such morphogens in the media and three-dimensional (3D) culture of cells (Sato et al., 2011a) (Figure 1.3B). Deposited by both epithelial and stromal cells, ECM proteins comprising the basement membrane are in contact with the basal side of the epithelium and provide biochemical and mechanical signals. The ECM proteins that are compatible to integrin receptors such as collagen, tenascin, and laminin form the connective tissue matrix in the lamina propria beneath the epithelial layer (Simo et al., 1991, Beaulieu, 1992, Mahida et al., 1997, Okuno et al., 2002). While collagen type IV is found throughout homogeneously, specific laminin subtypes exhibit differential distribution along the crypt-villus axis (Beaulieu and Vachon, 1994). Similarly, epithelial cells display just as varied expression pattern of integrin subunits (Beaulieu, 1992). For example, Paneth cells next to ISCs in the niche have high expression of β4 integrin and the resultant stiffness leads to crypt fission when the crypt is crowded by ISC proliferation (Langlands et al., 2016). This process is found to allow the number of crypt units to increase as the organ grows as well as repopulate the epithelial layer in response to wounding (Totafurno et al., 1987, Withers and Elkind, 1970, Park et al., 1997). In addition to epithelial cell anchoring to the ECM underneath, the physical properties of the ECM also regulate epithelial cell behaviour. Proliferation of normal epithelial cells has been shown to change in response to changes in the ECM (Fernandez-Sanchez et al., 2015).

The role of the ECM is also highlighted in *in vitro* intestinal organoid cultures. The efficiency of intestinal organoid formation is reported to be varied when ISCs are cultured in PEG hydrogels of varying stiffness (Gjorevski et al., 2016). It is also improved by Rho-associated protein kinase (ROCK) signalling inhibitor treatment which reduces anoikis-induced cell death of isolated epithelial cells (Sato et al., 2009). Moreover, small intestinal organoids injected into the damaged colonic mucosa of the recipient mouse were found to incorporate into the colon epithelia and maintain small intestinal identity, suggesting the intrinsic programme dictates the lineage commitment of the epithelial cells (Fukuda et al., 2014). On the other hand, reduced laminin α5 deposition, most commonly found in the villus basement membrane, led to loss of villi in the small intestinal mucosa, but the expression of small intestinal marker sucrase-isomaltase persisted (Mahoney et al., 2008). The incomplete transition from one to another suggests that while the ECM is important for ISC fate decision, the niche is broader then the structural support for ISCs and the cellular niche is also important for maintenance of the ISC niche (Lee et al., 2018).

Cellular constituents of the ISC niche include both epithelial cells and stromal cells providing cues necessary for ISC proliferation and self-renewal (Santos et al., 2018). Paneth cells have been denoted as a key cellular ISC niche component as they supply ligands for Wnt and Notch pathways as well as epidermal growth factor (EGF) to the ISCs creating a signalling gradient (Sato et al., 2011b). In lieu of Paneth cells in the small intestine, Reg4-expressing cells are revealed to be located next to ISCs in the colon and perform niche cell-like functions similar to Paneth cells, expressing Notch and EGF ligands (Sasaki et al., 2016). However, epithelial cells are not the only source of factors available to the ISCs.

Stromal cells such as fibroblasts and smooth muscle cells also participate in establishing morphogen gradients along the crypt-villus axis (Santos et al., 2018). Animal models with epithelial-specific depletion of Wnt proteins retain intestinal homeostasis highlighting the role of stromal cells in the niche (Kabiri et al., 2014, San Roman et al., 2014, Farin et al., 2012, Valenta et al., 2016). Pericryptal fibroblasts with high expression of α-smooth muscle actin (ACTA2) are found to reside in the lamina propria and provide Wnt ligands that promote ISC proliferation in the crypt

(Farin et al., 2012, Gregorieff et al., 2005). Alternatively, Gli^+ subepithelial mesenchymal cells in the ISC niche are found to provide stromal Wnt ligands (Valenta et al., 2016). Differentiation of ISCs is chiefly modulated by stromal cells in lamina propria at the tip of the villus expressing BMP4, which activates the BMP pathway to promote epithelial differentiation (He et al., 2004). A descending BMP signal gradient is created by pericryptal fibroblasts and smooth muscle cells at the crypt producing BMP antagonists, such as Gremlins and Chordin (Kosinski et al., 2007). Moreover, an $ACTA2^-$ mesenchymal population distinct from previously identified $ACTA2^+$ pericryptal fibroblasts has been reported to secrete BMP antagonist Gremlin-1 as well as RSPO1 (Stzepourginski et al., 2017). Although definitive correlation of stromal cell identity and signals each provide to the epithelium is yet unclear, stromal cells are an important suppliers of niche factors that regulate signalling pathways underlying ISC homeostasis (Trentesaux and Romagnolo, 2018).

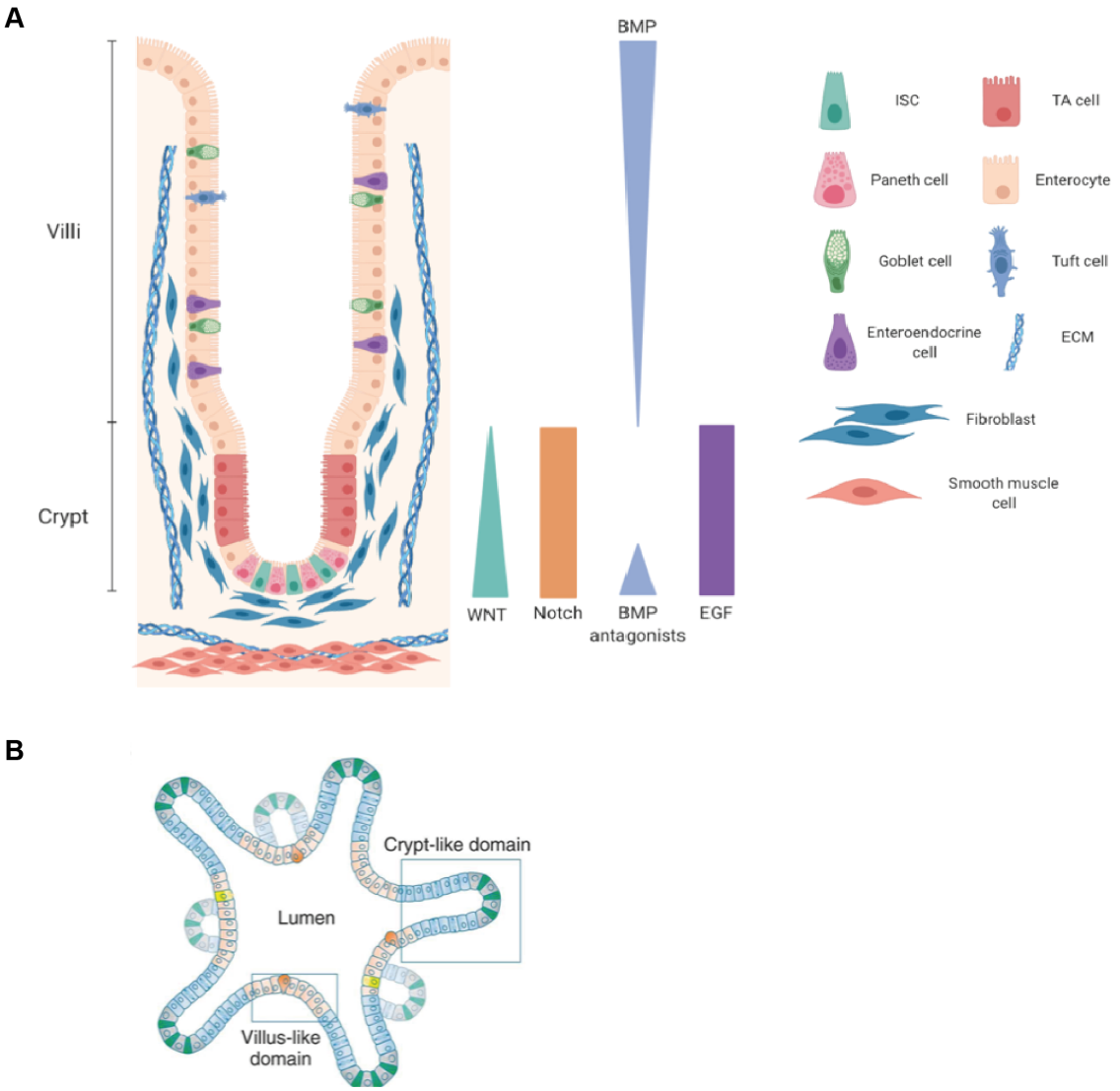

Figure 1.3 The intestinal stem cell (ISC) niche and *ex vivo* intestinal organoids.

(A) ISC niche and gradient of signalling pathways involved in ISC homeostasis. At the bottom of the crypt, LGR5-expressing ISCs are located next to Paneth cells, a differentiated secretory cell type of the epithelium. The ISCs either self-renew or generate transit-amplifying (TA) cells, which then differentiate into various functional cells such as Paneth cells, goblet cells, enteroendocrine cells, tuft cells, and absorptive enterocytes. The extracellular matrix (ECM) comprises the niche providing biomechanical and biochemical support. Mesenchymal cells such as fibroblasts and smooth muscle cells are cellular components of the ISC niche. Comprehensively, non-cellular and cellular niche regulate ISC behaviour via several signalling pathway activities, such as Wnt, Notch, BMP, and EGFR. Figure created with Biorender.com. (B) Schematic architecture of intestinal organoids. Small intestinal organoids show buddings where crypt-like domain and villus-like domain can be distinguished. Colonic organoids do show buds but not as clearly defined as small intestinal organoids. Figure modified from Rizk & Barker, 2012.

1.1.2 Signalling pathways underlying ISC homeostasis

Tissue homeostasis is a fine balance of proliferation and differentiation as well as cell death. Due to the high turnover rate, rigorous regulation of ISC homeostasis is key. Unsurprisingly, a number of signalling pathways are involved in moderating ISC maintenance during homeostasis and wound response. This section includes a brief overview of several pathways involved in ISC homeostasis and the Wnt signalling pathway is described in more detail.

1.1.2.1 Notch signalling pathway

The Notch pathway works to hone the ISC homeostasis together with the Wnt pathway (Korinek et al., 1998, van Es et al., 2005). Five Notch ligands (Delta-like (DLL) 1/3/4 and Jagged (JAG) 1/2) are transmembrane Notch ligands that bind to Notch receptors on receiver cells to trigger proteolytic cleavage events that eventually release Notch intracellular domain (NICD) from the receptor by γ-secretase complex (Kopan and Ilagan, 2009). After translocating to the nucleus, NICD interacts with a DNA binding protein, Recombination Signal Binding Protein For Immunoglobulin Kappa J Region (RBPJ), and recruits co-activators to initiate the tissue-specific Notch transcription programme (Jarriault et al., 1995, Kopan and Ilagan, 2009, Demitrack and Samuelson, 2016). Notch targets include Olfm4 and hairy and enhancer of split (Hes) family transcription factors including Hes1, that repress the transcription factor Atoh1 (Tamura et al., 1995, Jensen et al., 2000, VanDussen et al., 2012). Notch pathway activation suppresses expression of Delta and yields a lateral inhibition whereby Notch signals transmitted to adjacent receiver cells creates a feedback loop amplifying reverse Notch activities between the cells (Sancho et al., 2015) (Figure 1.4).

Notch signalling activation in ISCs has been found to be critical for ISC maintenance (Pellegrinet et al., 2011). High expression levels of Notch receptors are found in ISCs (Fre et al., 2011). Notch ligands are expressed in secretory cells, such as Paneth cells and Reg4+ cells in the colon, some of which directly interact with ISCs to provide niche signals (Pellegrinet et al., 2011, Sasaki et al., 2016). Notch signalling regulates progenitor proliferation as well as differentiation (Jensen et al., 2000,

Pellegrinet et al., 2011, van Es et al., 2005, Stanger et al., 2005). Progenitors with high Notch activity (receiver cells) differentiate to absorptive enterocytes while neighbouring progenitors with low Notch activity adopt a secretory identity (Sancho et al., 2015). Secretory progenitors are found to express Notch ligands to inhibit Atoh1-dependent secretory differentiation in adjacent cells (van Es et al., 2012b). Notch hyperactivation reduces Atoh1 expression and inflates the absorptive enterocyte population, whilst suppression of Notch signalling leads to secretory cell hyperproliferation (VanDussen et al., 2012, Stanger et al., 2005, Fre et al., 2005, VanDussen and Samuelson, 2010). As tight regulation of the pathway is required for tissue homeostasis, aberrant Notch activity is observed in intestinal cancers (Reedijk et al., 2008, Piazzi et al., 2011). While Notch hyperactivation promotes formation of adenoma, tumourigenesis in APC-mutant has been shown to be resistant to Notch inhibitor-induced cell differentiation, suggesting that canonical Wnt hyperactivation exerts stronger effect on cell behaviour (Fre et al., 2009, Peignon et al., 2011). The hierarchy is most likely context-dependent as the role of Notch in promoting ISC differentiation relies on its downstream effector Math1 which serves as a tumour suppressor and is often lost in many primary human tumours (van Es et al., 2010).

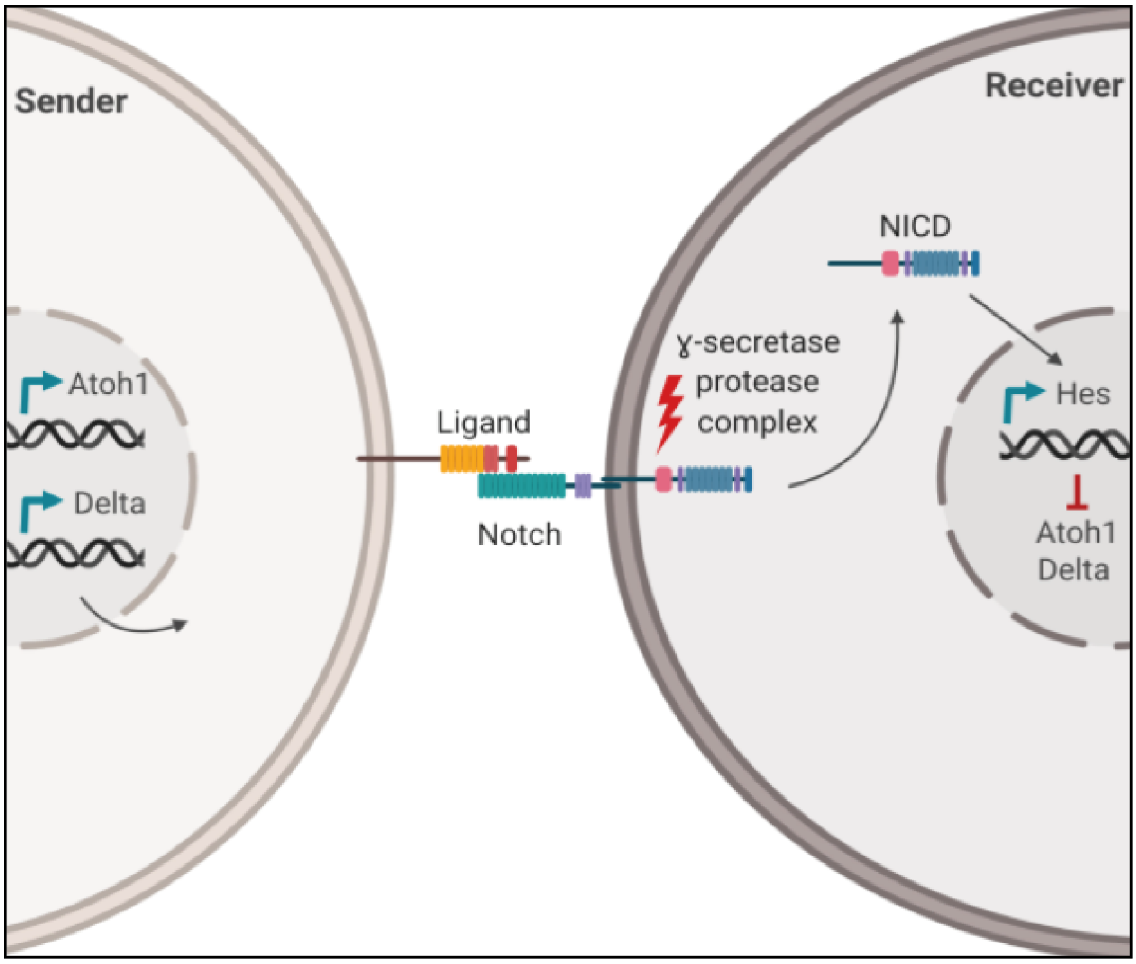

Figure 1.4 Schematic of Notch signalling pathway.

Notch ligands binding to Notch receptor on the neighbour cell initiates a cleavage cascade culminating in release of Notch intracellular domain (NICD) by γ-secretase complex. NICD then translocates into the nucleus and associates with DNA-binding proteins to start tissue-specific Notch transcription programmes. The expression level of Notch ligand Delta is reduced upon Notch activation, providing a feedback mechanism for lateral inhibition that enhances differential fate decision between adjacent cells demarked in part by sending and receiving Notch signal. Figure created with Biorender.com.

1.1.2.2 TGF-β/BMP signalling pathway

The transforming growth factor-β (TGF-β) superfamily of signalling includes the TGF-β pathway with transmembrane receptors divided into two subtypes, type I and II (Mullen and Wrana, 2017). TGF-β family ligands, including bone morphogenetic proteins (BMPs), bind to receptors and induce formation of a complex with two type I and two type II receptors initiating signal transduction through receptor-mediated phosphorylation of intracellular protein SMADs (Massagué et al., 2005, Miyazono et al., 2005, Feng and Derynck, 2005, Moustakas and Heldin, 2009, Hinck, 2012) (Figure 1.5). Activated regulated SMADs (R-Smads) including SMAD1/2/3/5/8 form complexes with SMAD4, also known as Co-Smad for being a common partner for all R-Smads, to modulate the transcription programme (Miyazono et al., 2005, Feng and Derynck, 2005, Moustakas and Heldin, 2009, Massagué, 2012). Resultant gene expression is context-dependent as it is determined by SMAD interaction with cell type-specific DNA binding partners (Mullen and Wrana, 2017). Conversely, SMAD6/7 have inhibitory functions at receptor level or at SMAD-SMAD interaction providing negative feedback (Miyazawa and Miyazono, 2017). It is suggested that TGF-β pathway involves Smad2/3, whilst BMP pathway preferentially activates Smad1/5/8 (Mullen and Wrana, 2017). The specific role of TGF-β in ISC biology remains unclear compared to that of BMP pathway.

In the intestine, BMP signalling exerts a crucial suppressive effect on Wnt-mediated ISC self-renewal to avoid hyperproliferation with a gradient opposite to that of Wnt activity (Clevers, 2013, Medema and Vermeulen, 2011, Yeung et al., 2011, Haramis et al., 2004, Kosinski et al., 2007). Smad-mediated suppression of stem cell genes such as *Lgr5* promotes epithelial differentiation (Haramis et al., 2004, He et al., 2004, Qi et al., 2017). A gradient of BMP activity along the crypt-villus axis is generated by mesenchymal cells that surround the crypt producing BMP antagonists, such as Noggin and Gremlin-1, and mesenchymal cells beneath the villi secreting BMP ligands BMP2/4 (Haramis et al., 2004, Hardwick et al., 2004, He et al., 2004, Kosinski et al., 2007). Loss of BMP receptor *Bmpr1a* enlarges the ISC compartment and ectopic expression of Noggin or Gremlin-1 induces abnormal crypt formation and expansion, indicative of its role in suppressing ISC niche expansion (He et al., 2004, Haramis et al., 2004, Davis et al., 2015). The risk of CRC has been found to be higher

with mutations in BMP components such as *SMAD4* and BMP receptor (Brosens et al., 2011, Howe et al., 1998, Howe et al., 2001).

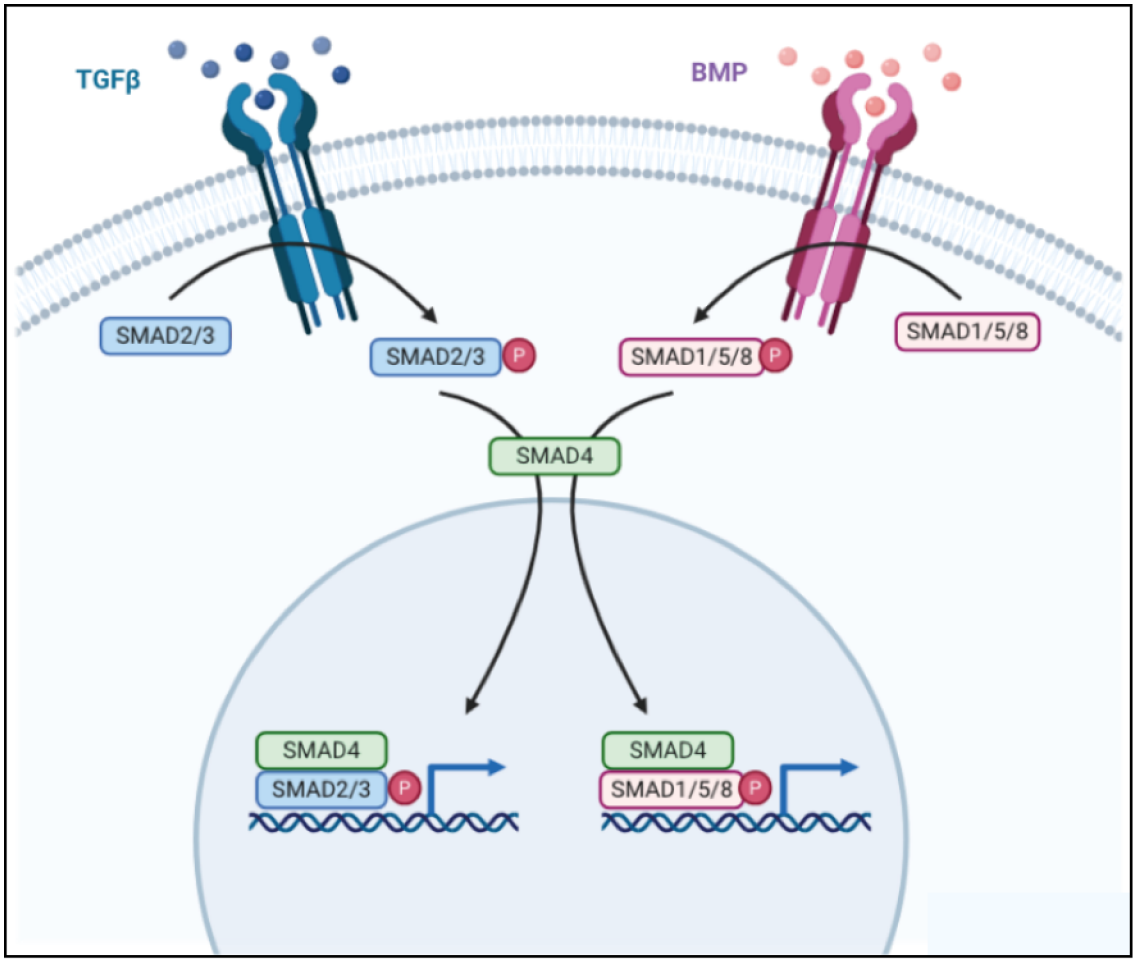

Figure 1.5 Schematic of SMAD-dependent TGF-β/BMP signalling pathway.

Ligands (TGF-β and BMPs) trigger the signal transduction cascade when they bind to type I or type II serine/threonine kinase receptors, resulting in heterotetrametric complex formation. The phosphorylation cascade culminates in phosphorylation of R-Smads, mainly SMAD2/3 in TGF-β-induced, and SMAD1/5/8 in BMP-induced signal transduction. Phosphorylated R-Smads form a complex with Co-Smad, SMAD4, and translocates into the nucleus where the complex associates with cell-specific cofactors to modulate the transcription. Figure created with Biorender.com.

1.1.2.3 EGFR signalling pathway

The EGF receptor (EGFR) pathway regulates key cellular processes in homeostatic tissue development (Alexander et al., 2015, Jeong et al., 2013, Arda-Pirincci and Bolkent, 2014). Activation of the pathway promotes autophosphorylation of receptor tyrosine kinase (RTK) and activation of multiple pathways reflecting the diverse outcomes achieved by EGFR activity (Wee et al., 2015, Tang et al., 2016). The downstream pathways include STATS, Ras/mitogen-activated protein kinases (Ras/MAPK), phospholipase C-γ/protein kinase C (PLC-γ/PKC), and phosphatidylinositol 3-kinase/AKT (PI3K/AKT) pathways (Brand et al., 2011) (Figure 1.6).

In the intestine, EGFR activity contributes to epithelial cell survival, proliferation, and migration primarily via Ras/MAPK pathway (Wilson and Gibson, 1999, Frey et al., 2004, Riegler et al., 1997, Suzuki et al., 2010). High expression of EGFR is observed in ISCs whilst the ligands are provided by Paneth cells (Sato et al., 2011b). EGF is found to be required for human intestinal organoid formation and survival (Matano et al., 2015). Moreover, p38 inhibitor is essential for long-term culture of intestinal organoids as EGF-mediated migration or proliferation is determined by p38 that modulates EGFR (Frey et al., 2004, Otsuka et al., 2010). Loss of p38 promotes epithelial proliferation facilitating long-term expansion of the epithelial cells in organoid cultures (Frey et al., 2006). EGFR inhibitor Lrig1 expressed by ISCs suppresses the pro-mitotic EGFR signal to maintain the homeostatic ISC compartment size, and loss of such negative regulation leads to enlargement of the proliferative compartment (Wong et al., 2012). Enterocytes are found to express EGFRs, and EGFR ligands available in the intestinal lumen cross over the epithelial barrier to activate the pathway during injury (Konturek et al., 1989, Wright et al., 1990).

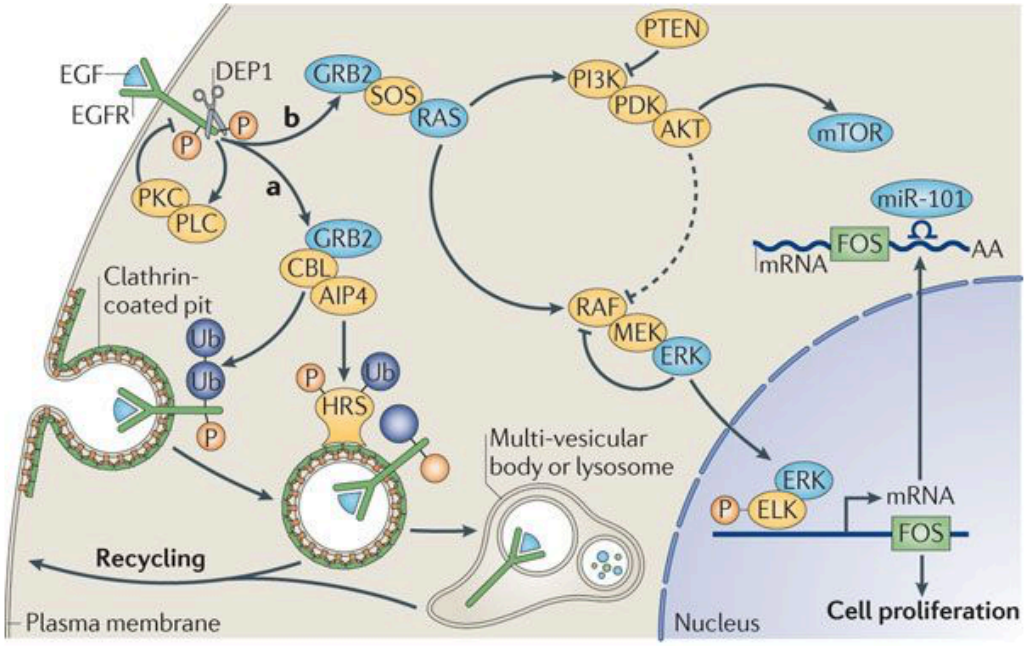

Figure 1.6 Schematic of EGFR signalling pathway.

Binding of epidermal growth factor (EGF) to EGF receptor (EGFR) initiates tyrosine kinase activity which is regulated by Tyr-specific phosphatases acting as inhibitors, for example, density-enhanced phosphatase (DEP1). Adaptors such as growth factor receptor-bound protein 2 (GRB2) recognising phosphorylated receptors act as signalling branching point (denoted as a and b). Ubiquitin (Ub) ligase CBL is recruited to pack receptors in endosomes, and AIP4 ubiquitylates clathrin binders such as HRS. Ubiquitin tags on the receptors and interaction with adaptors leads to lysosomal degradation of EGFR. On the other hand, RAS-mediated downstream signalling pathway involves kinase cascades such as RAF-MEK-ERK and PI3K-PDK-AKT. Nuclear translocation of ERK removes DNA-binding repressors or recruits transcription factors like ELK for target gene transcription including FOS. Selective microRNAs (miRNAs) serve as suppressors of signal-independent transcription. Upon mitogen activated protein kinase activation, these miRNAs are degraded resulting in transcription programme initiation. mTOR, mammalian target of rapamycin. Figure adapted from Avraham & Yarden, 2011.

1.2 Wnt signalling pathway

Wnt signalling is an evolutionarily conserved pathway that plays an essential regulatory role for a range of cellular processes during development and homeostasis (Clevers, 2006, Logan and Nusse, 2004). The Wnt signalling can be largely categorised into two pathways, based on the involvement of β-catenin: canonical (β-catenin-dependent); and non-canonical (β-catenin-independent). In the intestine, the canonical Wnt pathway has been revealed to be crucial for ISC proliferation, with the activity being the highest at the crypt base (Gregorieff et al., 2005, Farin et al., 2016). In the following section, Wnt signalling is summarised and the canonical pathway is described in more detail regarding its components, signal transduction, and regulation.

1.2.1 Non-canonical Wnt pathway

The non-canonical pathway does not require β-catenin and regulates cell movement during morphogenesis (Veeman et al., 2003). Several branches of signal transduction have been identified that are initiated by Wnt ligand binding to receptors that do not result in β-catenin-mediated transcription initiation (Figure 1.7).

Wnt/PCP (planar cell polarity) signalling underlies processes in tissue morphogenesis such as cell polarity and directional cell movement (Castanon et al., 2013, Wu et al., 2013). Wnt/PCP receptors include receptor tyrosine kinase-like orphan receptor-1/2 (ROR1/2) that forms a complex with Frizzled (FZD) when bound to Wnt proteins (Kelley, 2008, Krausova and Korinek, 2014). The complex recruits and activates Dishevelled (DVL) to then engage with hydrolyse guanosine trisphosphate (GTPase) Rho (Tree et al., 2002, Habas et al., 2001). The GTPases Rac1 and Rho activate ROCK and c-Jun N-terminal kinase (JNK) to regulate cytoskeletal dynamics as well as transcription programme via activating transcription factor 2 (ATF2) (Kikuchi et al., 2011). On the other hand, Wnt/Ca^{2+} signalling is responsible for Ca^{2+} increase by release from the endoplasmic reticulum (ER) or import from the extracellular space (Sheldahl et al., 2003). It involves activation of PLC when ROR1/2-FZD complex is formed upon Wnt ligand binding (Sheldahl et al., 2003). Subsequently, PLC promotes a Ca^{2+}-dependent transcription programme in

response to intracellular calcium fluxes, which activate the pathway components PKC, calcium/calmodulin-dependent kinase II (CamKII), and calcineurin (Sheldahl et al., 2003). The Wnt/Ca^{2+} signalling has been suggested to have pro-tumourigenic roles (Dissanayake et al., 2007). However, the definitive impact of non-canonical Wnt pathway in ISC homeostasis and cancer is largely unknown, and primary focus of this section will be on the canonical Wnt pathway.

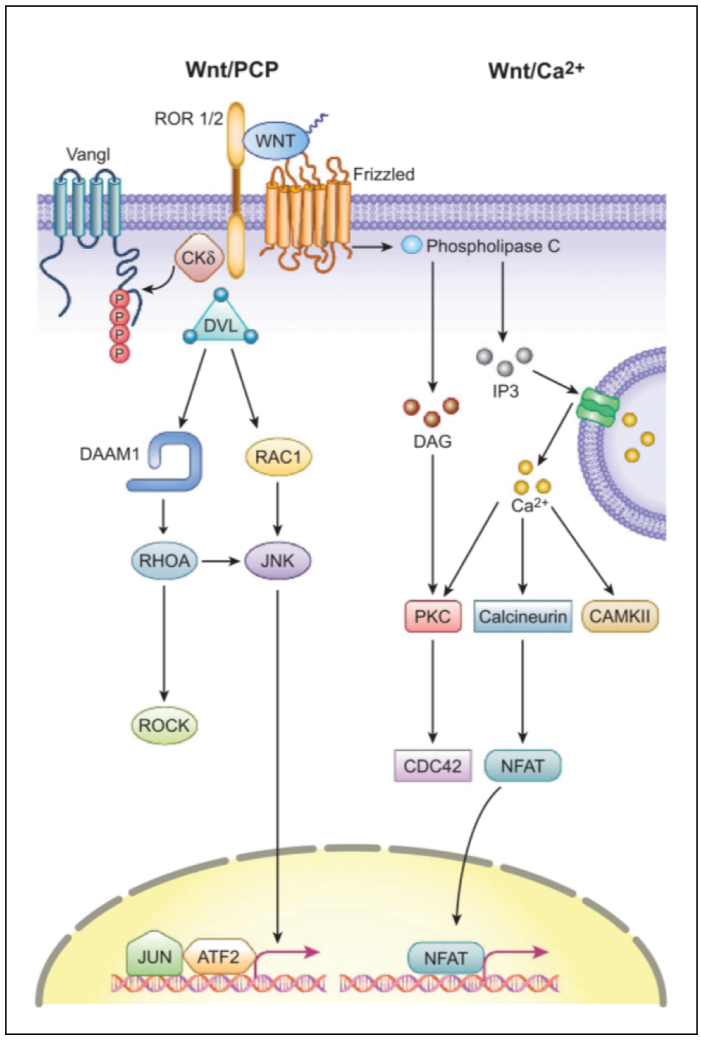

Figure 1.7 Schematic of non-canonical Wnt signalling pathway.

In Wnt/PCP signalling, the ROR-FZD receptor complex recruits DVL upon ligand binding, which in turn frees RHO from inhibition of DAAM1 (DVL associated activator of morphogenesis 1). RAC1 and RHO activate JNK and ROCK, initiating cytoskeletal rearrangements and ATF2-mediated transcription programme. Vangl is phosphorylated in WNT5-dependent activation of Wnt/PCP pathway. Upon Wnt/Ca^{2+} signalling activation, PLC triggers intracellular calcium fluxes, leading to calcium-

dependent responses at cytoskeletal and transcriptional levels. Figure adapted from Zhan et al., 2017.

1.2.2 Canonical/β-catenin-dependent Wnt signalling

The canonical Wnt signalling pathway constitutes the regulatory mechanism for ISC maintenance together with pathways described in the previous section. It participates in stem cell proliferation and cell fate decisions, and Wnt suppression results in ISC deficiency ablating the intestinal epithelium (van Amerongen and Nusse, 2009, Korinek et al., 1998, Kuhnert et al., 2004, van Es et al., 2012a). Inhibition of the canonical pathway *in vivo* has been found to lead to premature differentiation of stem cells resulting in ISC pool exhaustion (Yan et al., 2017). The *ex vivo* culture of intestinal organoids requires Wnt ligands, exogenous or Paneth cell-derived, and their withdrawal yields differentiated cells (Sato et al., 2011b). This section recaps our current understanding of β-catenin-dependent Wnt signalling transduction and regulation.

1.2.2.1 Canonical Wnt signal transduction

The canonical Wnt pathway hinges on regulating the intracellular concentration of β-catenin, a vital effector of the pathway, as accumulation of it leads to initiation of Wnt target gene transcription. The pathway is activated when Wnt ligands bind to receptors and trigger a series of intracellular signal transductions to eventually result in nuclear translocation of β-catenin (Figure 1.8). When no Wnt ligands are bound to low-density lipoprotein receptor-related protein 5/6 (LRP5/6) and FZDs, β-catenin degradation is promoted (van Amerongen and Nusse, 2009). The level of β-catenin is modulated by β-catenin destruction complex that is comprised of adenomatous polyposis coli (APC), AXIN, glycogen synthase kinase 3-alpha/beta (GSK3α/β), and casein kinase 1 (CK1).

AXIN is a scaffold protein that interacts with all members of the β-catenin destruction complex as well as β-catenin (Stamos and Weis, 2013). A Wnt negative feedback mechanism is in place as AXIN is a direct Wnt target gene, but the protein level is also targeted for degradation in response to β-catenin stabilisation via the activity of E3 ligases RNF146 and SIAH1/2 providing a positive feedback (Yamamoto et al., 1999, Ji et al., 2017, Zhang et al., 2011, Lustig et al., 2002). APC contains a highly conserved domain, the β-catenin inhibitory domain (CID), that has been denoted to be critical for reducing β-catenin levels since APC is responsible for binding to β-catenin in the destruction complex (Rubinfeld et al., 1993, Kohler et al., 2009, Roberts et al., 2011). CK1 phosphorylates β-catenin at Ser45 and primes it for modification by GSK3 (Liu et al., 2002, Amit et al., 2002). GSK3α/β have redundant functions in the destruction complex, phosphorylating β-catenin after CK1 at Thr41, Ser37, and Ser33, the -α and -β isoform showing tissue-specific expression patterns (Doble et al., 2007). Phosphorylated β-catenin is then ubiquitinated by F box/WD repeat protein, β-TrCP, and targeted for proteasomal degradation (Aberle et al., 1997, Liu et al., 2002). Without β-catenin nuclear translocation, transcription repressors Groucho/transducing-like enhancer protein (Gro/TLE) remain bound to transcription factor T cell-specific factor/lymphoid enhancer-binding factor (TCF/LEF) to suppress Wnt target gene transcription (Arce et al., 2009, Cavallo et al., 1998, Roose et al., 1998).

In the presence of canonical Wnt ligands, LRP5/6 and FZD form a heterotrimeric complex together with Wnt, and the destruction complex is subsequently recruited to the membrane (Gan et al., 2008, Li et al., 2012b). Scaffold protein DVL is responsible for mediating the recruitment as it binds to both FZD receptor and AXIN (Gao and Chen, 2010). The FZD-DVL-AXIN interaction dissociates β-TrCP from the destruction complex resulting in β-catenin accumulation in the cytosol (Li et al., 2012b). Translocation of β-catenin into the nucleus prompts TCF-mediated transcription of Wnt target genes by forming the β-catenin/TCF/LEF transcriptional complex (Rubinfeld et al., 1993, Behrens et al., 1996). In the nucleus, β-catenin acts as a co-activator in TCF/LEF binding to Wnt enhanceosome comprised of Wnt transcriptional enhancers (Fiedler et al., 2015, van Tienen et al., 2017). Conformational changes in the Wnt enhanceosome by β-catenin binding are suggested to promote ubiquitination of transcription repressors Gro/TLE and

subsequent chromatin remodelling leads to transcriptional activation (van Tienen et al., 2017, Flack et al., 2017).

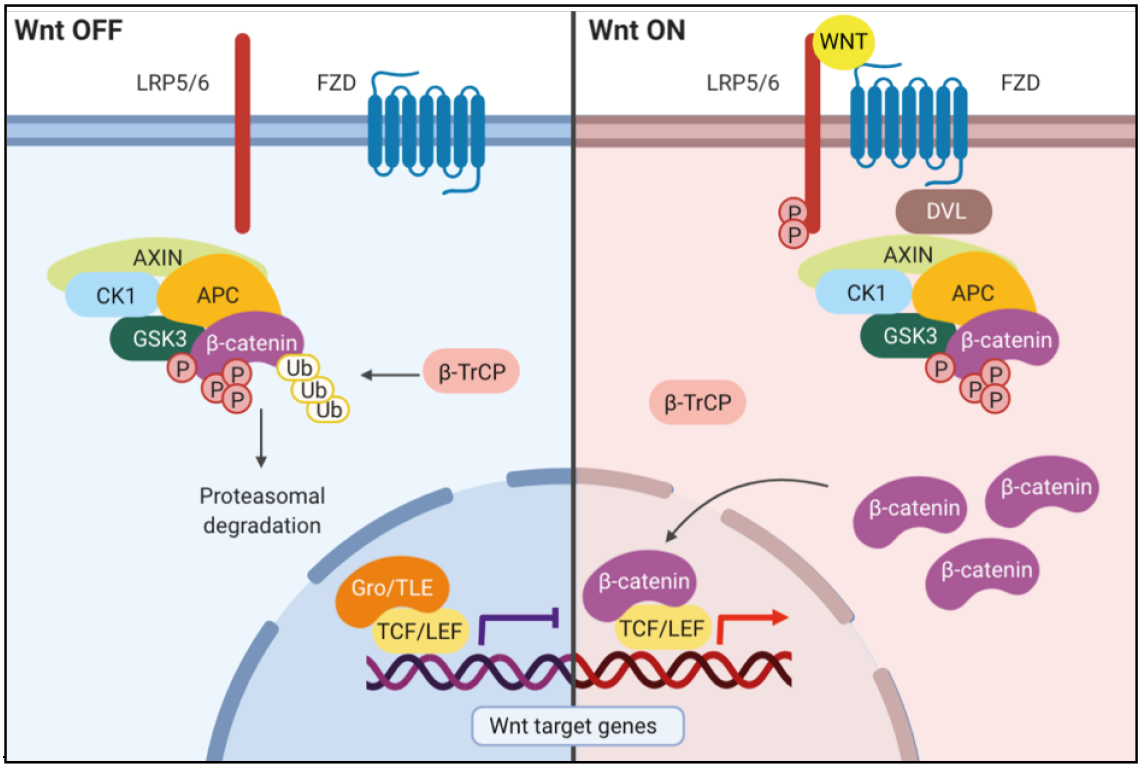

Figure 1.8 Schematic of the canonical Wnt signalling pathway.

In the absence of Wnt ligands, β-catenin destruction complex targets β-catenin for phosphorylation, and subsequent ubiquitination by β -TrCP leads to proteasomal degradation of β-catenin. Without β-catenin in the nucleus, a repressive complex of Groucho/transducing-like enhancer protein (Gro/TLE) and transcription factor T cell-specific factor/lymphoid enhancer-binding factor (TCF/LEF) suppresses Wnt transcription programme. When Wnt ligands are bound to low-density lipoprotein receptor-related protein (LRP) and Frizzled (FZD) receptors, β-catenin destruction complex is deactivated, and accumulated cytoplasmic β-catenin translocates into the nucleus. In the nucleus, β-catenin displaces Gro/LEF, associating with TCF/LEF to initiate transcription of Wnt target genes. Figure created with Biorender.com.

1.2.2.2 Wnt ligands and receptors

The mammalian Wnt family includes 19 Wnt ligands that are secreted glycoproteins manufactured in the ER, which convey extracellular signals to the cell via receptors on cell surface (Komiya and Habas, 2008) (Figure 1.9). Wnt production and secretion has been found to undergo post-translational modifications (PTMs) with important functional implications in secreting cells. In the ER, acyltransferase Porcupine attaches palmitoleic acid to Wnt proteins which acts as a binding motif for Wnt receptors (Kadowaki et al., 1996, Janda et al., 2012, Komekado et al., 2007, Rios-Esteves et al., 2014, Willert et al., 2003). Then, Wnt ligands with lipid PTM are transported to the cell membrane by transmembrane protein Wntless/evenness interrupted (Wls/Evi), or GPR177 in mammals, and the Golgi apparatus (Bartscherer et al., 2006, Banziger et al., 2006, Yu et al., 2014). They are recycled back to trans-Golgi network by intracellular membrane trafficking complex Retromer, from where they are shuttled back to the ER to transport more Wnt ligands (Coudreuse et al., 2006, Gasnereau et al., 2011, Yu et al., 2014).

Different secretion mechanisms have been described for how processed Wnt ligands reach the receiver cells to activate the pathway including solubilisation of Wnt proteins to dissociate from cell membrane (Mulligan et al., 2012). Alternatively, Wnt ligand transport by packaging into lipid protein particles or exosomes has been suggested (Neumann et al., 2009, Gross et al., 2012). Although it remains unclear how exactly Wnt ligands reach the receiver cells to activate the pathway, the modes of travel seem to confer differential ranges. WNT3A proteins bound to the membrane are found to generate a concentrated area of high Wnt activity (Farin et al., 2016). Conversely, Wnt ligands packaged in exosomes are found to have long-range Wnt signalling activation, for example epithelial Wnt proteins inducing sperm maturation in epididymis (Koch et al., 2015).

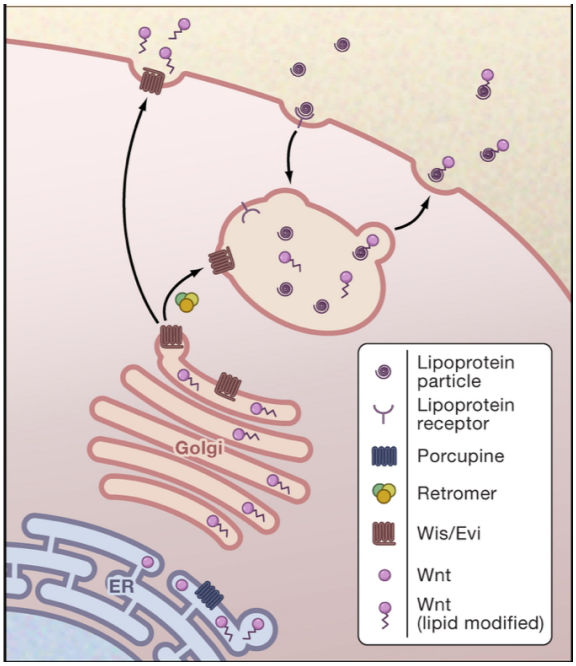

Figure 1.9 Schematic of Wnt secretion.

Wnt ligands in the ER are palmitoylated by Porcupine and then transported to the cell membrane for secretion by Wls/Evi (GPR177 in mammals). Packaging into the lipoprotein particles may be undertaken by endo/exocytic compartment involving the retromer complex. Figure adapted from Clevers, 2006.

Among the 19 different Wnt ligands, some are known to preferentially activate the canonical pathway while others are considered non-canonical ligands (Driehuis and Clevers, 2017). The activation of either canonical or non-canonical Wnt signalling is determined by the ligand and the receptor, rather than the Wnt protein alone, suggestive of another layer of regulation where cells control the receptor availability that the ligands encounter (Niehrs, 2012, Driehuis and Clevers, 2017). Wnt ligands WNT5A and WNT11 have been revealed to be capable of activating both canonical and non-canonical Wnt pathways as they can bind to a range of receptors including FZDs, and ROR1/2 in a context-specific manner (Gao et al., 2011, Heisenberg et al., 2000, Ying et al., 2008). Deletion of specific Wnt ligands yields distinct phenotypic outcomes, however a degree of functional redundancy of Wnt proteins has been

suggested (Stark et al., 1994, Chen et al., 2017a, Farin et al., 2012, Nusse and Clevers, 2017).

FZD receptors (FZD1-10 in human) are seven-pass transmembrane proteins with an extracellular N-terminal cysteine-rich domain (CRD) (Bhanot et al., 1996, MacDonald and He, 2012). Wnt ligands bound to the CRD allow FZD proteins to form a complex with single-pass transmembrane receptor LRP5/6 (Hsieh et al., 1999, Rulifson et al., 2000, Tamai et al., 2000, Pinson et al., 2000). LRP5/6 proteins form canonical Wnt co-receptors whose extracellular domain forms a complex with that of FZD with Wnt ligands (MacDonald and He, 2012). The phenotype of a transgenic model with *Lrp6* mutation is similar to that of Wnt mutants, highlighting its role in the canonical Wnt pathway (Pinson et al., 2000). Interestingly, Wnt ligands are found to have preferences over specific LRP6 extracellular domains suggesting that one LRP6 receptor may bind to more than one Wnt protein at a time (Bourhis et al., 2010). Indeed, blocking of LRP6 yields differential inhibition of Wnt ligand engagement depending on the ligand interface targeted (Ettenberg et al., 2010, Gong et al., 2010).

The canonical Wnt receptor complex interacts with AXIN and DVL (Bilic et al., 2007). Similar to their roles as components of β-catenin destruction complex, AXIN is recruited to the cell membrane after which GSK3 and CK1 phosphorylate the cytoplasmic tail of LRP (Davidson et al., 2005, Zeng et al., 2005). The interaction of DVL and AXIN which recruits AXIN to the cell membrane has also been shown to contribute to deactivating the β-catenin destruction complex (Fiedler et al., 2011). In addition to the multifaceted interplay of signal transduction components, the regulation of the pathway is complex at cell surface beyond Wnt ligands binding to their receptors.

1.2.3 Dynamic Wnt regulation at cell surface

Reflecting its role in key cellular processes, Wnt regulation is carefully carried out by multiple effectors in and out of the cell. The availability of these factors is crucial in moderating the Wnt pathway. Here, notable Wnt agonists and antagonists operating at cell surface level are summarised.

1.2.3.1 RNF43/ZNRF3 and LGR/RSPO

Modulation of the receptor level is one of the ways Wnt signalling is regulated. FZD receptor level is reduced by activities of RING finger 43 (RNF43) and zinc and RING finger 3 (ZNRF3), transmembrane E3 ubiquitin ligases that suppress Wnt activation by promoting lysosomal degradation of FZD via ubiquitination (Hao et al., 2012, Koo et al., 2012) (Figure 1.10). DVL has been found to be an essential component for RNF43/ZNRF3-FZD interaction (Jiang et al., 2015). As Wnt targets, RNF43/ZNRF3 represent a negative feedback mechanism of the canonical Wnt pathway (Koo et al., 2012, Takahashi et al., 2014). It has been suggested that such negative feedback upregulated upon pathway activation ensures that the activity is tightly controlled (de Lau et al., 2014).

Conversely, Wnt suppression by RNF43/ZNRF3-mediated receptor endocytosis has been shown to be mitigated by R-spondin (RSPO) binding to 7-transmembrane proteins LGR4/5/6 (de Lau et al., 2011, Koo et al., 2012). Both Wnt target genes as well as stem cell markers, LGR4/5/6 have an important role in augmenting Wnt activity (de Lau et al., 2011, Glinka et al., 2011). The LGR-RSPO interaction forms a complex with RNF43/ZNRF3 inhibiting FZD degradation (de Lau et al., 2011, Koo et al., 2012, Hao et al., 2012, de Lau et al., 2014). LGRs promote RSPO binding to RNF43/ZNRF3 rather than interacting directly with the E3 ligases (Chen et al., 2013). RSPO has been shown to enhance canonical Wnt activation as RSPO-overexpressing animals had abnormal expansion of crypts (Kim et al., 2005). In the niche, RSPO is mainly stromal in origin and functions as a potent Wnt positive regulator for LGR^+ cells (de Lau et al., 2011, Koo et al., 2012, Greicius et al., 2018, Kabiri et al., 2014). It has been reported that Wnt activation induces LGR5 expression, thus rendering the ISCs responsive to RSPO, which in turn promotes self-renewal and proliferation (Yan et al., 2017).

While LGRs are functionally similar, they show a differential expression pattern. For example, LGR5 is exclusively expressed by ISCs, whereas LGR4 is expressed across the crypt (de Lau et al., 2011). LGR6, on the other hand, is absent in normal

intestine, and instead is a marker for stem cells in the hair follicle (Snippert et al., 2010a). Notably, LGR5 is a marker for ISCs responsible for homeostatic turnover (Barker et al., 2007). The expression of LGR5 allows potentiation of Wnt activation in ISCs when RSPO is available in the microenvironment along with Wnt ligands (de Lau et al., 2011, Koo et al., 2012, Greicius et al., 2018, Kabiri et al., 2014). Adult ISCs expressing LGR5 can be stably expanded *ex vivo* in the long term as 3D organoid cultures, in media containing key niche factors such as Wnt and RSPO (Barker, 2014).

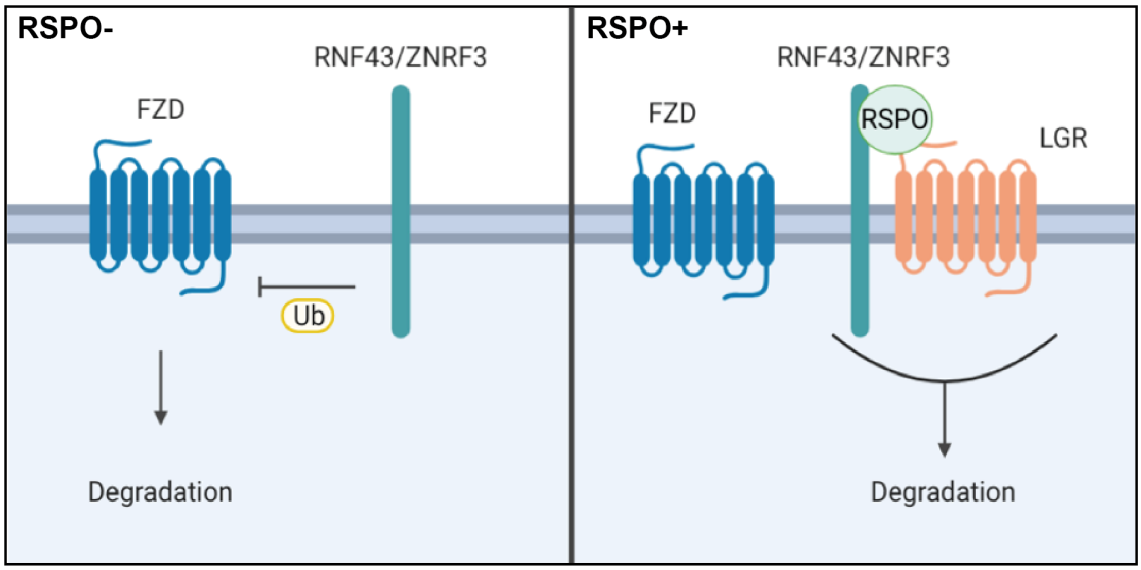

Figure 1.10 Wnt receptor FZD availability modulated by RNF43/ZNRF3, LGRs, and RSPO.

In the absence of R-spondin (RSPO), transmembrane E3 ubiquitin ligases RING finger 43 (RNF43) and zinc and RING finger 3 (ZNRF3) target Frizzled (FZD) for ubiquitination promoting its internalisation and degradation. When RSPO is bound to leucine-rich repeat-containing G protein-containing receptor (LGR) and RNF43/ZNRF3, the complex is internalised, inhibiting RNF43/ZNRF3-mediated FZD turnover. Subsequent stabilisation of FZD on cell membrane enhances Wnt activity when the pathway is activated in the presence of Wnt ligands. Figure created with Biorender.com.

1.2.3.2 Wnt protein-Wnt receptor interaction competitors

Alternative negative regulatory mechanisms by Wnt antagonists prevent receptor binding of Wnt proteins by interacting with Wnt receptors or the ligands (Figure 1.11). Dickkopf (DKK) is a group of secreted proteins constituting 4 members (DKK1-4) in vertebrates, of which three acts as Wnt antagonists except for DKK3 (Glinka et al., 1998, Krupnik et al., 1999, Mao et al., 2001, Mao and Niehrs, 2003). Wnt antagonist DKKs have been shown to bind to LRP5/6 and prevent formation of LRP-FZD complex (Glinka et al., 1998). The DKK-mediated Wnt suppression also involves transmembrane receptor Kremen, which has been found to interact with DKK1 and LRP6 to promote internalisation of the Wnt receptor LRP6 (Mao et al., 2002). The suppressive effect mediated by DKKs are specific to the β-catenin-dependent Wnt pathway as it targets LRPs (Bafico et al., 2001, Mao et al., 2001, Semënov et al., 2001). On the other hand, DKK3 is associated with the β-catenin-independent pathway and does not involve Kremen (Krupnik et al., 1999, Mao et al., 2001, Mao and Niehrs, 2003).

Wnt ligand binding to LRPs has also been suggested to be interrupted by WISE and Sclerostin (SOST) (Itasaki et al., 2003, Semenov et al., 2005). WISE, also known as Sclerostin domain containing 1, uterine sensitisation-associated gene-1, and Ectodin, competes with Wnt proteins to bind to LRP6 either in a secreted or intracellular form (Itasaki et al., 2003). However, the function of extracellular WISE is suggested to be context-dependent as it can also activate Wnt pathway during development (Driehuis and Clevers, 2017). In the ER, WISE is found to suppress LRP6 expression on the cell membrane (Guidato and Itasaki, 2007). Antagonistic action of SOST stems from its binding to LRP resulting in failure of LRP-FZD complex formation (Semenov et al., 2005).

Moreover, Wnt activity is inhibited by Wnt antagonists directly interacting with Wnt ligands. The first extracellular de-acylase to be described as a Wnt inhibitor, Notum, targets Wnt proteins to cleave their palmitoylation moiety removing the docking site for FZD (Kakugawa et al., 2015). Secreted frizzled-related proteins (SFRPs) have an N-terminal CRD domain as FZDs and a netrin-related motif (NTR) at the C-terminus (Hoang et al., 1996, Bhat et al., 2007). The CRD domain is reminiscent of that in

FZDs and allows SFRPs to compete with Wnt receptors for Wnt ligands (Dann et al., 2001). In addition, direct interaction of SFRP and FZD by CRD dimerisation has been suggested to inhibit the Wnt pathway by sequestering FZD receptors (Dann et al., 2001, Cruciat and Niehrs, 2013). Wnt-inhibitory factor 1 (WIF-1) exerts a Wnt suppressive effect by binding to Wnt proteins preventing the interaction with Wnt receptors necessary for Wnt activation (Surmann-Schmitt et al., 2009, Driehuis and Clevers, 2017).

Homeostatic ISC behaviour is regulated by several pathways and better understanding of them provides insights into intestinal diseases such as CRC. In particular, abnormal Wnt activation in ISCs has prominent implication in tumourigenesis (Barker et al., 2009, Schepers et al., 2012). Investigating the pathway dysregulation in CRC at multiple levels, both intracellular and extracellular, will offer novel therapeutic applications.

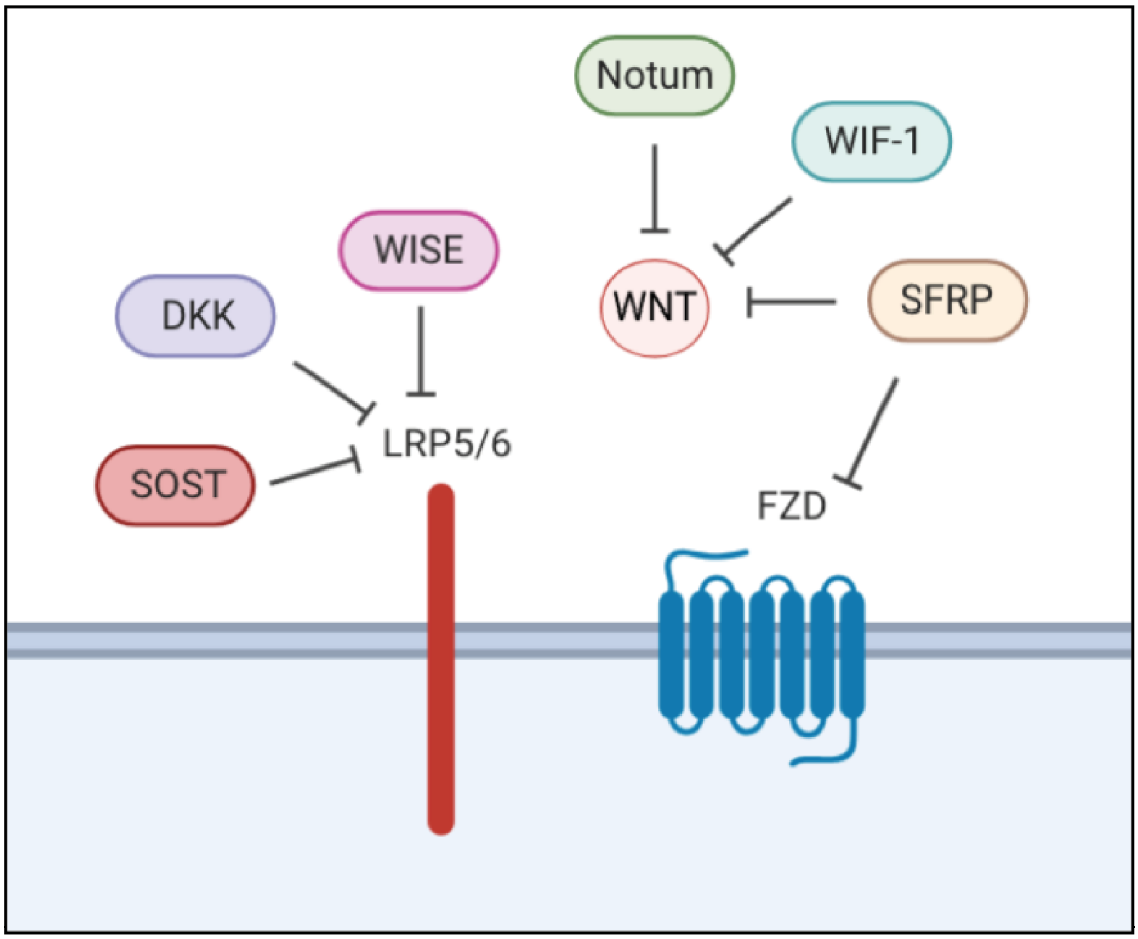

Figure 1.11 Robust regulation of Wnt pathway by secreted factors.

Secreted factors WISE, Dickkopf (DKK), and Sclerostin (SOST) interact with LRP competing with Wnt ligands to suppress Wnt activation. Notum, Wnt-inhibitory factor 1 (WIF-1), and Secreted frizzled-related proteins (SFRPs) negatively regulate the canonical Wnt pathway by interacting with Wnt proteins directly. In addition, SFRPs dimerise with Frizzled (FZD) receptors thus preventing Wnt ligand binding. Figure adapted from Driehuis and Clevers, 2017. Figure created with Biorender.com.

1.3 Colorectal cancer biology

Colorectal cancer is one of the most common cancers worldwide and is the second leading cause of cancer-related mortality (Bray et al., 2018). The majority of CRCs progress by accumulating mutations in tumour suppressor genes and oncogenes (Cristobal et al., 2017). Common mutations found in CRCs include functional loss of tumour suppressors and activating mutations in pathways regulating an array of cell behaviour, including the canonical Wnt pathway (Wood et al., 2007). Aberrant inactivation of APC or activation of β-catenin act as driver mutations in CRC and are often followed by alterations in KRAS and PI3K pathways (Bos et al., 1987, Powell et al., 1992, Janssen et al., 2006, Cristobal et al., 2017). Additionally, mutations that inactivate tumour protein 53 (TP53) or genes of the SMAD family are found to accumulate in cancer cells exacerbating the disease (Vogelstein et al., 1988, Rodrigues et al., 1990). Although such mutational events often come in a sequential pattern in CRCs, the accumulation seems to hold more weight than the sequence (Fearon and Vogelstein, 1990, Cristobal et al., 2017).

CRC is a highly varied disease as heterogeneity is prevalent with regards to the mutation as well as the signalling pathways involved (Markowitz and Bertagnolli, 2009). Consequently, the pathology, molecular and phenotypic profiles, and response to therapy are heterogeneous (Tenesa and Dunlop, 2009). Recent technological advances have enabled comprehensive characterisation of CRCs to better understand the molecular profiles of CRCs and associated patient outcomes (Guinney et al., 2015). Here, Wnt dysregulation in CRC and CRC molecular subtypes are summarised. Furthermore, tumour microenvironment (TME) with a particular focus on cancer-associated fibroblasts (CAFs) and their role in tumour progression are described.

1.3.1 Wnt hyperactivation in CRC

The link between tumourigenesis and the Wnt pathway was first elucidated when exogenous activation of Wnt1 by transgenic overexpression or viral insertion resulted in mammary tumour formation (Nusse and Varmus, 1982, Tsukamoto et al., 1988). Soon after, mutation in a major component of canonical Wnt pathway, APC, was

implicated in a hereditary colon cancer (Kinzler et al., 1991, Nishisho et al., 1991). Loss of APC function was found to upregulate β-catenin activation demonstrating a causal relationship between Wnt and CRC (Korinek et al., 1997). It is now known that mutations in the Wnt pathway are one of the main events that drive tumourigenesis of CRC (Segditsas and Tomlinson, 2006, Network, 2012).

Underscoring the functional significance of the pathway, mutations in *APC* and *β-catenin* are one of the earliest mutations in the majority of CRCs and initiate crypt hyperplasia with abnormal upregulation of Wnt target genes (Polakis et al., 1999, Reya and Clevers, 2005, Humphries and Wright, 2008). High nuclear localisation of β-catenin serves as a poor prognosis marker in CRC (Baldus et al., 2004). APC inactivation mutations are now identified in approximately 80% of sporadic CRCs (Morin et al., 1997, Cheng et al., 2019). Different mutations in *APC* have been found to cause differential degrees of Wnt hyperactivation as well as tumour location in the large intestine (Christie et al., 2013, Buchert et al., 2010). Investigations using reversible APC knock-out (KO) in animal models have revealed that APC dysregulation is key in maintaining tumourigenic hyperproliferation (Dow et al., 2015). Hyperactivation of the Wnt pathway leads to overexpression of Wnt target genes that facilitate CRC progression. For example, upregulation of Wnt targets c-MYC and CYCLIN D1 have been linked to exacerbation of CRC (He et al., 1998, Tetsu and McCormick, 1999). Furthermore, β-catenin signalling upregulates transcription factors regulating genes of epithelial-to-mesenchymal transition (EMT), subsequently promoting invasion and metastasis (Yang et al., 2006).

Although less common than APC mutations, mutations of other Wnt components have been identified in CRCs (Figure 1.12). Mutually exclusive to APC mutations, loss of RNF43 accounts for approximately 20% of CRCs (Giannakis et al., 2014). These CRC cells are found to be sensitive to therapeutic approaches targeting Wnt secretion as the dysregulation of the pathway is at cell surface level, hence still dependent on Wnt ligands for proper Wnt activation (Seshagiri et al., 2012, van de Wetering et al., 2015). Fusion proteins containing *RSPO2* or *RSPO3* resulting in aberrant overexpression are also detected in non-APC mutant CRCs promoting Wnt hyperactivation (Seshagiri et al., 2012, Hashimoto et al., 2019). Mutations in TCF family protein are found in some CRCs but the functional impact of the mutations on

Wnt activity are not yet clearly defined (Duval et al., 1999, Shimizu et al., 2002, Bass et al., 2011, Cuilliere-Dartigues et al., 2006, Network, 2012). Moreover, Wnt hyperactivation in CRC may be in part due to epigenetic modifications. WNT5A expression has been revealed to be epigenetically silenced in CRCs, abolishing its suppressive effect on canonical Wnt activity (Ying et al., 2008). *AXIN2* has been shown to be epigenetically silenced in some CRCs conveying a similar activating effect on Wnt pathway as APC mutations (Koinuma et al., 2006). Downregulation of secreted Wnt antagonists such as DKK, WIF-1, and SFRP by hypermethylation has been observed to lead to enhanced Wnt activity (Suzuki et al., 2004, Taniguchi et al., 2005, Qi et al., 2006, Rawson et al., 2011, Voorham et al., 2013, Silva et al., 2014).

Wnt hyperactivation has also been identified to result from upregulation of factors not directly involved in Wnt signal transduction. For example, upregulation of BCL9, a co-activator of the TCF/LEF transcription complex for β-catenin-dependent Wnt transcription programme, is commonly found in cancer (Takada et al., 2012). Increased expression of Proliferating cell nuclear antigen (PCNA)-associated factor in CRC has been suggested to promote recruitment of histone methyltransferase EZH2 to the TCF/LEF transcription complex resulting in hyperactivation of β-catenin signalling (Jung et al., 2013). Differentiated intestinal epithelial cells are found to de-differentiate into ISCs upon NF-kB pathway activation which enhances Wnt pathway, resulting in tumour formation (Schwitalla et al., 2013).

Moreover, investigation of upregulated genes in CRC has revealed a novel participant in the Wnt pathway. Tribbles pseudo-kinase 3 (TRIB3), highly expressed in some CRCs with poor prognosis, is a Wnt target gene that provides a positive feedback loop by promoting β-catenin-TCF/LEF interaction in the nucleus (Hua et al., 2019, Miyoshi et al., 2009). As a stress sensor, TRIB3 is upregulated in response to hypoxic, inflammatory, ER, and metabolic stresses (Ohoka et al., 2005, Prudente et al., 2012, Eyers et al., 2017). TRIB3 stabilises the β-catenin-TCF/LEF complex and increases Wnt target gene expression, many of which promote proliferation (Zheng et al., 2008, Hua et al., 2019).

The abnormal activation of the canonical Wnt pathway in CRC is responsible for proliferation of cancer cells, EMT, and metastasis. Notably, aberrant Wnt activation

is also present in CRC even without activating mutations in the pathway (Polakis, 2007, Yang et al., 2016). The CRC pathology, as well as the Wnt pathway, are emerging to be complex with yet unknown players. The microenvironment is attracting much attention as another contributor to tumour progression. It is key to consider the multidimensional roles of the TME and stromal cells to fully appreciate the complexity of CRC and Wnt hyperactivation. Recent advances in sequencing technologies have enabled characterisation of CRCs to scrutinise the tumour composition and gene expression pattern capturing the changes in cancer cells as well as the TME (Network, 2012, Guinney et al., 2015).

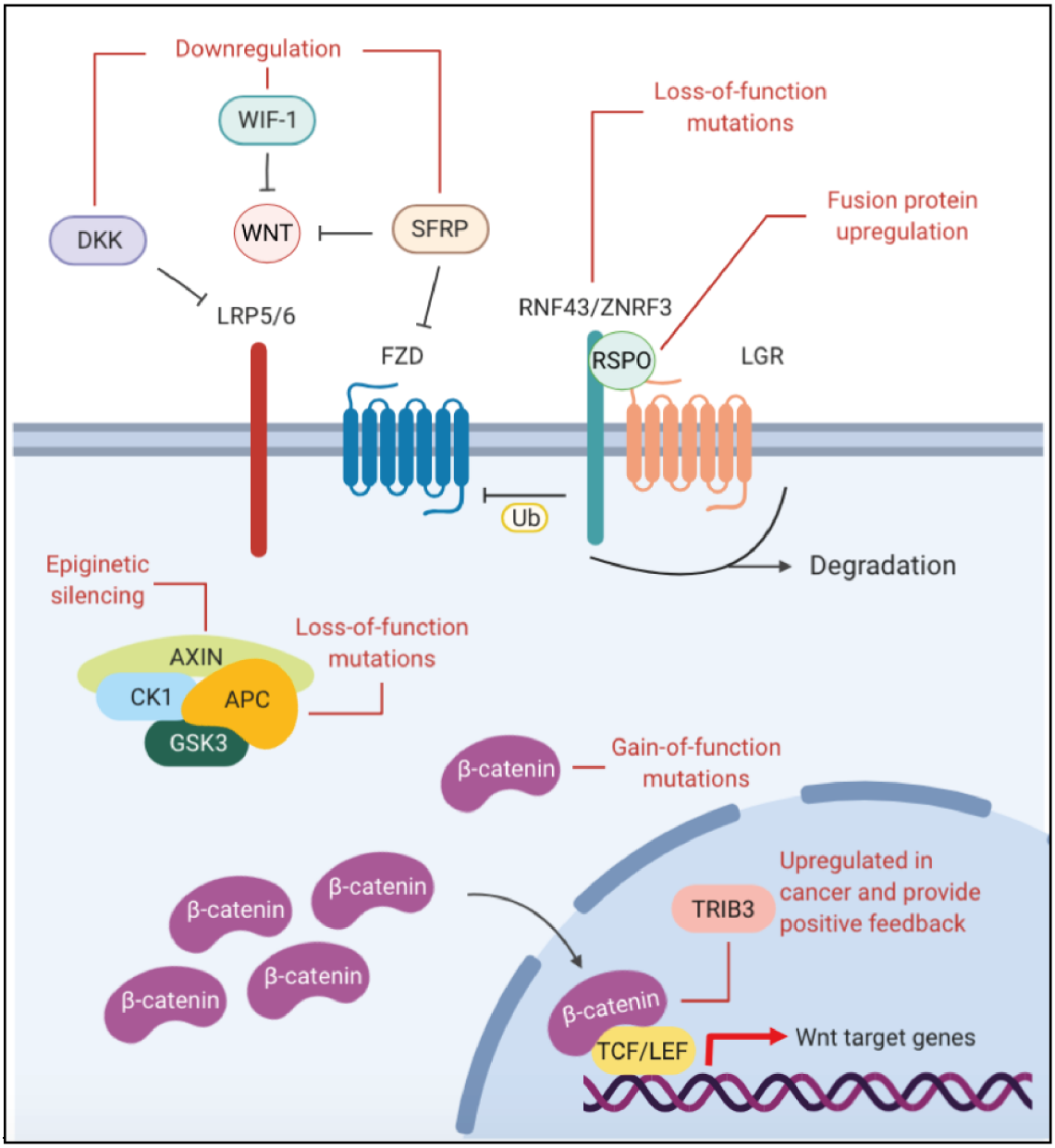

Figure 1.12 Summary of selective known mutations and alterations of Wnt components observed in CRC.

Secreted Wnt antagonists DKK, WIF-1, and SFRP are downregulated, and Wnt target *AXIN2* is epigenetically modified in CRC. Negative Wnt regulator RNF43/ZNRF3 and β-catenin destruction complex component APC are identified to harbour loss-of-function mutations in CRC. Canonical Wnt effector β-catenin has gain-of-function mutations that facilitate its accumulation in the absence of Wnt ligands. Fusion proteins of Wnt potentiator RSPO are abnormally upregulated in CRC. TRIB3 is a Wnt target upregulated in cancer that forms a positive feedback loop by stabilising β-catenin-TCF/LEF interaction. Figure created with Biorender.com.

1.3.2 CRC molecular subtypes

Colorectal cancers have been largely classified into microsatellite stable (MSS) CRCs that harbour oncogenic mutations such as APC loss, and those with microsatellite instability (MSI) that does not originate from cells with oncogenic mutations (Boland and Goel, 2010). Impaired DNA mismatch repair machinery in MSI CRCs results in hypermutation at sequence repeats or microsatellites, and hypermethylation of the genome that frequently leads to downregulation of tumour suppressors (Boland and Goel, 2010). MSI early-stage CRCs can subsequently obtain APC or other mutations thus rendering classifications muddled with MSS CRC.

Recent meta-analyses of high-throughput sequencing data from a large cohort of CRC patients have enabled classification of CRCs into four molecular subtypes (Guinney et al., 2015). The four consensus molecular subtypes (CMS1-4) are proposed with distinct characteristics in gene expression profile and tumour phenotype integrated with additional published data such as mutations, epigenetic changes, copy number variations, and patient outcome (Guinney et al., 2015) (Figure 1.13). This classification allows a more informed categorisation of CRC molecular profile with regards to patient details compared to a previous division of MSS and MSI that mainly focused on alterations in cancer cells since the CMS system takes gene expression changes in the TME into consideration as well (Isella et al., 2015, Colangelo et al., 2017). Further studies dissecting the expression profiles in cancer cells and stromal cells have revealed that genes associated with poor prognosis are in fact upregulated in stromal cells rather than cancer cells (Isella et al., 2015, Calon et al., 2015).

The molecular profiles of the CMS differ as well as patient outcomes. The majority of MSI tumours show traits of CMS1, such as defective DNA mismatch repair, hypermethylation, low somatic copy number alterations (SCNAs), and high immune infiltration (Guinney et al., 2015). The high immune infiltration and mutational load of CMS1 are thought to translate to good patient outcome observed in this subtype (Becht et al., 2016). CRCs of CMS2 are characterised by high proportion of oncogenic copy number gains and tumour suppressor copy number losses, and differentiation of epithelial cells by Wnt and MYC activations during tumourigenesis

(Guinney et al., 2015). Metabolic adaptation and KRAS activation define CMS3 whilst CMS4 includes some MSI showing prominent upregulation of EMT genes, angiogenesis, and TGF-β activation (Guinney et al., 2015). CMS4 CRCs show the worst patient outcome due to high immune evasion in the TME, which is comprised mainly of CAFs that promote metastasis and suppress activity of immune infiltrates in the tumour (Becht et al., 2016). Despite the upregulation of lymphoid gene expression observed in CMS4, antigen-presenting cells fail to mature and in turn T cells are not activated, leading to poor relapse-free and overall survival (Becht et al., 2016). Therefore, it is crucial to also consider the contribution of the TME and stromal cells in CRC to fully appreciate the disease mechanism(s).

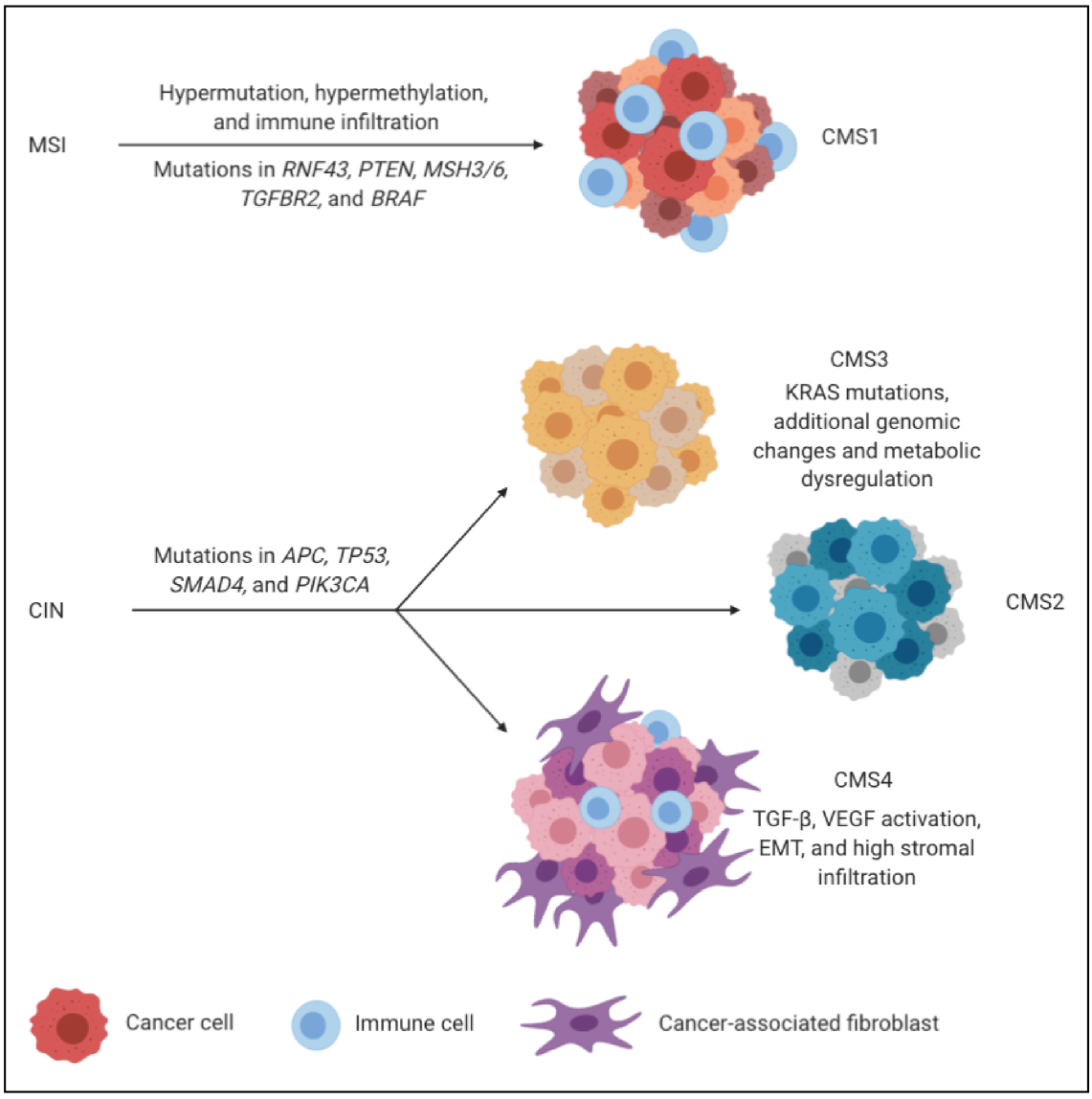

Figure 1.13 Consensus molecular subtypes of CRC.

Majority of tumours with microsatellite instability (MSI) are found to be CMS1 distinguished by hypermutation, hypermethylation, and high immune infiltration along with mutations in genes described above (*APC* mutations not as common as in CMS2-4). Most microsatellite stable (MSS) tumours and some MSI tumours with chromosomal instability (CIN) can be classified as either CMS2, 3, or 4. CMS2 CRCs show high Wnt activation by *APC* mutation. CMS3 tumours show metabolic deregulation, whereas CMS4 CRCs are characterised by high stromal infiltration, activation of transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), and epithelial-to-mesenchymal transition (EMT). Figure adapted from Dienstmann et al., 2017, and created with Biorender.com.

1.3.3 Cancer-associated fibroblasts in CRC microenvironment

The TME has characteristics distinct from its normal counterpart. While many studies have identified key mutations in cancer that result in dysregulation of pathways responsible for stem cell proliferation and differentiation, stromal cells in the TME such as endothelial cells, immune cells, and CAFs are now beginning to be recognised for their roles in tumour progression (Wood et al., 2007, Network, 2012, Calon et al., 2015, Becht et al., 2016). The poorest patient prognosis associated with CMS4 also underscores the contribution of stromal cells on tumour malignancy since it is the subtype showing the highest stromal infiltration (Colangelo et al., 2017). In particular, CAFs have been implicated in tumourigenesis by enhancing cell proliferation and invasion. The complex activities that CAFs perform aid tumour cells to succeed in pro-tumourigenic microenvironment (LeBleu and Kalluri, 2018).

Once dubbed as “wounds that never heal,” tumour stroma and wounds share similarities such as fibroblast activation, and altered ECM deposition and remodelling (Dvorak, 1986, Foster et al., 2018). Growing tumours also show similar changes in the microenvironment to healing wounds including fibronectin (FN) deposition and crosslinking, and immune cell recruitment (Schafer and Werner, 2008). The majority of CAFs express ACTA2 and essentially carry out what $ACTA2^{+}$ myofibroblasts do during wound healing but in a chronic manner to confer pro-tumourigenic effects (Shiga et al., 2015). Compared to normal fibroblasts, CAFs show increased deposition of collagen and other ECM proteins as well as secretory factors that alter cancer cell behaviour (Xing et al., 2010, Shiga et al., 2015). Moreover, CAFs are more proliferative and retain the activated state for longer, making them one of the most common cellular components of the TME (Kalluri and Zeisberg, 2006, Pietras and Ostman, 2010).

While normal fibroblasts develop from primitive mesenchyme, variation in CAF origin has been reported. Resident fibroblasts can be recruited and activated to adopt CAF phenotype (Fukino et al., 2004). Epithelial cancer cells may undergo EMT to transdifferentiate into CAFs via a series of oxidative stress-induced mutational events (Radisky et al., 2005, Kalluri and Zeisberg, 2006, Cirri and Chiarugi, 2011). Similar transdifferentiation of endothelial cells into CAFs has been suggested via

endothelial-to-mesenchymal transition whereby endothelial cells in the TME lose endothelial marker expression and upregulate fibroblast markers such as ACTA2 (Zeisberg et al., 2007). Moreover, it has been shown that mesenchymal cells residing in the bone marrow may differentiate into CAFs (Quante et al., 2011).

In normal colon, fibroblasts comprise the majority of stromal population in the lamina propria carrying out ECM deposition and maintenance of the basement membrane (Colangelo et al., 2017). Tumourigenesis is often triggered when the communication between these fibroblasts and epithelial cells via multiple signalling pathways are dysregulated (Karagiannis et al., 2012). Cancer cell-derived reactive oxygen species, TGF-β, interleukins (IL-4/6), and prostaglandin E (PGE) have been reported to induce activation of fibroblasts into CAFs in CRC (Cirri and Chiarugi, 2011, Hawinkels et al., 2014). ACTA2-expressing pericryptal myofibroblasts that mainly deposit ECM proteins for wound healing when activated upon tissue injury are considered to be the major source of CAFs in CRC; the two cell types have similar transcriptomic profiles (Gabbiani et al., 1971, Chang et al., 2004, De Wever et al., 2008). However, CAFs show a more intense secretory phenotype producing growth factors, ECM proteins, and ECM remodelling enzymes (Paunescu et al., 2011). It has also been revealed by single cell RNA-sequencing of CRC samples that CAFs are distinguishable from normal fibroblasts by their participation in the TGF-β signalling pathway (Li et al., 2017).

Although functionally and phenotypically distinct, defining conclusive CAF gene marker(s) has proven difficult due to overlaps with other cell types, which most likely reflects the heterogeneous cellular source of CAFs (Bu et al., 2019). For example, upregulation of fibroblast-specific protein (FSP1) is observed in CAFs but it is also a marker for several immune cell types including macrophages (Osterreicher et al., 2011). Nevertheless, relatively distinct upregulation of ACTA2 and fibroblast-activation protein (FAP) has been reported, and rather than an exclusive set of CAF markers, a collection of genes upregulated in CAFs has been identified to hold prognostic value in CRC (Herrera et al., 2013). Indeed, the gene expression pattern of CAFs is capable of predicting response to treatments, relapse, and/or survival of CRC patients, which is indicative of their important role in tumour progression (Berdiel-Acer et al., 2014b, Berdiel-Acer et al., 2014a).

Unlike normal myofibroblasts in homeostatic healing wounds, CAFs promote migration, invasion, and proliferation of cells resulting in deviation of cells from the initial remodelling site and expansion to neighbouring areas (Bauer et al., 2010, Xing et al., 2010, Pidsley et al., 2018). One of CAF-mediated tumourigenic effects arises from CAFs modulating the ECM by aberrant synthesis and remodelling (Faouzi et al., 1999, Erdogan et al., 2017). Much of collagen in the tumour has been shown to be secreted by CAFs as well as laminin that promotes cancer cell migration (Faouzi et al., 1999, Fullar et al., 2015). CAFs also produce ECM remodelling enzymes such as lysyl oxidase (LOX) and matrix metalloproteinases (MMPs), which can alter ECM stiffness (Pickup et al., 2013, Torres et al., 2015). The LOX family of enzymes include LOX and LOX-like (LOXL) 1-4 and induces collagen crosslinking by oxidation, increasing the stiffness of the TME (Wang et al., 2016). In CRC, higher expression of LOXL2 in CAFs compared to normal fibroblasts (NFs) from the same patient has been associated with poor prognosis (Torres et al., 2015). LOXL2 overexpression by CAFs is also associated with enhanced invasion of gastric cancer cells (Kasashima et al., 2014). Commonly found in the TME due to increased collagen deposition and crosslinking, aligned collagen fibres are suggested to provide a pathway for cells to travel (Nebuloni et al., 2016). MMPs are zinc-containing endopeptidases that allow digestion of ECM and migration of cells to distant sites, and upregulation of many of the 24 mammalian MMPs has been observed in multiple cancers (Vandenbroucke and Libert, 2014, Liu et al., 2019). However, the role of individual MMPs has not been clearly defined as of yet; some are associated with poor patient outcome while others are positively correlated with patient survival (Iyer et al., 2012).

Moreover, it has been demonstrated upregulation of FN in human tumours can be attributed to CAFs in addition to cancer cells (Attieh et al., 2017, Erdogan et al., 2017). FN is associated with poor prognosis as it enhances cell cohesion, invasion, and migration of cancer cells in multiple cancers (Menzin et al., 1998, Serres et al., 2014, Yi et al., 2016). Increased FN fibrillogenesis and contractility by CAFs confer invasiveness to cancer cells (Attieh et al., 2017). Dysregulated ECM composition and assembly by CAFs generates a TME of an altered architecture that promotes cell invasion and tumour progression (Malik et al., 2015). The stiffness has also been suggested to provide a mechanical positive feedback loop in which CAFs maintain

the activated phenotype due to stiffness, subsequently inducing the ECM to become stiffer (Calvo et al., 2013).

In addition to ECM remodelling that alters biomechanical cues, CAFs influence cancer cells via direct cell contact or paracrine factors (Liu et al., 2019). Direct CAF-cancer cell interaction is attributed to greater motility of cancer cell (Choe et al., 2013). CAFs have been described to mediate invasion by forming adhesion complexes of E-cadherin/N-cadherin with cancer cells (Labernadie et al., 2017). On the other hand, CAFs produce secretory factors to modulate cancer cell behaviour such as hepatocyte growth factor (HGF), EGF, and macrophage migration inhibitory factor (MIF) (Cirri and Chiarugi, 2011, Hawinkels et al., 2014). CAF-derived HGF, as well as osteopontin (OPN), have been implicated in cancer stem cells de-differentiating from cancer cells (Todaro et al., 2014). Moreover, increased secretion of Tenascin-C (TNC) by CAFs stimulated with HGF or TGF-β has been implicated in enhancing invasiveness and metastasis of cancer cells (De Wever et al., 2004). Fibroblast growth factor 1 (FGF1) produced by CAFs increases the mobility and invasive capacity of cancer cells (Henriksson et al., 2011). Secretion of fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor A (VEGFA) by CAFs promotes angiogenesis in the TME facilitating nutrient delivery to cancer cells (De Palma et al., 2017). Soluble factors of the cytokine/chemokine family are also upregulated in CAFs contributing to tumour progression. Angiogenesis is also promoted by CAF-derived C-X-C motif chemokine 12 (CXCL12) that recruit endothelial cells to the tumour, and neutralising antibody treatment has yielded reduction in tumour volume (Orimo et al., 2005).

1.4 Hypothesis

Tumours are now recognised to be a tissue comprised of cancer cells with unrestrained proliferative capacity and stromal cells within the TME that generate the physical support for cells to reside in, supplying altered biomechanical and biochemical cues (Hanahan and Weinberg, 2011, Quail and Joyce, 2013). As the

major cellular component of the TME, CAFs are responsible for remodelling the TME as well as secreting factors that modulate cell behaviour to promote malignancy.

One of the crucial pathways driving tumour progression in CRC is the canonical Wnt pathway, reflective of its import in ISC homeostasis (Vermeulen et al., 2010). Wnt hyperactivation by stabilisation and accumulation of β-catenin is commonly observed regardless of mutations in the pathway components (Polakis, 2007, Yang et al., 2016). CRCs with mutations in APC or β-catenin have been shown to be dependent on Wnt ligands for Wnt pathway activation (Voloshanenko et al., 2013, Scholer-Dahirel et al., 2011). Tumour progression in CRCs may require exogenous Wnt stimulation, either by enhancing receptor sensitivity in *APC*/*β-catenin* mutants, or by augmenting extracellular signals as an alternative Wnt activating mechanism. One of the major sources of Wnt ligands is the TME. Heterogeneous Wnt activity in epithelial cancer cells has been observed within a tumour, cells with higher activity residing close to the stroma (Guinney et al., 2015, Baldus et al., 2004). Of note, CMS4 with the highest stromal infiltration is associated with the poorest patient outcome highlighting the contribution of CAFs in tumour progression. I hypothesise that CAF-derived factors include yet unidentified paracrine Wnt regulators.

To explore potential paracrine Wnt regulator(s) secreted by CAFs in CRC, a public transcriptomic data of a large CRC patient cohort was analysed (Network, 2012). A set of genes reported to be CAF markers and several ECM components are revealed to have similar upregulated expression pattern following hierarchical clustering analysis. This gene set is referred from now on as “CAF-enriched cluster” containing CAF genes and stromal factors. Notably, CAF markers in the cluster are associated with poor patient outcome indicative of their impact in tumour progression (Calon et al., 2015). I hypothesise that the cluster includes putative Wnt regulators whose upregulation in CRC similar to such poor prognosis markers indicates alternative pro-tumourigenic Wnt activation and/or potentiation.

1.5 Aim

The aim of my PhD is to characterise paracrine Wnt regulation by factors upregulated in CAFs that contribute to aberrant Wnt activation in CRCs even without activating mutations in the Wnt pathway. Furthermore, I seek to build an *ex vivo* model to better study the crosstalk between epithelial cancer cells and CAFs.

A candidate Wnt regulator, PERIOSTIN (POSTN), is identified and potential CAF-mediated effect on Wnt activity is investigated. Specific aims of the project are as follows: 1) to establish patient-derived fibroblasts as a reliable *ex vivo* models, 2) to determine the role and underlying molecular mechanism of POSTN in the Wnt pathway, and 3) to tissue-engineer an *in vitro* 3D co-culture system of epithelial cells and fibroblasts.

Chapter 2. Materials and Methods

2.1 Cell line culture

HEK293T and HCT116 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) GlutaMAX (Gibco) supplemented with foetal bovine serum (FBS) at 5% final concentration and 100 units/ml penicillin (Gibco) and 100 µg/ml streptomycin (Gibco). Cells were passaged using TrypLE (Gibco) and maintained in a humidified atmosphere of 5% CO_2 at 37°C.

2.2 Human primary intestinal organoid culture

Small intestinal organoid cultures established from human biopsies were provided by Laween Meran and Isobel Massie.

Fresh CRC tissues were collected at University College London Hospital with informed consent. For primary culture establishment, the sample was cut into small pieces and resuspend in digestion medium containing 2 µg/ml Hyaluronidase, 1 mg/ml Collagenase II, and Y-27632 (Sigma) in Advanced DMEM supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, HEPES (Invitrogen), and Glutamax (Invitrogen, Ad-DF+++). The mix was incubated in a shaker at 37°C for 1 hour and passed through 70 µM cell strainer. The remaining tissue pieces were further used for fibroblast isolation as described below. The flow-through was then added with FBS at a final concentration of 5% and centrifuged for 10 minutes with break on 3 at 1500 rpm. The cell pellet was then washed with Advanced DMEM three times. The final pellet resuspended in Cultrex® BME Type 2 RGF PathClear (Amsbio) was seeded in 24 well plate and cultured as previously described (Sato et al., 2011a). Normal and tumour organoids were isolated from the same patient when possible.

Both small intestinal and colonic organoids were cultured in human organoid expansion media containing WNT3A, RSPO1, and Noggin conditioned media, nicotinamide (Sigma), N-Acetylcysteine (Sigma), EGF (Invitrogen), gastrin (Sigma), SB202190 (p38 inhibitor, Sigma), A83-01 (TGF-β inhibitor, Tocris Bioscience), and

primocin (Invitrogen). Noggin and RSPO1 conditioned media were generated in HEK293T cells, and WNT3A media in L cells. Newly passaged organoids were treated with Y-27632. Images of organoids were acquired using EVOS FL Cell Imaging System (Life Technologies).

2.3 Human primary fibroblast culture

To generate patient-derived primary fibroblasts, remnant tissue pieces from CRC organoid isolation were placed on 6 well plates and slowly immersed in fibroblast growth media, DMEM GlutaMAX (Gibco) supplemented with FBS at 5% final concentration, insulin transferrin solution (Invitrogen) at 1% final concentration, and 100 units/ml penicillin and 100 µg/ml streptomycin. Media was replaced every two days for 7 – 10 days. When fibroblasts attached to the cell plate were observed, original tissues were removed and fibroblasts were re-plated in a new dish using TrypLE. Primary fibroblasts were maintained in DMEM supplemented as described previously, and used for experiments until they reached passage number 10. Human normal colon fibroblasts CCD-18Co (ATCC® CRL-1459™) were cultured in the same media condition described above for primary fibroblasts.

2.4 MTT assay

Viability was examined using Thiazolyl Blue Tetrazolium Bromide (MTT, Sigma). Culture medium was replaced with media containing 1.2 mM MTT and incubated for 1 hour at 37°C. The dye was solubilised with acidified isopropanol (40 mM HCl). Optical density at 560 nm was measured at indicated time points.

2.5 Contractility assay

Fibroblasts were trypsinised and resuspended in culture medium at 1 x 10^6 per ml concentration. Rat tail collagen I (Thermo Fisher Scientific) supplemented with 1x Minimum Essential Media (MEM, Gibco) was neutralised with sodium hydroxide. Resuspended cells were mixed in neutralised collagen I and aliquoted into individual

wells of 96 well plate. After 1 hour in the incubator to set the collagen I mix, gels were released from the edges of the well. Fibroblast culture medium was added on top and maintained for 14 days. Images were taken regularly and contraction over time was quantified using Fiji.

2.6 RT-qPCR

RNA was extracted using RNeasy® Kit-Qiagen, according to the manufacturer's protocol. 0.5 – 1 µg of RNA was reverse transcribed to cDNA with cDNA synthesis kit (Thermo Fisher Scientific) following the manufacturer's instructions. Quantitative real-time PCR (RT-qPCR) were carried out in a 12 µl reaction mixture comprised of 5 µl 2X SYBR Green and 500 nM of each primer (see primers in Table 1). The reaction mixture without template cDNA was used as a control. Samples were normalised to housekeeping genes *β-ACTIN* or *Peptidylpropyl isomerase B* (*PPIB*).

2.7 TCF-TOPFlash assay

Cells were plated at 30% confluency and transfected at 60% confluency in 48 well plate the day before and transfected using Polyethylenimine (PEI, Polysciences) as per the manufacturer's instructions. 24 hrs after seeding, cells in each well were transfected with 100 ng TOPFlash or 100 ng FopFlash TCF luciferase-reporter plasmids. The TOPFlash construct carries ten binding motifs for TCF, cloned upstream of a minimal promoter while FopFlash have mutations (Korinek et al., 1997). Transfection efficiency was normalised by renilla luciferase transfected at 10 ng per well together with either TOPFlash or FopFlash. Cells were then treated overnight with control, WNT3A conditioned media, or WNT3A conditioned media with RSPO1 conditioned media when indicated 24 hours post-transfection. After overnight treatment, cells were lysed with luciferase lysis buffer (Promega), and the activity of luciferase reporter was measured using the Dual-Luciferase-reporter assay system (Promega) with a microplate luminometer (Berthold Technologies).

TOPFlash assay with primary fibroblasts was carried out using HEK293T Wnt luciferase reporter cells that contain TOPFlash construct of seven TCF response

elements and renilla (Li et al., 2013). Fibroblasts and reporter cells were seeded at 1:1 ratio and harvested 24 hours later in luciferase lysis buffer. Alternatively, reporter cells were treated with fibroblast conditioned media the day after seeding and lysed 24 hours later. Cell lysates were processed as described above using a luminometer and normalised to renilla luciferase activity.

2.8 Immunoprecipitation and western blotting

Cells were seeded at 30% confluency in 6 well plate for transfection. 24 hours after seeding, the cells were transfected with 2 μg of expression plasmids as indicated using PEI following the manufacturer's instructions. The cell lysate was harvested 24 hours post-transfection for immunoprecipitation (IP) or 48 hours post-transfection for protein level examination in lysis buffer composed of 150 mM NaCl, 1 mM EDTA, 30 mM Tris at pH 7.5, 0.1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol (DTT), 1 % Triton X-100, 10% Glycerol, EDTA-free protease inhibitor cocktail tablets (Roche), and phosphatase inhibitor cocktail tables (Roche). When indicated, cells were treated with 10 μM of proteasomal inhibitor MG132 for 4 hours before lysate collection.

For IP, cell lysates were centrifuged for 30 minutes at 14000 rpm 4°C and an aliquot of the supernatant was taken as input. The rest were pre-cleared with IgG-agarose beads (Millipore) at 4°C for 2 hours minimum. After centrifugation to pellet the beads, the cleared cell lysates were incubated with antibodies at a concentration of 1 μg/ml or beads conjugated with FLAG (Sigma, A2220) at 4°C overnight. The next day, a minimum 2-hour incubation with IgG-agarose beads were carried out following antibody incubation overnight to form immunocomplexes and washed with lysis buffer. When samples were incubated with beads already conjugated with FLAG, they were washed with lysis buffer. The wash cycle of centrifugation and addition of fresh lysis buffer was repeated for six times, each no less than 10 minutes. After the last wash, beads were resuspended in appropriate volume of lysis buffer and resolved in 10% sodium dodecyl sulfate–polyacrylamide gels (SDS-PAGE) to detect proteins as described below.

For protein detection, cells lysate was centrifuged for 30 minutes at 14000 rpm. The resulting supernatants were used for protein quantification by Bradford assay. Same amount of cellular protein from each sample condition was resolved in 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked using 5% milk (OXOID) in Tris-buffered saline (50mM Tris, 150mM NaCl) supplemented with 0.1% Tween-20 (Sigma, TBST) for 1 hour. They were then incubated overnight at 4°C with primary antibodies diluted in blocking solution (see Table 2 for antibodies used). The next day, after a brief wash with TBST, species-matched HRP-conjugated secondary antibodies (GE Healthcare) diluted in blocking buffer were added and incubated for 1 hours at room temperature. Chemiluminescence ECL Prime Western Blotting Substrate (GE Healthcare) was used to detect antibody binding. Images were acquired with Amersham Imager 600 RGB (GE Healthcare) and processed using Adobe Illustrator. For β-ACTIN detection, primary antibody diluted in TBST were incubated for 15 minutes and washed for 30-minutes before immunoblots were detected using Immobilon Crescendo Western HRP substrate (Millipore, WBLUR0100).

2.9 Flow cytometry

HEK293T cells were seeded at 30% confluency and transfected with FZD5-V5 and POSTN overexpression plasmids the next day using PEI. 48 hours post-transfection, cells were trypsinised and counted. 4 x 10^5 cell suspension in PBS supplemented with 1% BSA, 10% FBS, and 1% sodium azide (resuspension buffer) was aliquoted in V-bottomed 96 well plate for anti-FZD5 antibody staining diluted in resuspension buffer. Following 30-minute incubation with primary antibody at room temperature, the plate was centrifuged at 1400 rpm for 2 minutes at 4°C. The supernatant was removed and cells were resuspended in secondary antibody conjugated to Alexa Fluor 488 diluted in 3% BSA in PBS and incubated for 30 minutes at room temperature in the dark. Centrifugation wash with resuspension buffer in PBS was repeated for three times. After the final wash, the cells were resuspended in PBS and 3 μM DAPI and proceeded for flow cytometry with BD LSR Fortessa (BD Biosciences, San Jose). Alexa Fluor 488 was excited using a 288 nm laser and measured in a 530/30BP and DAPI was excited using a 355 nm laser and measured

in a 450/50BP. The analysis was performed using FlowJo (BD); dead cells, doublets, and debris were excluded from the final analysed population.

2.10 Transwell co-culture

Fibroblasts were trypsinised and seeded on 24 well plate at a confluency of 5000 cells per well. Small intestinal organoids seeded at high confluency four days before the experiment set up were passaged without mechanical dissociation and split in 1:5 dilution onto transwell inserts. The co-culture was maintained in 75% human organoid expansion media and 25% fibroblast growth media for seven days. Organoid growth was imaged every two days using EVOS. On day 7, organoids were incubated with 10 μM 5-ethynyl-2'deoxyuridine (EdU, Life Technologies) for 2 hours and fixed in 10% buffered formaldehyde for 2 hours and processed for further analyses. Others were harvested directly in RLT buffer (Qiagen) for RT-qPCR.

2.11 Generation of POSTN KO CAFs

Primary fibroblasts were counted and prepared in reaction mix of 5 x 10^5 cells per nuclefection. The nucleofection was carried out with Neon™ Transfection System (Invitrogen) following the manufacturer's instructions. Briefly, counted cells were resuspended in the resuspension buffer and sgRNAs (Synthego) and Cas9 protein (Invitrogen) were added in appropriate ratio. After 30 minutes incubation to allow formation of Cas9-gRNA ribonucleoprotein, the cells were nucleofected using Neon transfection device (Invitrogen). Nucleofected cells were harvested for Sanger sequencing and immunoblot analyses, and expanded for experiments in fibroblast growth media. The sgRNAs used to target POSTN were 5'-UGCCCAGAGUGCCAUAAACA-3', 5'-AGUGCCAUAAACAUGGUCAA-3', and 5'-GUGCCAUAAACAUGGUCAAU-3'.

2.12 Generation of POSTN-expressing fibroblasts

HEK293T cells were transfected with psPAX2 and pMD2.G together with lentiviral construct containing POSTN pWPI-POSTN-GFP. The supernatant was harvested every morning and afternoon on day 1 and day 2 post-transfection. Four collections of viral supernatants were pooled and concentrated using centrifugal filters (Sartorius, Vivaspin20) by centrifugation at maximum speed until 200-250 µl of concentrate remained. The concentrated viral supernatant was aliquoted and stored at 80°C. Human colonic fibroblast cells (CCD18-Co, ATCC® CRL-1459™) were seeded in 6 well plate at 60% confluency and viral supernatant with 1X Polybrene (Sigma, H9268) was added. Three days post-transduction, cells were used for WB analyses and later in *in vitro* experiments.

2.13 Construction of 3D culture system with collagen I scaffold

Collagen I was neutralised as described previously (2.5). Primary fibroblasts were then resuspended in the neutralised collagen. The mix was aliquoted and left to polymerise in the 37°C incubator for 15 minutes. To dehydrate the scaffolds, sterile absorbers (Lonza) were applied for 15 minutes at room temperature. The resulting collagen sheet embedded with fibroblasts were used for 3D model experiments.

Primary human organoids were dissociated and seeded on top of the collagen sheets at a confluency of 1-to-1 ratio to primary fibroblasts. The media composition was 25% fibroblast growth medium and 75% human organoid growth medium, with Y-27632 withdrawn after the first media change. The 3D culture was expanded for two weeks in submersion, and maintained in air-lifting culture afterwards.

Samples were incubated with EdU for 2 hours and fixed in 10% buffered formaldehyde. Harvested samples were processed and embedded in paraffin for subsequent analyses via immunohistochemistry and immunofluorescence. For RNA extraction, samples were lysed directly in RLT buffer (Qiagen) and extracted using RNeasy FFPE Kit (Qiagen) per manufacturer's instructions.

Gene	Forward	Reverse
β-ACTIN	ACGGCCAGGTCATCACCATTG	CGGAGTACTTGCGCTCAGGAGGA
PPIB	ATTCCATCGTGTAATCAAGGAC	TAGATGCTCTTTCCTCCTGTG
ACTA2	TCCCAGACATCAGGGGGTGATG	GGTGCCAGATCTTTTCCATGTCGT
FAP	ATTTATGCTGGTCGCCTGTT	GGTGGATCTCCTGGTCTTTG
CALD1	GAAGAGCAGAGGAGGAAGCA	ATCTTCTGGCGTTTCTCAGC
FN	TCAAAGCAAGCCCGGTTGTTATG	GATCCAGCAAGTCTCTTCAGCTTCAGGTTT
POSTN	GCACTCTGGGCATCGTGGGA	AATCCAAGTTGTCCCAAGCC
LGR5	ACCTGAAAGCCCTTCATTCA	TGCTATGGTCCACACTCCAA
AXIN2	AGTGTGAGGTCCACGGAAAC	CTTCACACTGCGATGCATTT
CMYC	TCTCCTTGCAGCTGCTTAG	GTCGTATCGAGGTCATAG
TCF4	ACGAGGGCGAACAGGAGGAG	TGGGCGAGAGCGATCCGTTG
TCF7	GGTTCACAGGCCTCTGCAGAC	CTTGTGTCTTCAGGTTGCGG
ALPI	TTTAACCAGTGCAACACGA	TCCTACTGACTTTCCTGCT
MUC2	GACACCATCTACCTCACCCG	TGTAGGCATCGCTCTTCTCA
CDH1	GGGCAGAGTGAATTTTGAAG	AGAAACGGAGGCCTGATGGG
LOX	GGCACAGTTGTCATCAACATTACC	GACTCAATCCCTGTGTGTGTGCAGTAC
OLFM4	CTTTCCAAAGTGAGGGAATATGTC	GATGTCAATTCGGACAGTTAGG
LYZ	GGATGTGTTTGGCCAAATGGGAG	CGATTTCTCCATGCCACCCACTGCA

Table 1. RT-qPCR primers

Antibodies	Host species	Dilution	Source
β-ACTIN	Mouse	1:20000	Sigma (A3854
ACTA2	Rabbit	1:1000	Abcam (ab5694)
CALD1	Rabbit	1:200	Sigma (HPA008066)
FAP	Mouse	1:500	Abcam (ab54651)
POSTN	Rabbit	1:500	Santa Cruz (sc-67233), Abcam (ab219056)
c-MYC (9E10)	Mouse	1:500	Santa Cruz (sc-8026)
V5	Mouse	1:1000	Abcam (ab27671)
FLAG	Mouse	1:1000	Sigma (A2220)
FZD5	Mouse	1:200	Invitrogen (MA5-17080)
SOX9	Rabbit	1:500	Millipore (AB5535)
CYCLIN D1	Rabbit	1:75	Cell Signalling (2978)
COLLAGEN I	Rabbit	1:500	Abcam (ab34710)
FN	Rabbit	1:100	Abcam (ab23750)
Alexa-Fluor488	Mouse	1:500	Invitrogen (A11029)
Alexa-Fluor568	Rabbit	1:500	Invitrogen (A11036)

Table 2. List of antibodies

2.14 Immunohistochemistry

Constructed scaffolds were fixed in 10% buffered formaldehyde for 2 hours and embedded in paraffin. 4-5 μm sections were deparaffinised with xylene and rehydrated by a series of ethanol treatment. Antigens were retrieved by 20-minute

treatment of either 0.01M citrate buffer (pH6) or Tris-EDTA buffer (10 mM Tris base, 1 mM EDTA solution, pH9) at high temperature. Sectioned samples were then blocked in 1% BSA and incubated overnight with desired antibodies at 4°C. Afterwards, samples were incubated with secondary antibodies for 1 hour and washed with PBS. Sections were stained with diaminobenzidine (DAB) via incubation with peroxidase substrate. Images of detected protein expression was acquired by a bright-field microscope.

2.15 Immunofluorescence

Cells were grown on poly-L-lysine-coated (Sigma) glass coverslips in 24 well plate and fixed with 4% paraformaldehyde (PFA) for 15 minutes. After three PBS washes, they were blocked with 1% BSA in PBS for 15 minutes before an overnight incubation with primary antibodies at 4°C. The next day, cells were washed with PBS three times and incubated with secondary antibodies conjugated to Alexa-Fluor 488/568 at room temperature for 1 hour in dark. Finally, cells were incubated with 15 µM DAPI for 10 minutes after three PBS washes. The coverslips were washed with PBS three times and mounted with ProLong™ Gold Antifade Mountant (Thermo Fisher Scientific) on glass slides. The section samples of collagen I 3D co-culture were first deparaffinised using xylene and rehydrated via a graded serial treatment with ethanol. Antigen retrieval was performed at high temperature for 20 minutes in either 0.01M citrate buffer or Tris-EDTA buffer. Samples were then blocked in 1% BSA and incubated overnight with desired antibodies at 4°C. After 1-hour incubation at room temperature with secondary antibodies conjugated to Alexa-Fluor, sections were stained with DAPI for 10 minutes. Mountant was added on top and sections were covered with cover slips. Images were acquired using a Leica SPE confocal microscope and processed using Leica LASX.

2.16 Atomic force microscopy

Atomic force microscopy (AFM) measurements were done with Hamid Mohammadi using JPK NanoWizard I (JPK Instruments, Berlin, Germany), mounted on an inverted microscope system (Nikon, Tokyo, Japan). Cantilevers with polystyrene

spherical tip (25 μm in diameter, Novascan, USA) and nominal spring constant of 0.06 N/m were used to measure the stiffness of the samples. Cantilevers were calibrated by acquiring a force curve on the glass slide surface followed by spring constant calibration using JPK software thermal noise fluctuation method. For stiffness measurements, cantilever was lowered at speed of 5 μm/s indenting about 2 μm into the sample with constant set point force. Measurements consisted of around 20 indentations per sample. The Hertz-model (elastic model approximation) was used for calculation of the Young's modulus (i.e. apparent elastic modulus) from each extend curve.

2.17 Xenograft model

Primary fibroblasts and CRC organoids were mixed in Ad-DF+++ and BME in 1:1 ratio and injected into both flanks of SCID mice aged between 7 to 11 weeks. At the end of the experiment, mice were peritoneally injected with EdU (0.3 mg per 10 g of mouse weight) from a 10 mg/ml stock solution 2 hours before tumours were collected, weighed, and photographed before being processed for histology. All animal procedures were performed according to Project License constraints and Home Office guidelines and regulations.

2.18 Statistical analysis

All data are presented as the mean ± the standard error. Statistical analyses were performed with GraphPad Prism software. Normal distribution of data was determined by Kolmogorov-Smirnov test or Shapiro-Wilk test and the statistical significance was determined by student's unpaired two-tailed t-tests to consider both positive or negative changes in experimental groups compared to the defined control group, where *p* values lower than 0.05 were considered statistically significant.

Chapter 3. Establishment of patient-derived fibroblasts and screening for potential paracrine Wnt regulators

3.1 Introduction

Mutations in the Wnt pathway are often found in CRC, which reflects the importance of this pathway on the regulation of proliferation and differentiation of stem cells in the crypt. Recently classified molecular subtypes of CRC can often be distinguished by the presence of certain oncogenic mutations, such as *APC*, *KRAS* and *TP53* (Guinney et al., 2015, Network, 2012). The Wnt signalling is hyperactivated in CMS2 CRCs that are featured with *APC* mutations. However, high Wnt activity is observed across CRCs regardless of tumourigenic mutations in the Wnt signalling pathway (Polakis, 2007, Yang et al., 2016). It remains unclear how hyperactivation of Wnt signalling, resulting in hyperproliferation, is instigated in non-APC mutant CRCs (i.e. CMS1, 3 and 4).

As described previously, ISCs in the small intestine have an epithelial source of Wnt ligands as they are located proximally to Paneth cells (Sato et al., 2011b). An equivalent epithelial Wnt supply has not been described in colon. Rather, ISCs in the colon are found to be largely dependent on stromal Wnt ligands for activation of the pathway (Rothenberg et al., 2012, Sasaki et al., 2016). Interestingly, despite the prevalence of *APC* and *β-catenin* mutations, some CRCs may still require Wnt ligand binding for Wnt activation (Voloshanenko et al., 2013, Groden et al., 1995). Indeed, several studies have shown that cancer cells located closer to stromal cells show higher Wnt activation, suggesting that CRCs may still be dependent on exogenous Wnt signal (Guinney et al., 2015, Brabletz et al., 2001, Vermeulen et al., 2010). In particular, CMS4 CRCs have high stromal cell infiltration, while *APC* and *β-catenin* are mostly unaltered in this cancer subtype. Conceivably, the non-APC mutant CMS4 CRCs may rely on an alternative mechanism for Wnt activation that involves CAFs.

An increasing body of evidence highlights the complex and important role of CAFs in the TME. CAFs are known to play an active role in ECM remodelling, which in turn

alters the TME to promote cancer progression (Xing et al., 2010, Shiga et al., 2015, LeBleu and Kalluri, 2018). Abnormal ECM can facilitate vascularisation, alter immune cell infiltration, and promote cellular transformation and metastasis (Lu et al., 2012). In addition to ECM remodelling, the secretome of CAFs is likely another key element for tumour progression (Tommelein et al., 2015). CAFs in CRCs are found to function as cancer stem cell (CSC) niche to enhance their tumourigenicity by activating the Wnt pathway (Vermeulen et al., 2010). It has been demonstrated that CAFs surrounding tumours secrete HGF, which triggers the β-catenin-dependent transcription programme allowing cancer cells to selectively adopt a CSC identity and functionality accompanied by high Wnt activity depending on their proximity to the stroma (Vermeulen et al., 2010). In addition, co-culture of CAFs and CRC spheroids showed increased secretion of Wnt ligands in CAFs (Horman et al., 2017). Exosomes derived from CAFs contain WNT3A and enhance Wnt activity in CRC epithelial cells (Hu et al., 2015b). It has also been described that Wnt secretion is required to sustain high Wnt activation in CSCs despite their Wnt activating mutations, while perturbation in Wnt ligand secretion reduced clonogenicity *in vitro* and tumourigenesis *in vivo* (Voloshanenko et al., 2013). Altogether, the data suggest the important role of CAFs in paracrine Wnt activation to promote tumour progression.

I hypothesise that CRCs, in particular CMS1 with most MSI CRCs, and CMS4 with the highest stromal infiltration, may exploit CAFs in the TME for alternative route of paracrine Wnt activation. This project aims to identify the putative paracrine factors secreted by stromal cells surrounding the tumour that may contribute to the pathological Wnt activation in CRCs. To explore potential CAF-derived Wnt regulators in a more biologically relevant context, patient-derived primary fibroblasts were generated in this study and the CAF-specific properties were confirmed in *ex vivo* culture. Putative stromal Wnt regulator was first selected from expression analysis of The Cancer Genome Atlas (TCGA) data and literature review. The Wnt regulatory role of the selected candidate was further examined using the patient-derived primary fibroblasts.

3.2 Results

3.2.1 TCGA hierarchical clustering reveals a cluster of stromal cancer markers

The Cancer Genome Atlas has undertaken genomic, transcriptomic, and exomic analyses of a large cohort of cancer patients, including CRC, with the aim to shed light on molecular characteristics and the pathology (Network, 2012). The publicly available data generated by TCGA provides a valuable dataset of molecular profiles of cancer patients and has enabled analyses and discoveries of novel correlations between dysregulated molecular features and cancer. A comprehensive list of publications taking advantage of the TCGA dataset is compiled and accessible via the official TCGA programme at the National Cancer Institute, the United States of America. In-house hierarchical clustering analysis of the expression profile from the TCGA data revealed a cluster of genes differentially upregulated in a subpopulation of CRCs (Figure 3.1). This cluster consisted of genes coding for ECM proteins (e.g. *COL1A1/2*, *COL3A1*, *COL4A1/2*, *COL5A1*, etc.), ECM remodelling enzymes (*MMP2*, *MMP11*, *LOX*, *LOXL1*, etc.), and some stromal-expressing proteins (*FN*, *THY-1*, etc.). CAF gene upregulation was also found in this cluster, such as *FAP, CALD1,* and *IGFBP7* that have been previously associated with poor prognosis of CRC patients (Calon et al., 2015). This cluster was therefore named as “CAF-enriched cluster”.

In addition to the ECM genes and CAF markers, a number of secretory components of the Wnt pathway were also noted in this cluster, including the Wnt ligands *WNT2* and *WNT5A*, and the inhibitors *SFRP2*, *SFRP4*, and *DKK2*. Interestingly, one of the CAF markers POSTN has been previously associated with Wnt signalling and tumour progression in breast cancer (Malanchi et al., 2011). It has been shown that POSTN was secreted by fibroblasts to establish the metastatic niche by activating Wnt signalling in migrating cancer stem cells (Malanchi et al., 2011). Despite the upregulated expression in CRCs, the underlying mechanism of POSTN in CRC progression and Wnt regulation remains largely uncharacterised (Bao et al., 2004, Kikuchi et al., 2008, Kikuchi et al., 2014, Calon et al., 2015). I hypothesise that POSTN may function as paracrine Wnt regulator to promote pathological Wnt activation and tumour progression in CRCs.

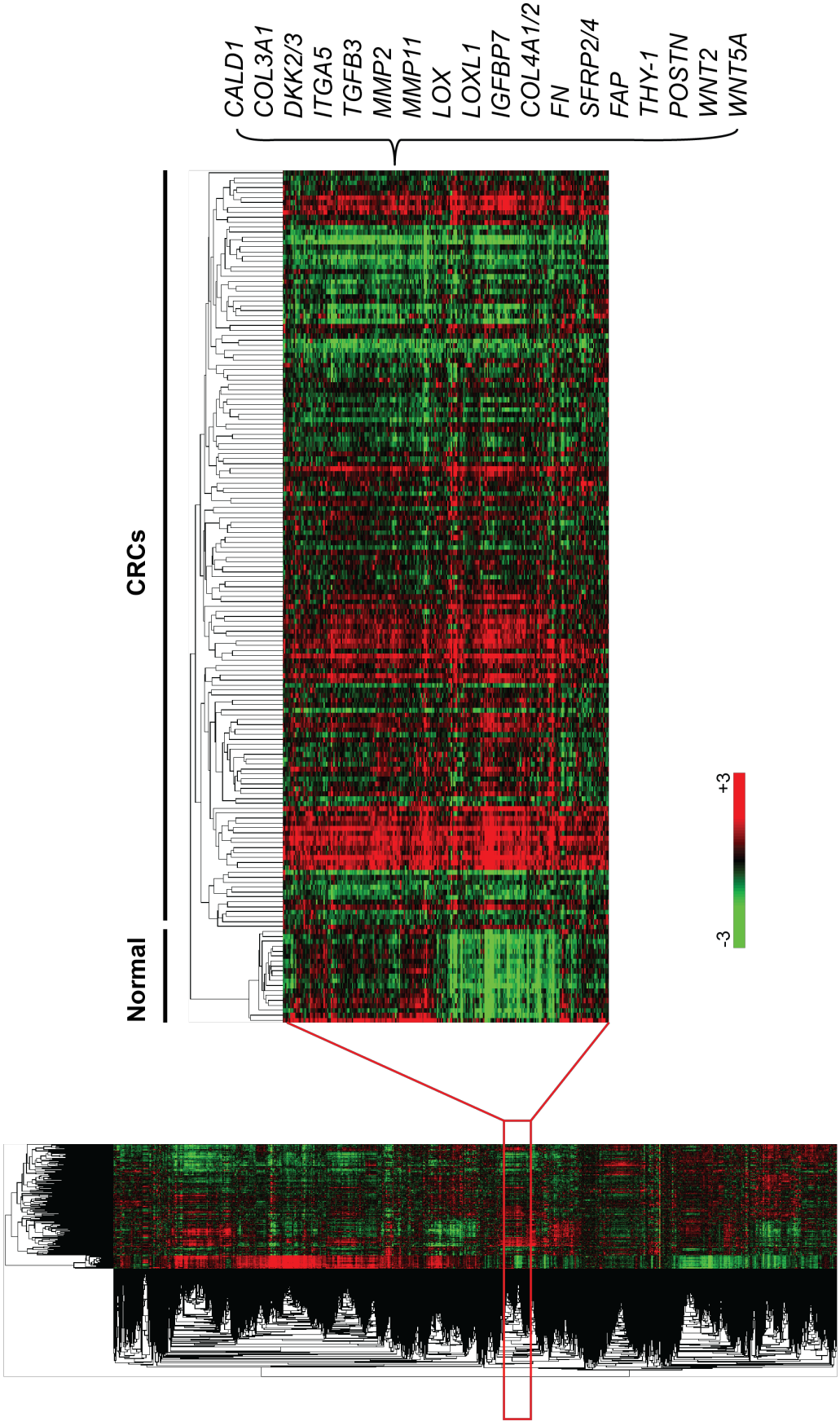
CALD1
COL3A1
DKK2/3
ITGA5
TGFB3
MMP2
MMP11
LOX
LOXL1
IGFBP7
COL4A1/2
FN
SFRP2/4
FAP
THY-1
POSTN
WNT2
WNT5A
CRCs
Normal
+3
-3

Figure 3.1 "CAF-enriched cluster" from TCGA hierarchical clustering.

Heat map demonstrating hierarchical clustering analysis of 19 normal and 142 CRCs obtained from TCGA expression data set. The "CAF-enriched cluster" is zoomed in on the right, and selected CAF-associated genes in the cluster are listed. Scale bar indicates a log2 ratio from -3 to +3 that shows changes in gene expression. Data are acquired using TreeView software. Upregulated expression in red, downregulated expression in green.

3.2.2 Patient-derived primary fibroblasts retain their CAF properties in *ex vivo* culture

To study paracrine Wnt regulation in CRC, paired primary normal fibroblasts (NFs) and CAFs were first generated from resected patient tissue (Figure 3.2A). Tissue samples were mechanically dissociated before being further processed enzymatically for isolation of proliferative epithelial cells to establish normal and tumour organoid culture, and the tissue fragments were later plated on a cell culture dish directly for explant culture (Figure 3.2B). Patient-derived CRC organoids from four patients were successfully established and one pair was utilised in *in vivo* studies described in Chapter 5. The established patient-derived NFs and CAFs were characterised to confirm that the paired populations were still distinguishable after *in vitro* culture. In total nine patient-derived NF and CAF pairs were established and tested for their characteristics. However, due to variable expandability, not all of the lines were available to be characterised and utilised throughout the project, and patient lines that did not show any difference between NF and CAF populations in multiple assays were excluded from the study (Figure 3.3). MTT assays showed that CAFs were slightly more proliferative than NFs albeit not significant (Figure 3.2C). Collagen contraction assays further demonstrated that the contractility was generally higher in CAFs than in NFs (Figure 3.2D). The contraction assay serves as an indirect readout for their ECM remodelling capacity (Calvo et al., 2013).

Next, I examined whether the stromal markers associated with poor CRC prognosis were upregulated in my patient-derived CAFs as reported previously in the literature (Calvo et al., 2013, Calon et al., 2015, Sugimoto et al., 2006). The mRNA transcript

levels and protein cell lysates were analysed to examine the expression levels of the reported CAF markers (Figure 3.4). Indeed, most of these CAF markers were upregulated in the established CAFs as compared to the paired NFs from the same patients albeit diagnosis was not consistent due to limited sample availability (Figure 3.4A). However, the mRNA expression of these stromal CAF markers varied among the individuals. For instance, the upregulated *ACTA2* expression levels was the highest in Patient 3 CAFs but was relatively modest in Patient 21. Most of the CAF markers were significantly upregulated in CAFs derived from Patient 17 and 22, while the expression change was negligible in Patient 21. The variation of CAF markers expression could possibly be caused by the heterogenous cell populations in the CAFs or the CRC patient subtypes. Of note, increased expression of ACTA2 and FAP was consistently noted in CAFs at the protein level (Figure 3.4B).

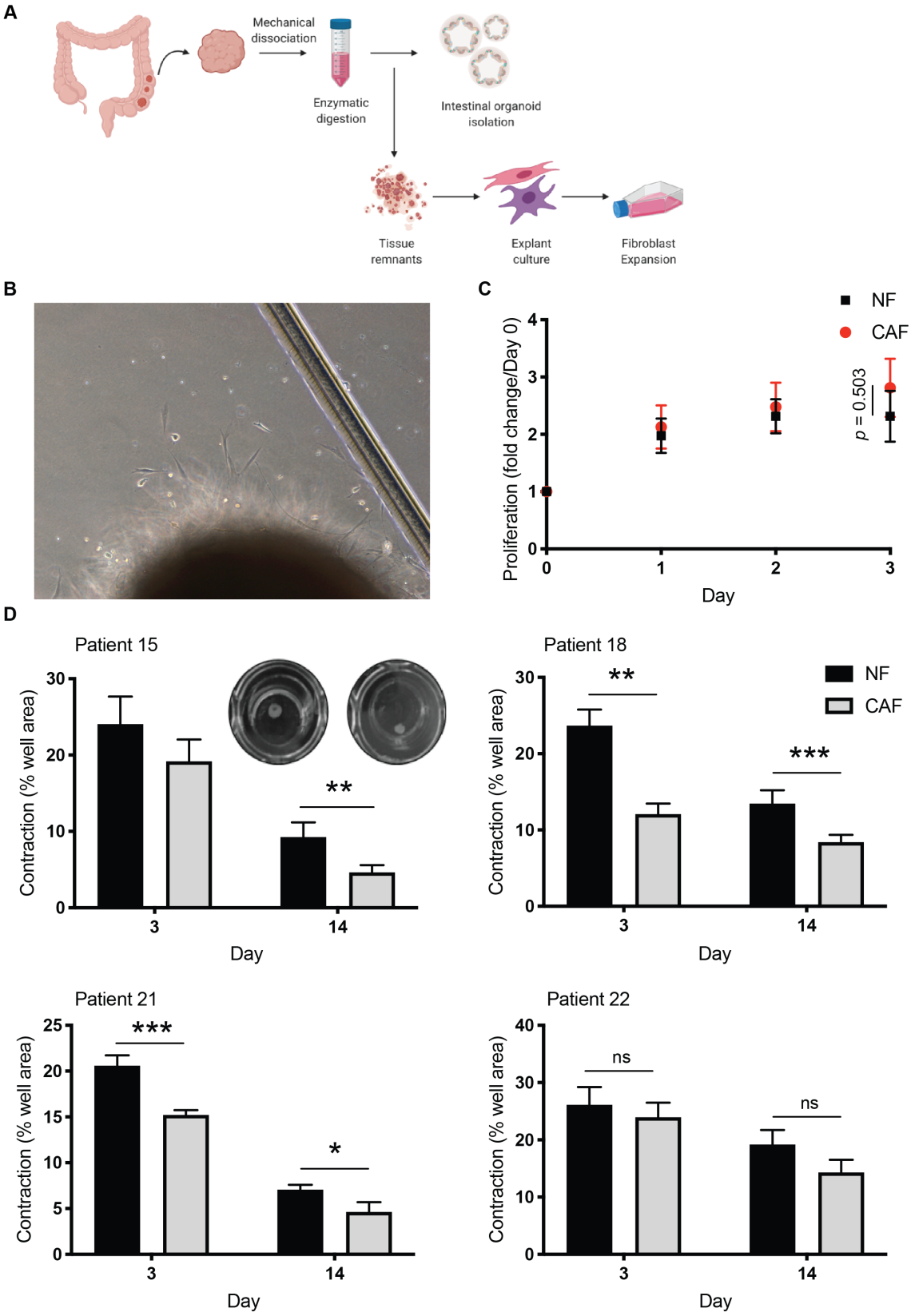

Figure 3.2 Establishment and characterisation of patient-derived primary fibroblast populations.

(A) Schematic workflow of patient-derived primary cell culture establishment from resected CRC tissue. Enzymatically digested tissues after mechanical homogenisation were processed to isolate primary organoid cultures and remnant tissues were then plated on cell culture dish for explant culture. Attached fibroblasts outgrowing from the tissue were then passaged and expanded. (B) Representative image of explant culture for primary fibroblast isolation on day 7. (C) Quantification of proliferation of NFs and CAFs at day 1, 2, and 3 after seeding. Data are shown as fold change from day 0 and N = 8 biological replicates ± SD. No significant difference observed between two populations ($p = 0.503$). (D) Quantification of contractility of paired NFs and CAFs seeded in collagen hydrogels at day 3 and 14. Each patient N = 3 technical replicates ± SD. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ compared with NFs at the same time point. Inset images in Patient 15 show representative contraction by fibroblasts at day 14.

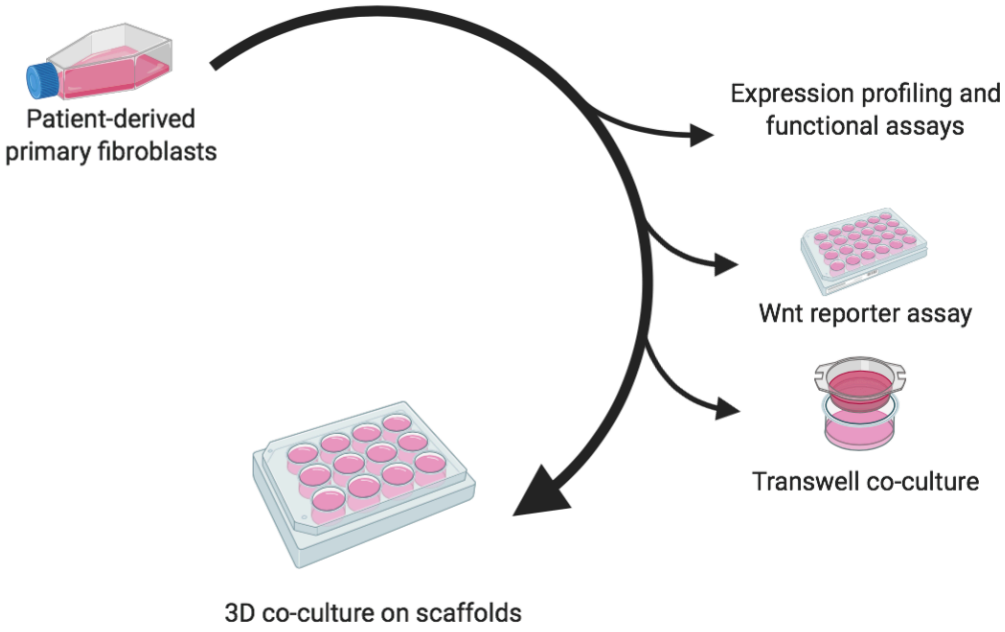

Figure 3.3 Schematic workflow of patient-derived primary fibroblasts.

Primary fibroblasts established from CRC patient samples were characterised and utilised in functional and Wnt reporter assays. Patient-matched paired NFs and CAFs that failed to expand or did not show differences were excluded from further use for co-cultures in transwell and 3D collagen I scaffold (Chapter 5).

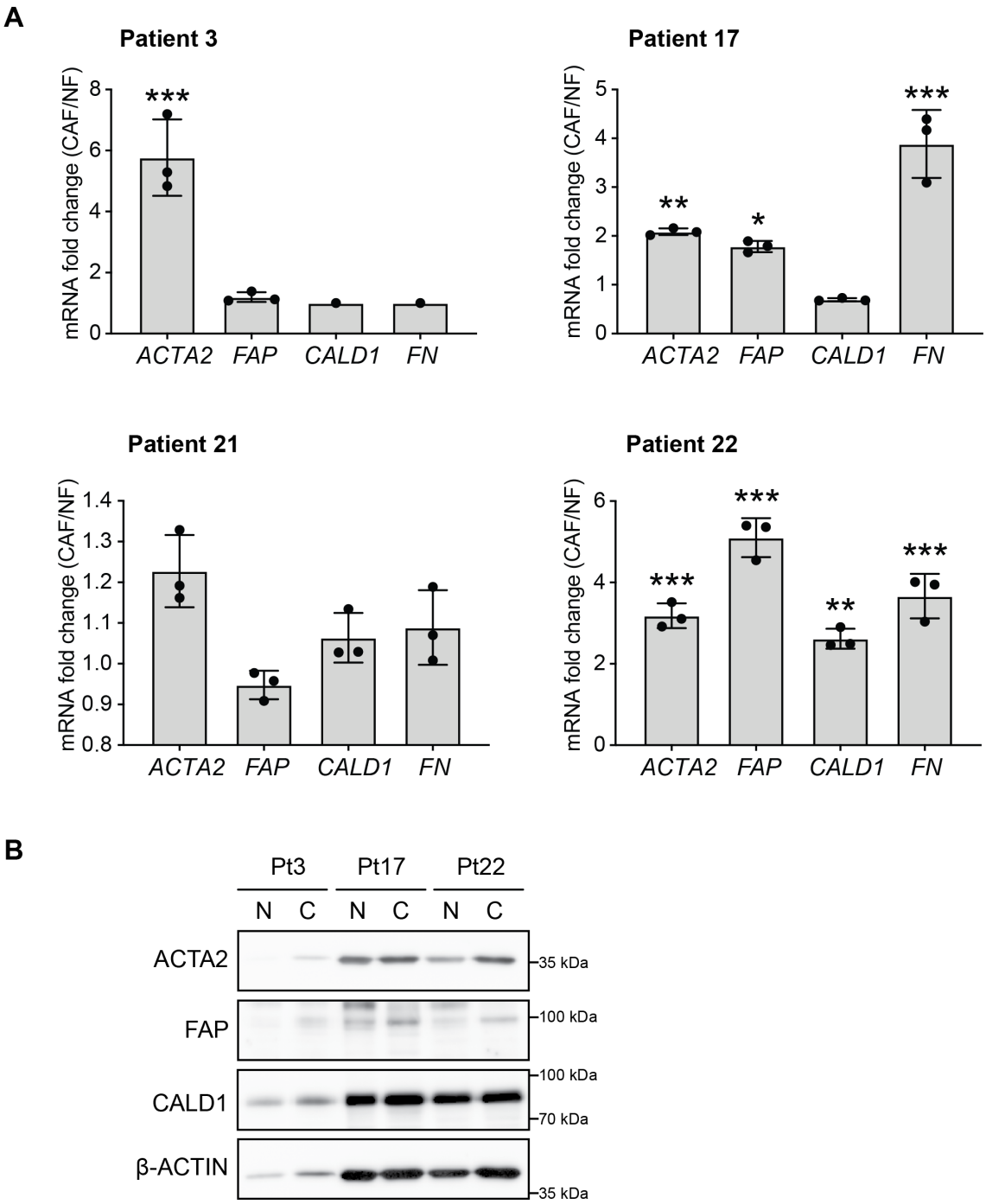

Figure 3.4 CAF marker expression profiles of established primary fibroblasts.

(A) Quantitative RT-qPCR analyses of paired patient-derived fibroblasts from four patients. Reported CAF markers ACTA2, FAP, CALD1, and known fibroblast marker FN were examined. Data are presented as fold change compared to NF and N = 3 technical replicates ± SD. *p<0.05, **p<0.01, and ***p<0.005 of relative mRNA levels compared to normal counterparts after normalisation to *β-ACTIN* or *PPIB* levels. (B) Western blot of CAF markers in three paired primary fibroblasts.

3.2.3 CAFs can promote Wnt activation

One of the key hypotheses in this study is that some CRCs may be dependent on paracrine Wnt regulators secreted by CAFs for Wnt activation and tumour progression. To address whether CAFs are able to promote Wnt activation, Wnt reporter cells were used in which TCF-TOPFlash reporter was integrated to measure the Wnt activity. Two independent approaches were taken to test the Wnt activity of the fibroblasts: 1) co-culture of fibroblasts with Wnt reporter cells or 2) treatment of the fibroblast conditioned media (Figure 3.5A). This allowed distinguishing potential contact-independent mechanisms via secretory factors as the only source of modulators in the conditioned media, while co-culture captured both secretory factors and cell contact-dependent mechanisms, including potential factors induced by the co-culture that enabled reciprocal signalling between the two cell types.

TCF-TOPFlash analyses of the co-culture experiment showed that the Wnt activity was indeed significantly higher in CAFs than in their NF counterparts (Figure 3.5B). Conditioned media derived from the fibroblasts also showed consistent increase of Wnt signalling by CAFs than by NFs. Overall there was a differential degree of Wnt potentiation by CAFs compared to patient-matched NFs, while the Wnt activity levels varied among individual CAF lines. The consistent upregulation of Wnt signalling in both co-culture and conditioned media conditions suggests that the paracrine Wnt activation from CAFs is likely induced by secretion and contact-independent mechanism. The differential Wnt activities among the individual CAF lines could be an indicative of patient-to-patient variation whereby the patients may be of different molecular subtypes with different mutation load and TME composition.

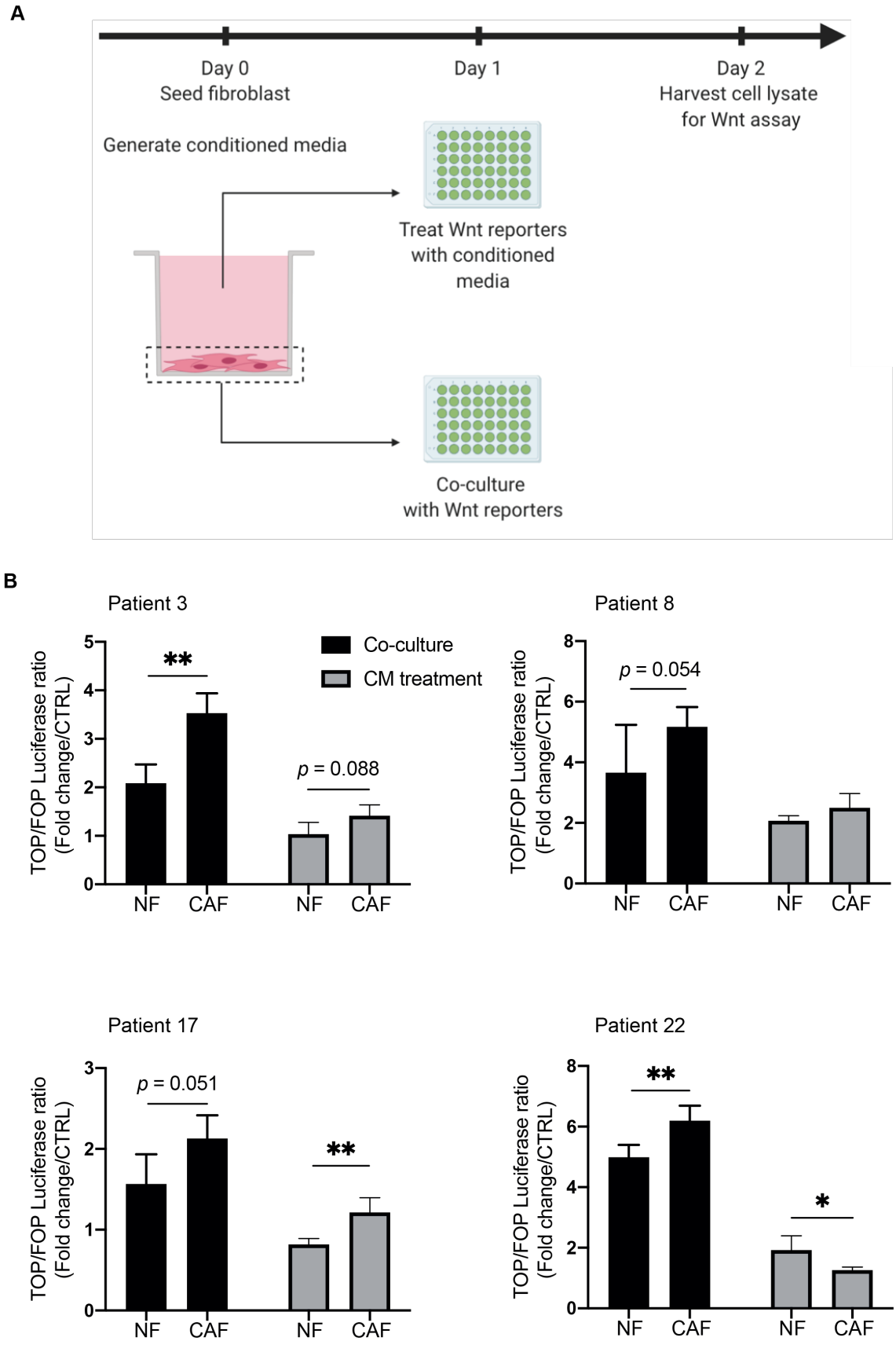

Figure 3.5 Wnt activation enhanced by CAFs.

(A) Schematic workflow of TCF-TOPFlash assay with HEK293T Wnt reporter cells. Reporter cells were either cultured together with fibroblasts or treated with fibroblast conditioned media for one day. (B) Relative TCF-TOPFlash reporter activity shown as fold change compared to reporter only control (co-culture) or fibroblast-free conditioned media control (CM treatment). N = 3 technical replicates ± SD of each patient, *p<0.05, and **p<0.01.

3.2.4 Overexpression of POSTN in human CRC fibroblasts

One of the CAF markers, POSTN has been previously described as poor prognosis marker for CRC, while the mechanism is largely unknown (Bao et al., 2004, Kikuchi et al., 2008, Kikuchi et al., 2014, Calon et al., 2015). Since the TCGA analysis showed upregulation of POSTN in the CAF-enriched cluster, I decided to first examine if POSTN is overexpressed in the CRC patient-derived CAFs.

Immunohistochemistry showed a modest stromal expression of POSTN in the normal colonic tissues, while the expression level was significantly increased in the CRC tissues (Figure 3.6A). Importantly, the staining showed that the upregulated expression of POSTN in CRC was indeed exclusive in the surrounding stromal cells rather than tumour cells (Figure 3.6A). To confirm that the upregulated expression is in the CAFs, I further analysed the POSTN expression in my primary patient-derived fibroblasts. RT-qPCR analysis of 5 paired CAF and NF samples showed that the transcriptional level of *POSTN* was upregulated in 3 out of 5 CAFs (Figure 3.6B). Specifically, Patients 3, 17, and 22 showed significant upregulation of *POSTN* in CAFs compared with NFs, whilst no upregulation was observed in Patients 8 and 21. Consistently, western blot analysis confirmed the upregulated expression of POSTN at protein level in the CAFs derived from Patient 3, 17, and 22 (Figure 3.6C). Similar to the TCGA data, differential expression of POSTN was also observed in the established CAFs from different patients, suggesting that POSTN expression may be upregulated in a subtype of CRCs (i.e. CMS4).

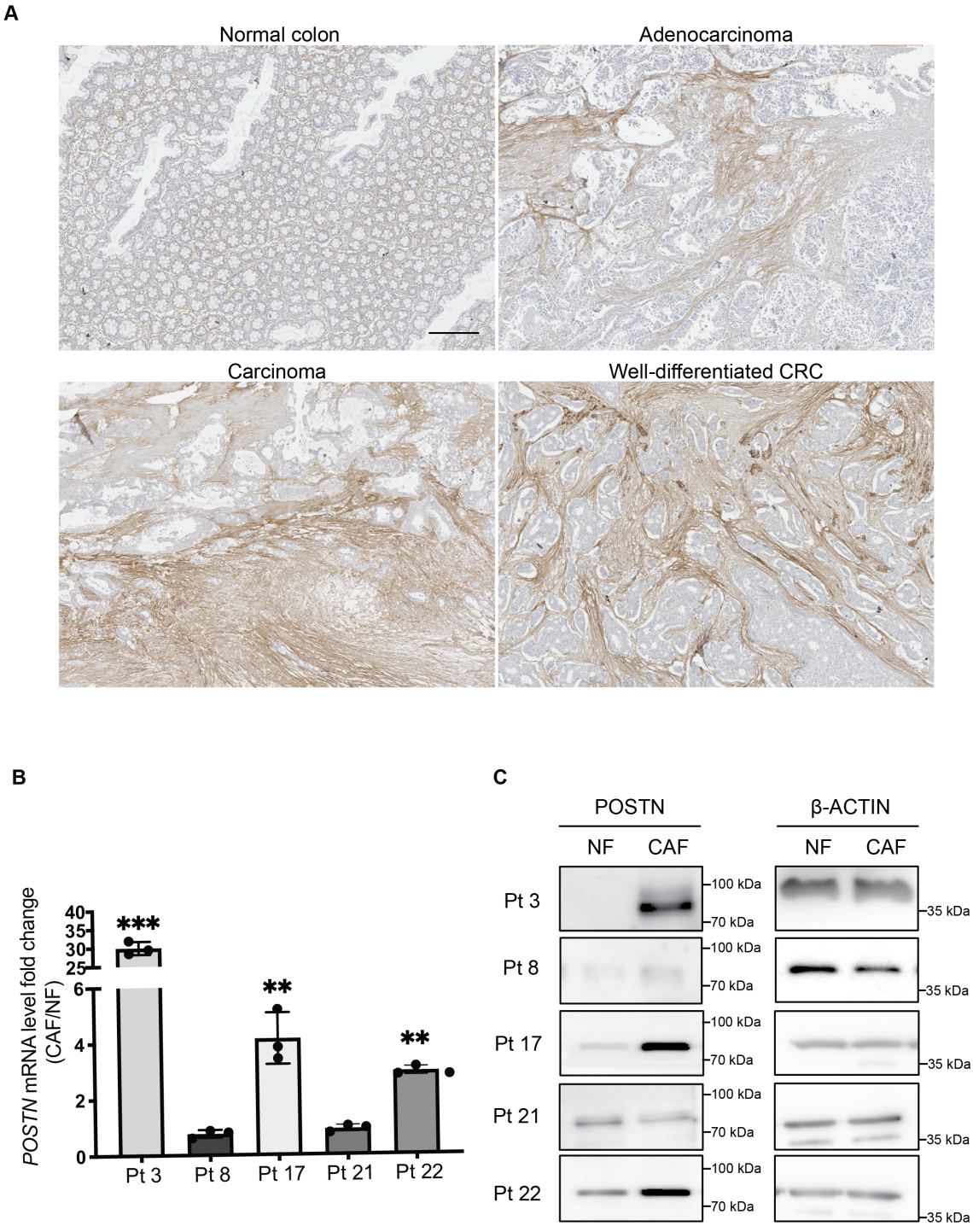

Figure 3.6 Expression of POSTN in CRC.

(A) Representative images of POSTN immunohistochemistry in normal colonic and CRC tissue sections. Scale bar, 200 µm. (B) RT-qPCR analyses of *POSTN* in paired patient-derived primary fibroblasts. Data are presented as fold change compared to mRNA level in NFs after normalisation to *β-ACTIN* levels. N = 3 ± SD technical replicates of each patient. **p<0.01, and ***p<0.005. (C) Western blot of POSTN in paired NFs and CAFs.

3.3 Discussion

Wnt activation is crucial to drive hyperproliferation and tumour progression in CRCs. Besides the well-known intrinsic *APC* and *β-catenin* mutations in cancer cells, it is still unclear if extrinsic factor(s) from the surrounding stromal cells also contribute to Wnt activation and tumourigenesis. To address the paracrine Wnt signalling from stromal cells, I first screened for the CAF signature from a large cohort of CRC samples. Hierarchical clustering of the TCGA expression dataset revealed a CAF-enriched cluster of genes that are preferentially upregulated in a subset of CRCs. Many stromal factors were present in this cluster, including genes encoding for ECM proteins, ECM remodelling enzymes, stromal genes, and CAF-enriched genes such as *FAP*, *IGFBP7, CALD1* and *POSTN*. A recent comprehensive study has reported that increased expression of many of these genes in CAFs are associated with poor prognosis of CRC regardless of clinical variables (Calon et al., 2015).

Interestingly, multiple secretory components of the Wnt pathway were also observed in this cluster, including *WNT2*, *WNT5A*, *DKK2/3* and *SFRP2/4*. Identification of *WNT2* and *WNT5A* in this cluster likely reflect the contribution of non-canonical Wnt signalling in tumour progression. In pancreatic cancer, metastatic cancer cells that showed high efficiency in anchorage-independent sphere formation have been shown to highly express WNT2 (Yu et al., 2012). Upregulation of WNT5A along with its receptor FZD2 in cancer has been implicated in EMT as FZD2-neutralising antibody confers suppressive effect on the growth and metastasis of the tumour (Gujral et al., 2014). Moreover, context-dependent role of stromal Wnt ligands WNT2 and WNT5A in the canonical Wnt pathway has been reported. WNT2 has been described to be a canonical Wnt ligand in various cancers including CRC (Huang et al., 2015, Jung et al., 2015). A marked upregulation of *WNT2* in CRC CAFs among Wnt components has been suggested as well as CAF-derived WNT2 promoting cancer cell invasion (Kramer et al., 2017, Aizawa et al., 2019). WNT2B is a close homologue of WNT2 which is expressed in the mesenchyme and has been shown to be one of ISC niche factors by activating canonical Wnt pathway to promote intestinal organoid culture (Farin et al., 2012). On the other hand, WNT5A is upregulated in stromal cells near epithelial cells in murine colonic injury models, the Wnt ligand suppressing the proliferation of neighbouring epithelial cells by TGF-β

signalling (Miyoshi et al., 2012). Sphere formation of colonic organoids *ex vivo* is reduced after WNT5A treatment even in the presence of WNT3A and RSPO1 (Miyoshi et al., 2012). Conversely, WNT5A has been reported to be a canonical Wnt ligand when receiver cells have higher expression of FZD4 and LRP6 (Mikels and Nusse, 2006).

High levels of DKK2 are observed in CRC, as well as other cancers, and promote cell proliferation and invasion through Wnt activation (Matsui et al., 2009, Xu et al., 2016a). DKK3 has been reported to be hypomethylated and its expression downregulated in CRC but information regarding the underlying mechanism and its role in the Wnt pathway is limited, although *in silico* analysis and *in vitro* studies have suggested that DKK3 can interact with Wnt receptor Kremen to activate the Wnt pathway (Hayashi et al., 2012, Shao et al., 2017, Nakamura and Hackam, 2010, Mohammadpour et al., 2016). On the other hand, SFRP2/4 in cancer is better characterised. Whilst commonly regarded as Wnt antagonists, SFRPs 1-5 are in fact found to have differential effects on Wnt activation, and consequently, on patient prognosis (Vincent and Postovit, 2017). A definite Wnt antagonistic role has been described for SFRP1 where its loss of expression, by promoter methylation or loss of heterozygosity, correlated with increased Wnt activation in cancers (Ugolini et al., 1999, Suzuki et al., 2002). Conversely, SFRP2 and SFRP4 are demonstrated to be of stromal origin, and their upregulation in tumours is indicative of EMT in multiple cancers (Vincent and Postovit, 2017). Analysis of the dataset has also uncovered that SFRP2/4 upregulation is associated with poor prognosis in CRC (Vincent and Postovit, 2017). The tight correlation of SFRP2/4 expression pattern with known CAF proteins collectively renders them markers for poor CRC prognosis and supports the notion that the “CAF-enriched cluster” is a gene set of stromal-derived factors that promote tumour progression (Calon et al., 2015, Vincent and Postovit, 2017).

The presence of Wnt regulators in this cluster suggests that paracrine Wnt activation in the TME may drive hyperproliferation of cancer cells and promote tumour progression. Since this cluster consists of known poor prognostic markers and Wnt agonists whose upregulation in the cancer stroma has been described, I speculate that this cluster includes CAF-derived factors that promote Wnt activation and tumour progression. In particular, I hypothesise that putative CAF-secreted Wnt regulators

are present in the cluster that may be responsible for paracrine Wnt activation. After literature research on the CAF-enriched markers with association to Wnt signalling, I selected POSTN as putative paracrine Wnt regulator in CRC for further characterisation. POSTN is a stromal secretory protein that can interact with integrins to promote cell adhesion and proliferation (Yan and Shao, 2006a) (Hamilton, 2008, Nishiyama et al., 2011). The upregulation of POSTN in multiple cancers has been associated with cancer cell invasion, metastasis, and poor patient outcome (Jang et al., 2016, Nuzzo et al., 2016, Bao et al., 2004, Kikuchi et al., 2008, Kikuchi et al., 2014, Calon et al., 2015). Despite its upregulated expression and its association to poor prognosis, the underlying mechanism in CRC remains elusive. Previous study has reported that POSTN is secreted by stromal cells to promote breast cancer metastasis by activating Wnt signalling via binding to Wnt ligands (Malanchi et al., 2011). However, it is unclear if POSTN also plays a role in Wnt activation and tumour progression in other cancer types such as CRC.

In order to investigate stromal Wnt regulators in CRC, primary fibroblasts were first established from CRC patients. I demonstrated that distinct characteristics of normal and cancer-associated populations were preserved in my primary fibroblast cultures. In general, CAFs had a greater capacity for ECM remodelling with slightly enhanced proliferation as previously described (Faouzi et al., 1999, Erdogan et al., 2017, Calvo et al., 2013). Cancer cells have been previously reported to lack the ability to remodel the ECM (Calvo et al., 2013). Therefore, CAF-specific ECM remodelling capacity reported so far indicates that increased mobility or invasiveness due to ECM remodelling stems from CAF activity rather than epithelial cancer cells and is in line with how CAF activity in the TME promotes tumour progression (Calvo et al., 2013).

Moreover, expression profiles of the patient-derived fibroblasts showed variation. As RT-qPCR analysis was carried out on a cell population level, this likely reflects cell heterogeneity at a single cell level. It should also be noted that CAF identity can be designated based on various parameters such as marker expression levels and ECM remodelling capacity, but they are often heterogeneous and marker expression profile seldom faithfully reflects their behaviour (Sugimoto et al., 2006, Calvo et al., 2013). Therefore, the collective distinction between NFs and CAFs in their marker expression profiles and functionality, rather than positivity of a certain marker, served

as a benchmark to conclude that my patient-derived fibroblasts were indeed distinguishable. Demarcation of the two primary fibroblast population may be assisted by additional characterisations such as ACTA2 fibre imaging, RNA-sequencing, and proteomics of the fibroblasts. In particular, RNA-sequencing and proteomics can provide a more comprehensive view of the cell identity and/or state, rather than having to depend on the expression of a few select markers. Whilst variation was observed in both the expression profile and in the degree of disparity between normal and cancer-associated pairs, such heterogeneity has previously been observed both *in vivo* and *in vitro* (Sugimoto et al., 2006, Calvo et al., 2013). Furthermore, the heterogeneity within primary CAF cultures has been reported (Herrera et al., 2013, Berdiel-Acer et al., 2014c). This can be caused by differential origin of CAFs, degree of activation, or distinct molecular cancer subtypes among the patients (Herrera et al., 2013, Worthley et al., 2010). CAF identity of primary fibroblasts may depend on the crosstalk with epithelial cancer cells which may be lost or mitigated upon isolation, or on epigenetic modifications in which case the identity is likely more stable. In the absence of patient information, it is difficult to attribute the heterogeneity observed in primary fibroblasts on specific factor(s) conclusively. Overall, the results show that the patient-derived fibroblasts recapitulate the *in vivo* properties, representing a suitable *ex vivo* model for the study.

I believe that my explant culture protocol for generation of fibroblasts allows better representation of cellular heterogeneity. Although it is difficult to rule out contamination of NFs with CAFs during tissue resection, this is the standard protocol used by many others to establish primary fibroblasts that recapitulate the cellular and functional complexities *in vivo* for studying the crosstalk between epithelial cells and fibroblasts in cancers. Many researchers immortalised the primary fibroblasts by introducing the catalytic subunit of human telomerase holoenzyme after culture establishment for long-term experimental use (Calvo et al., 2013, Arandkar et al., 2018, Aizawa et al., 2019). This can increase the number of feasible subcultures as primary fibroblasts often become senescent beyond a certain passage number. Indeed, in my hands, it was not possible to expand primary fibroblasts beyond passage 10. However, I chose to utilise non-immortalised primary fibroblasts in our experiments to avoid potential physiological alteration to the cells as prolonged

passaging of these primary fibroblasts could potentially lead to loss of *in vivo* cellular heterogeneity or bias to sub-clonal expansion.

To determine if fibroblast-mediated paracrine Wnt activation exists in CRC, I examined the Wnt activity in the primary NFs and CAFs. Whilst most CAFs promoted higher Wnt activity than NFs, the level of Wnt activation varied amongst the samples. As discussed above, this may likely be attributed to patient-to-patient variation as well as the molecular subtype of each CRC patient with differential TME composition. In addition, the data showed that CAFs exhibited higher Wnt activity than NFs in both co-culture and conditioned media treatment approaches, indicating that the paracrine Wnt activation by CAFs can be induced by secretory factors and not entirely contact-dependent.

In summary, I established patient-derived primary fibroblasts that recapitulated *in vivo*-like disparity and the CAF-derived Wnt potentiation. I further confirmed that the selected candidate POSTN was indeed upregulated in CAFs. To study the putative Wnt regulatory role of POSTN in CRC, paired primary fibroblast lines with increased Wnt activation and POSTN expression in CAFs compared to their normal counterparts were selected for subsequent analysis. I evaluated the molecular mechanism of POSTN in Wnt regulation (Chapter 4), and generated a biomimetic model system to assess the interaction between fibroblasts and epithelial cells (Chapter 5).

Chapter 4. Characterisation of POSTN as a potential Wnt regulator

4.1 Introduction

PERIOSTIN was first discovered in a murine osteoblast cell line as a potential cell adhesion protein in 1993, and was later defined to be one of TGF-β-inducible proteins (Takeshita et al., 1993, Horiuchi et al., 1999). The gene encodes a matricellular protein of 836 amino acids consisting of a signal peptide for secretion, an emilin (EMI) domain denoting a small N-terminal cysteine-rich module, and four fasciclin-like domains (FAS1) followed by a hydrophilic C-terminal region (Kii et al., 2010, Doliana et al., 2000, Hoersch and Andrade-Navarro, 2010). The FAS1 repeats facilitate interaction with cells by binding to integrins expressed on plasma membranes (Zinn et al., 1988, Hoersch and Andrade-Navarro, 2010), whereas the EMI domain and C-terminal region are reported to interact with ECM proteins such as TNC, FN, and collagens (Kii et al., 2010, Takayama et al., 2006, Norris et al., 2007). POSTN is found to facilitate collagen crosslinking by acting as a scaffold for BMP-1, which cleaves and activates collagen crosslinker LOX by binding to both for BMP-1 and collagen (Maruhashi et al., 2010). Animal studies have also uncovered its contribution to development and regeneration as POSTN-deficient mice suffer from aberrant collagen fibrillogenesis, low bone mass, and fatigue loading injury leading to severe bone damage (Norris et al., 2007, Bonnet et al., 2013).

Studies have attributed the regulation of POSTN expression to several transcription factors. Upregulation of POSTN transcription following SMAD2/3 phosphorylation has been described indicating that TGF-β activation results in POSTN expression (Chen et al., 2017b). Overexpression of c-Fos is shown to result in higher POSTN expression (Kashima et al., 2009). The transcription factor p73 has been confirmed to bind to the POSTN promoter to upregulate POSTN expression (Landre et al., 2016). Moreover, a response element to transcription factors TWIST-1/2 found in the POSTN promoter has been validated by a reporter assay in osteoblasts (Oshima et al., 2002). Further studies in disease models have demonstrated that Twist family transcription factors regulate POSTN expression. Fibroblasts derived from patients

with Setleis syndrome of aberrant facial development carry mutant TWIST2 that is no longer capable of inducing POSTN expression (Franco et al., 2011). In addition, analyses of TCGA data reveal that the POSTN expression pattern clusters with that of TWIST in a lung cancer cohort, and RNAi-mediated silencing of TWIST in prostate cancer cells results in POSTN downregulation (Hu et al., 2015a, Hu et al., 2017).

In cancer, POSTN has been revealed as a marker for metastasis and tumour progression, associated with poor prognosis and short relapse-free survival of multiple cancers, such as prostate, lung, liver, ovarian, pancreatic, breast, and colorectal (Nuzzo et al., 2012, Riener et al., 2010, Sung et al., 2016, Soltermann et al., 2008, Kanno et al., 2008, Malanchi et al., 2011, Ratajczak-Wielgomas et al., 2016, Oh et al., 2017, Xu et al., 2016b). However, there are contrasting data in the literature about the correlation of changes in POSTN expression level and prognosis. For example, a low epithelial but high stromal POSTN expression in prostate cancer has been associated with short survival whilst in liver cancer, a high epithelial POSTN expression correlated to reduced overall survival (Nuzzo et al., 2012, Riener et al., 2010). In ovarian cancer, despite high POSTN expression in both epithelial cancer cells and tumour stroma, upregulation of POSTN in epithelial cancer cells has had no significant link to patient prognosis (Sung et al., 2016). Overall, it is commonly believed that upregulation of POSTN expression in the tumour stroma is associated with tumour progression and poor prognosis (Gonzalez-Gonzalez and Alonso, 2018).

A higher POSTN expression in the tumour stroma is associated with tumour invasion, proliferation and EMT, suggesting that POSTN may play a role in the tumour niche to promote tumour progression (Malanchi et al., 2011, Hu et al., 2017, Hu et al., 2015a). In periodontal cells, POSTN has been shown to promote resistance to hypoxia-induced cell death, and similar mechanism is suggested to be at play as POSTN upregulation is observed in carcinoma cells in hypoxic conditions in addition to increased cell survival (Bao et al., 2004, Aukkarasongsup et al., 2013). POSTN has also been suggested to activate MAPK/ERK pathway and promote cancer cell proliferation (Kikuchi et al., 2014, Kotobuki et al., 2014).

Furthermore, POSTN has been suggested to be integral in promoting metastasis. Exogenous POSTN has been shown to promote EMT as seen in the increased

expression of EMT markers such as Vimentin and N-cadherin, while downregulation in that of E-cadherin, and the EMT accompanies increase in cancer cell mobility and proliferation, resulting in augmented metastatic activity with the activation of EGFR signalling pathway (Yan and Shao, 2006b, Hong et al., 2010). In addition, upregulation of POSTN is also associated with an increased activity of MMP2/9, indicative of the ECM remodelling that CAFs undertake compared to their normal counterparts (Yan and Shao, 2006b). Transplanted melanoma cells have shown to be strongly attracted to osteoblasts producing POSTN, which is reduced upon RNAi-mediated POSTN suppression, and metastatic cell-derived exosomes are found to contain high concentration of POSTN (Fukuda et al. 2015, Vardaki, et al. 2016). It has been shown that POSTN is capable of binding to integrins to promote cell adhesion and migration (Gillan et al., 2002). Consistently, cancer cell invasion and metastasis can be reduced by neutralising POSTN (Gillan et al., 2002, Zhu et al., 2011). In CRC, integrin binding of POSTN has been shown to activate the PI3K/AKT pathway and contribute to metastasis and chemoresistance of CRC cells (Bao et al., 2004, Xiao et al., 2015). Also, POSTN expression promotes angiogenesis and lymphanogenesis via AKT signalling pathway in multiple cancers (Kudo et al. 2012). Another route by which POSTN support tumour growth is immune evasion, as POSTN has been suggested to enhance recruitment of tumour-associated macrophages in glioblastoma (Zhou et al., 2015). Collectively, it is believed that POSTN is one of the ways to modulate the microenvironment that supports cancer cell migration and proliferation (Bao et al., 2004, Gillan et al., 2002, Zhu et al., 2011, Malanchi et al., 2011, Kikuchi et al., 2014).

While POSTN upregulation in various cancer types and the potential functional significance of the upregulation have been described, the role of POSTN in the Wnt pathway remains elusive. The TGF-β-induced POSTN expression in fibroblasts has been shown to promote metastases of circulating breast cancer cells (Malanchi et al., 2011). The study further showed that POSTN interacted with Wnt ligands and augmented Wnt signalling (Malanchi et al., 2011). Moreover, POSTN has been associated with phosphorylation of both LRP6 and GSK3β during osteogenic differentiation of mesenchymal stem cells (Zhang et al., 2017). However, it is still unclear if POSTN potentiates Wnt signalling in CRC, and the corresponding underlying mechanism remains ambiguous.

In the previous chapter, I confirmed that POSTN is upregulated in CAFs derived from CRC patients that show higher Wnt activity than NFs. Here, I aimed to explore the role of POSTN in Wnt signalling and investigate the potential underlying molecular mechanism of how the protein is implicated in the pathway. In this chapter, HEK293T cells were utilised for most *in vitro* studies as they allowed advantageous expandability relative to primary fibroblasts as well as functionally competent canonical Wnt pathway while primary fibroblasts were subject to patient-dependent mutations that could confound the mechanistic investigation.

4.2 Results

4.2.1 POSTN can enhance, but not induce, Wnt activation

To study the putative Wnt regulatory role of POSTN, I first carried out gain-of-function studies to express POSTN with TCF-TOPFlash reporters in HEK293T cells and measure the luciferase Wnt reporter activity (Figure 4.1A). Expression of POSTN in HEK293T cells did not alter the Wnt activity in the absence of Wnt stimulation. On the other hand, POSTN could enhance Wnt activation when treated with exogenous WNT3A alone or in combination with RSPO1, although the increase in WNT3A only condition varied and failed to show a consistent change. Condition with the addition of RSPO1 served as an indirect readout of Wnt enhancement at play *in vivo* to provide a relative comparison of potential effect of POSTN. Nevertheless, the pattern of potentiation in the WNT3A-treated conditions suggested that the Wnt-activating role of POSTN is dependent on the presence of Wnt ligands.

The Wnt activating effect of POSTN was further examined in three CRC cell lines, SW480, LS174T and HCT116. Interestingly, the Wnt-activating role of POSTN was observed only in HCT116 cells and not in SW480 or LS174T cells (Figure 4.1B). HCT116 cells are MLH1 low CRC cells that harbour an activating mutation in one allele of β-catenin but no mutations in APC (Koo et al., 2012, Morin et al., 1997). Conversely, SW480 cells have homozygous APC truncation, and LS174T cells have homozygous β-catenin mutation. The data indicated that POSTN was not able to potentiate Wnt activation in APC- or β-catenin-mutated cells, suggesting that the Wnt

regulatory role of POSTN is likely at the receptor level. Interestingly, it has been previously shown that HCT116 cells have autocrine Wnt secretion, which may explain the POSTN-induced Wnt activation (Bafico et al., 2004). To validate if HCT116 cells possess autocrine Wnt secretion, expression of different epithelial Wnt ligands were examined (Figure 4.1C). RT-qPCR analysis revealed that multiple Wnt ligands were expressed in HCT116 cells at significantly higher levels than in HEK293T cells likely responsible for higher baseline Wnt activity in HCT116 cells without exogenous WNT3A treatment. Since HCT116 cells have a WT copy of β-catenin and are able to respond to Wnt induction, I then further treated the cells with WNT3A and/or RSPO. Indeed, treatment of WNT3A and/or RSPO was able to activate Wnt signalling in HCT116 cells which is consistent with the HEK293T data (Figure 4.1D). Together, I concluded that POSTN is able to potentiate Wnt signalling at the receptor level in the presence of Wnt ligands, while POSTN alone is insufficient to induce Wnt activation.

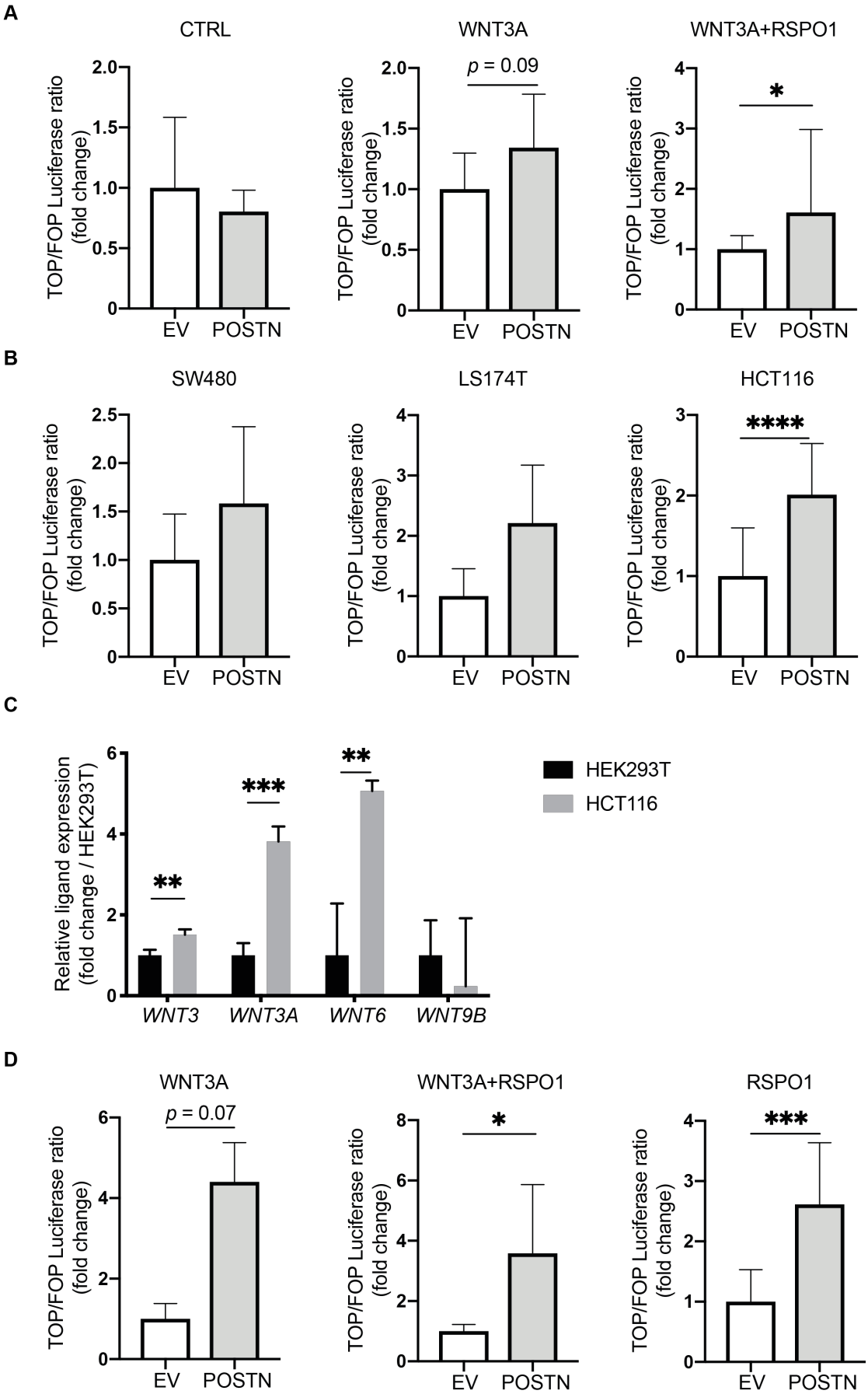
A
CTRL
WNT3A
WNT3A+RSPO1
p = 0.09
*
TOP/FOP Luciferase ratio (fold change)
EV
POSTN
B
SW480
LS174T
HCT116
****
C
Relative ligand expression (fold change / HEK293T)
HEK293T
HCT116
**
***
**
WNT3
WNT3A
WNT6
WNT9B
D
WNT3A
p = 0.07
WNT3A+RSPO1
*
RSPO1
***

Figure 4.1 Wnt activity potentiation by POSTN.

(A) Relative luciferase Wnt reporter activity in HEK293T cells with empty vector (EV) or POSTN overexpression after treatment with control conditioned media (CTRL), 50% WNT3A conditioned media (WNT3A), or 50% WNT3A and 10% RSPO1 conditioned media (WNT3A+RSPO1) as indicated. TCF-luciferase signals were normalised to renilla luciferase activities. Data are N = 3 ± SD, and *p<0.05. (B) Relative luciferase Wnt reporter activity in SW480, LS174T, and HCT116 cells with or without POSTN overexpression and treated with control conditioned media. Data are N = 3 ± SD, and **** p<0.0001. (C) RT-qPCR analyses of epithelial Wnt ligands in HEK293T and HCT116 cells. Data are shown as fold change of expression in HCT116 compared to HEK293T after normalisation to β-actin expression levels, and N = 3 ± SD. **p<0.01, and ***p<0.001. (D) Relative luciferase Wnt reporter activity in HCT116 cells with or without POSTN overexpression and treated with 50% WNT3A conditioned media (WNT3A), or 50% WNT3A and 10% RSPO1 conditioned media (WNT3A+RSPO1) as indicated. Data are N = 3 ± SD, *p<0.05, and *** p<0.001.

4.2.2 POSTN can interact with cell surface-expressed canonical Wnt receptors

The current data suggest that the Wnt regulatory role of POSTN is at cell surface level and is likely dependent on the presence of Wnt ligands. Interestingly, similar Wnt enhancing effect was observed in the Wnt agonists RSPO proteins, which are mostly secreted by stromal cells *in vivo* (de Lau et al., 2011, Koo et al., 2012, Greicius et al., 2018, Kabiri et al., 2014). To determine how POSTN potentiates Wnt activation, potential interaction with the known canonical Wnt receptors (LRP6 and FZD4/5/7) and the transmembrane receptors of the Wnt-RSPO axis (LGR4/5/6 and RNF43) were examined.

Lack of available antibodies capable of detecting endogenous receptors necessitated overexpressing the desired Wnt receptors or transmembrane proteins with or without POSTN for the assays. Immunoprecipitations (IPs) were performed with HEK293T cells after co-overexpression. Firstly, IP of MYC-tagged LRP6 showed that POSTN potentially interacted with LRP6 (Figure 4.2A). Reverse IP using POSTN antibody further confirmed their interaction. On the other hand, no interaction

between POSTN and FZD4/5/7 receptors was detected in POSTN IP or FZD4/5/7 tag IP experiments (Figure 4.2B).

Next, I evaluated if POSTN interacted with the transmembrane receptors for the Wnt-RSPO axis, LGR4/5/6 and RNF43. Unlike FZD receptors, binding of POSTN to all three LGRs was detected (Figure 4.2C). Among the three LGRs, the strongest interaction was detected with LGR6 regardless of overexpression efficiency in the input. In addition, POSTN also seemed to interact with RNF43 (Figure 4.2D). Interestingly, strong signal was detected in the co-IP of RNF43 and POSTN after proteasomal inhibition, suggesting protein stabilisation was necessary to assess interaction of POSTN the E3 ligase. Together, the data demonstrate that POSTN is capable of binding to Wnt receptors LRP6, LGR4/5/6 and RNF43.

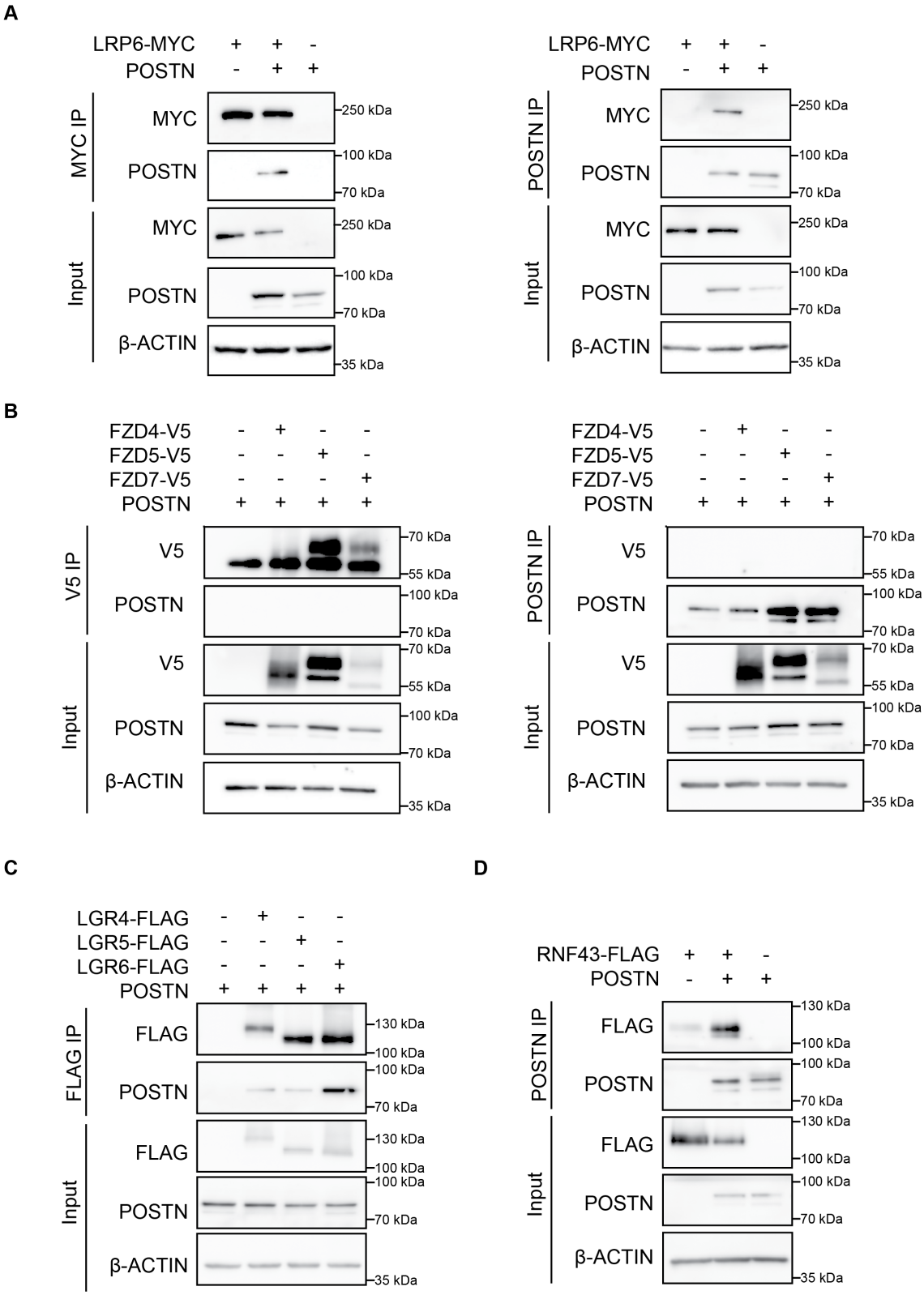

Figure 4.2 Potential interaction of POSTN with canonical Wnt receptors and associated transmembrane proteins.

Immunoprecipitation of either POSTN or tag protein after co-overexpression of POSTN and tagged Wnt receptors, LRP6 (A), FZDs (B), or tagged transmembrane

Wnt regulators LGRs (C) and RNF43 (D). Interactions with LRP6, LGRs, and RNF43 were detected whereas none was observed with FZD receptors. Depicted RNF43 IP was performed after 4 hours of MG132 treatment.

4.2.3 POSTN can stabilise FZD5 but not its putative binding partners

Following the IPs to identify putative binding partners, the protein levels of any of the Wnt receptors were assessed to address whether they were modulated by POSTN. POSTN and the Wnt receptors were co-overexpressed and the protein level of the Wnt receptors was examined by whole cell lysate western blot. Despite the interaction, LGR4/5/6 and LRP6 protein levels were not affected by ectopic expression of POSTN (Figure 4.3).

On the other hand, co-expression of FZD receptors with POSTN showed interesting observation. Despite the lack of interaction between POSTN and FZD receptors, a consistent stabilisation of FZD5, but not FZD4/7, was observed by approximately 2-fold when co-expressed with POSTN (Figure 4.4A). To directly visualise POSTN-mediated FZD5 stabilisation, immunofluorescence (IF) was performed in HEK293T cells overexpressing FZD5 together with EV control or POSTN (Figure 4.4B). Interestingly, FZD5 expression intensity appeared to be stronger in the POSTN-expressing cells, which is consistent with the western blot data. However, the IF data is arguably more qualitative than quantitative.

To confirm that POSTN mediates stabilisation of FZD5 protein at the cell surface level, I carried out flow cytometric analyses on HEK293T cells overexpressing FZD5 in the absence or presence of POSTN (Figure 4.4C). This approach allowed a quantitative examination of the protein level on the cell membrane by staining the cells with anti-FZD5 antibody without permeabilisation. The number of FZD5-positive cells did not change with POSTN expression, but the intensity of FZD5 signal detected from populations of the same size was significantly stronger when POSTN was co-overexpressed. The results indicate that POSTN expression increases FZD5

abundance on the cell membrane. Altogether, the data suggest that POSTN does not interact directly with FZD5, but may stabilise it indirectly.

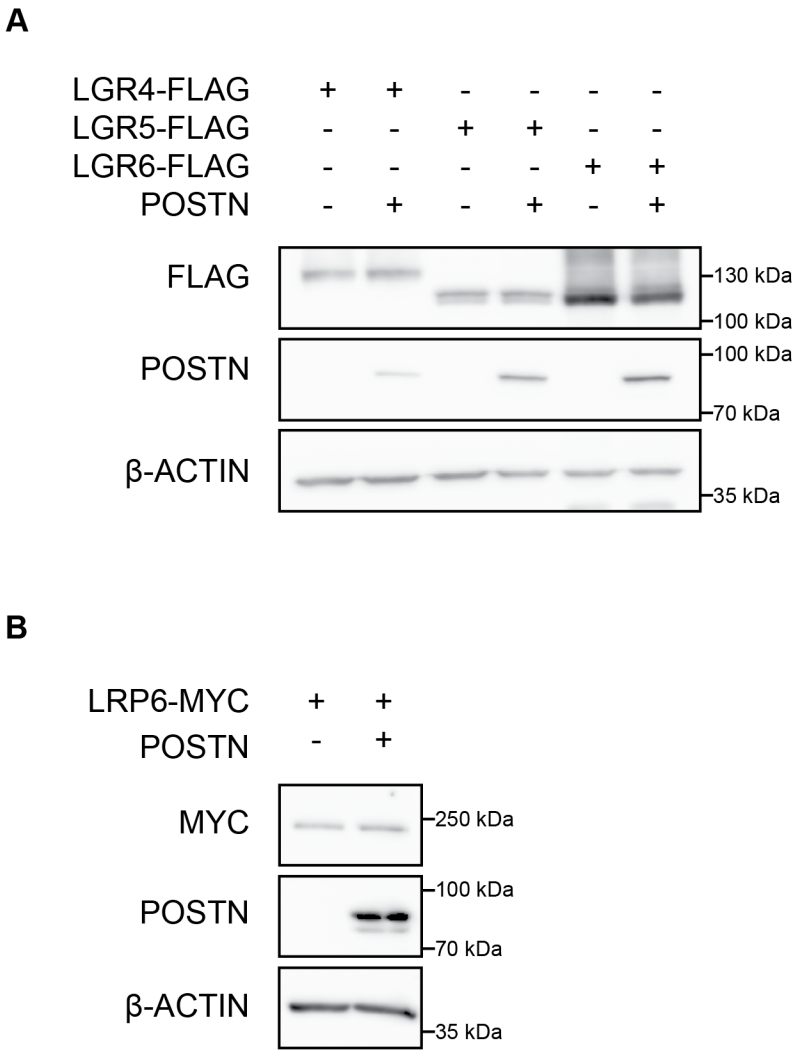

Figure 4.3 Levels of LGRs and LRP6 in the presence of POSTN.

Western blot of LGRs (A) or LRP6 (B) after co-overexpression with POSTN in HEK293T cells.

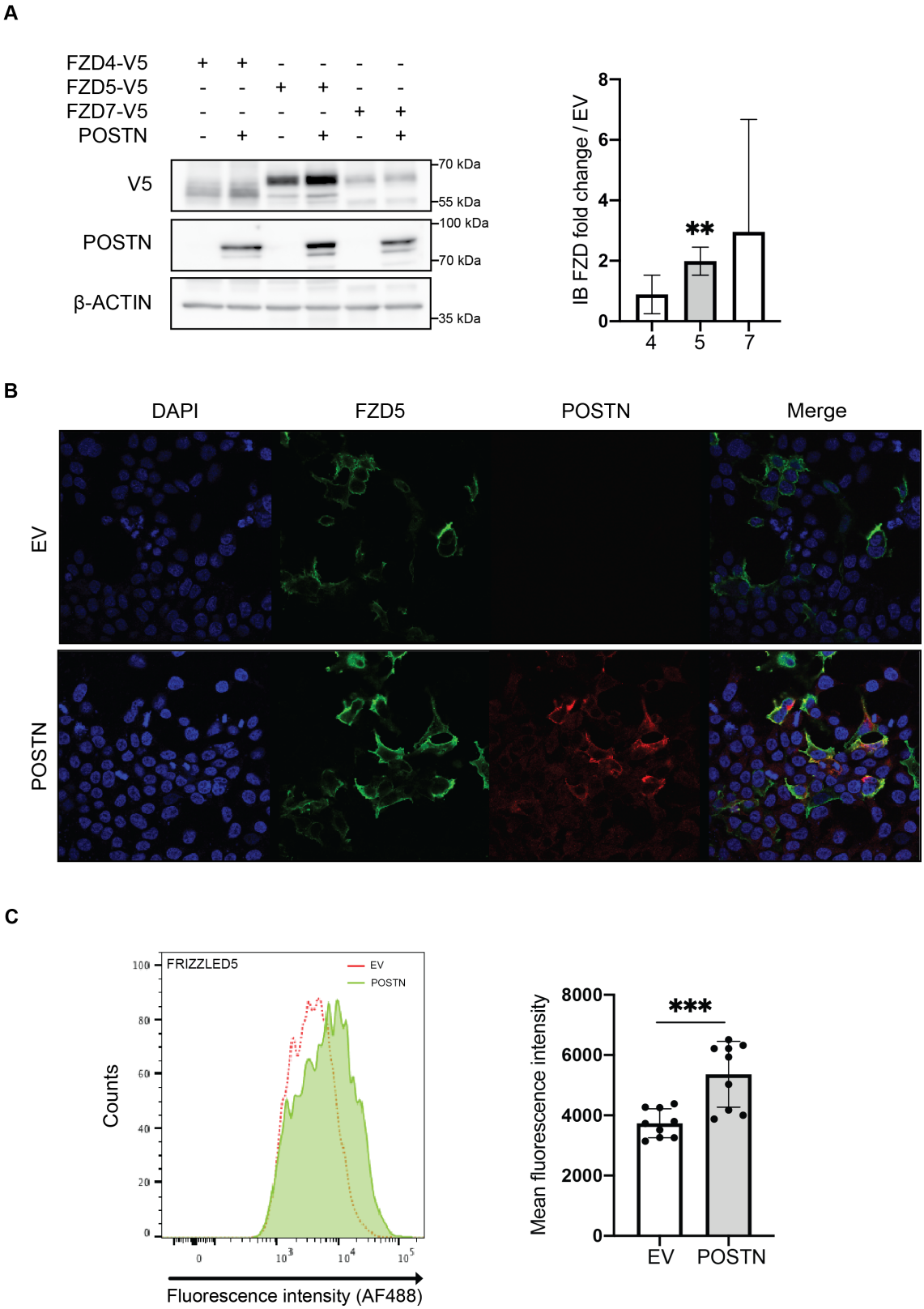

Figure 4.4 FZD5 stabilisation in POSTN-expressing cells.

(A) FZD levels detected by western blot of whole cell lysate after co-overexpression with empty vector (EV) or POSTN in HEK293T cells. The relative protein level normalised to β-actin with or without POSTN overexpression is shown. N = 3 or 4 ±

SD and ** p <0.01. (B) Representative immunofluorescent staining of tagged FZD5 and POSTN in HEK293T cells after overexpression of FZD5 with either EV or POSTN. (C) Flow cytometric analysis of surface FZD5 level after co-overexpression with EV or POSTN in HEK293T cells. Histogram is representative of three independent experiments. Mean fluorescence intensity of FZD5-positive population in either EV or POSTN co-overexpressed cells is shown. N = 3 ± SD and *** p<0.001.

4.2.4 POSTN may target RNF43 to potentiate Wnt activation

The lack of evidence showing a direct interaction between POSTN and FZD5 prompted an investigation into whether POSTN was stabilising FZD5 indirectly by modulating RNF43, an E3 ligase that targets FZD5 for degradation (Koo et al., 2012). I previously demonstrated that POSTN interacted with LGRs and RNF43 (Figure 4.2). Since a stronger co-IP between POSTN and RNF43 was detected after proteasomal inhibition, I tested if the POSTN affected the protein level of RNF43 (Figure 4.5). Indeed, HEK293T cells co-overexpressing POSTN showed a lower level of RNF43 when compared to cells overexpressing RNF43 with EV, which was confirmed by the quantification of relative protein levels detected by western blot (Figure 4.5A). The data suggest that POSTN may possibly enhance FZD5 expression by destabilising its E3 ligase RNF43 expression.

Next, I tested if POSTN can rescue RNF43-mediated Wnt inhibition. The TCF-TOPFlash reporter activities in HCT116 cells overexpressing RNF43 with EV or POSTN were compared. Consistent to my earlier data, POSTN expression alone increased Wnt activation. On the other hand, RNF43 overexpression significantly reduced Wnt reporter activity as expected (Figure 4.5B). In contrast to the hypothesis, co-overexpression of POSTN did not show any rescue effect on RNF43-mediated Wnt suppression. Similar data was observed when HCT116 cells were treated with exogenous WNT3A. The results suggest that POSTN may possibly stabilise FZD5 expression via decreasing RNF43 level, while such effect was insufficient to potentiate Wnt signalling. Further investigation is required to determine whether RNF43 is indeed involved in the underlying mechanism of POSTN that promotes FZD5 stabilisation.

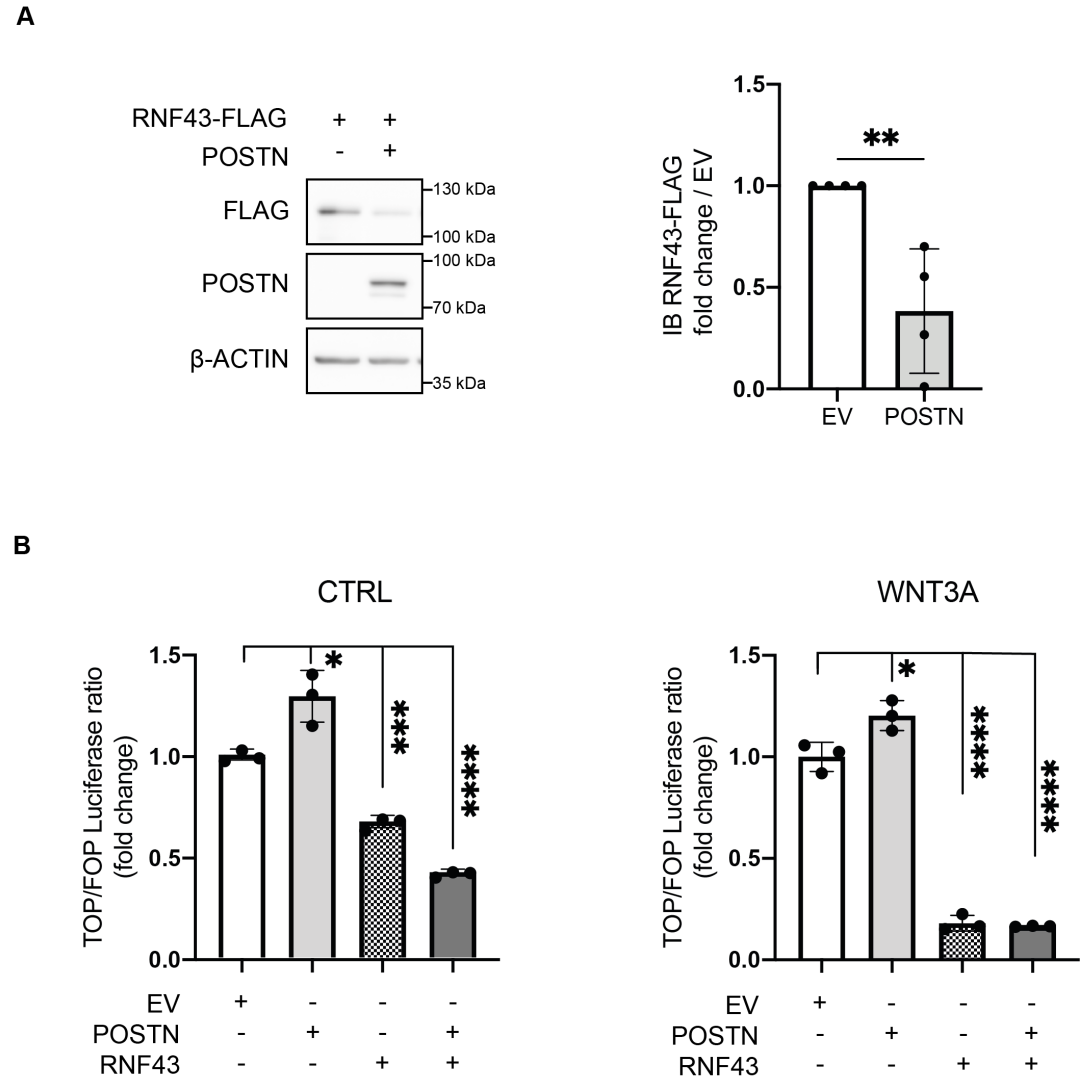

Figure 4.5 Contrasting effect of POSTN on RNF43 in Wnt pathway.

(A) Whole HEK293T cell lysate western blot after RNF43 overexpression with either EV or POSTN showing reduced RNF43 protein level after POSTN co-overexpression. Quantification of relative protein level detected after normalisation to housekeeping protein GAPDH or β-ACTIN. N = 4, and ** $p<0.01$. (B) Relative luciferase Wnt reporter assay in HCT116 cells overexpressing either EV, POSTN, and/or RNF43 treated with control or WNT3A conditioned media as indicated. POSTN overexpression failed to rescue Wnt activity suppression in RNF43 overexpressing cells. Data are representative of N=3 ± SD, * $p<0.05$, *** $p<0.001$, and **** $p<0.0001$.

4.3 Discussion

Abnormal Wnt activation is a common feature across CRCs notwithstanding mutations in the pathway. I hypothesise that stromal factors upregulated in CAFs may include paracrine Wnt regulators that contribute to such aberrant Wnt activity. In Chapter 3, a gene cluster was identified including CAF markers such as *FAP*, *IGFBP7*, and *CALD1* showing upregulated expression patterns (Calon et al., 2015). From the cluster, I selected POSTN to investigate as a potential Wnt regulator since it has been implicated to activate Wnt pathway in breast cancer and I demonstrated that it was indeed upregulated in CRC tumours and CAFs (Malanchi et al., 2011).

In this chapter, the putative Wnt regulatory mechanism of POSTN was explored by overexpression POSTN in cell lines. It should be noted that POSTN overexpression did not accurately reproduce physiological level of upregulated POSTN upregulation in cancer, as well as cellular context. However, the reproduction of physiological level of POSTN was not the priority, rather the examination of potential role of POSTN in the canonical Wnt pathway. Use of recombinant POSTN protein to divide POSTN sender cells and receiver cells did not yield effect in preliminary *in vitro* assays. It is possible that recombinant proteins were not stable or the assay time point was not optimised to assess the effect of the protein treatment. The demarcation of the specific effect derived from secreted POSTN was addressed by utilising POSTN-producing fibroblasts in TCF-TOPFlash assays in Chapter 5. Furthermore, cell lines were used to first determine POSTN-mediated effect on Wnt activity and the underlying mechanism. They offered several advantages over CRC patient-derived primary fibroblasts such as relatively unlimited expandability and fully functional canonical Wnt pathway without mutations in its components.

Firstly, I showed that POSTN is capable of enhancing Wnt activation in the presence of WNT3A in HEK293T cells. However, POSTN overexpression was enough to enhance Wnt activation in HCT116 without exogenous WNT3A (Figure 4.1B). This does not indicate that POSTN acts independently of Wnt ligands as HCT116 cells likely have autocrine Wnt secretion (Figure 4.1C). Higher Wnt activity observed in control condition may be the endogenous Wnt activation in HCT116 cells strengthened by POSTN rather than POSTN-induced Wnt activation. Further works

of examining the Wnt reporter activity after inhibition of Wnt secretion such as with Porcupine inhibitor LGK974 will confirm Wnt ligand dependence of POSTN.

The gain-of-function TCF-TOPFlash assays showed that POSTN fails to enhance Wnt activity in CRC cell lines, SW480 and LS174T, that harbour homozygous mutations in β-catenin destruction complex (Figure 4.1B). Therefore, I focused on potential interaction with Wnt receptors and transmembrane regulators present on cell membrane as they likely come in contact first with POSTN secreted by CAFs. Co-IP of POSTN with LRP6, LGRs and RNF43 was observed (Figure 4.2). As described previously, LRP6 and FZD receptors form a complex when bound to Wnt ligands to inhibit β-catenin destruction complex (Gan et al., 2008). When the potential effect of the interaction was examined on protein stabilisation, LGRs and LRP6 levels were not affected in POSTN-expressing cells. Surprisingly, despite an apparent lack of direct interaction between FZDs and POSTN (Figure 4.2), FZD5 showed a consistent increase in its protein level after POSTN overexpression and flow cytometric analyses corroborated that the FZD5 receptors were more abundant on the cell surface when the cells were co-overexpressing POSTN (Figure 4.4). It should be noted that both proteins were overexpressed due to surprisingly weak detection of endogenous FZD5 by the available antibody. Assays without any exogenous overexpression using more specific antibodies after optimisation of endogenous protein detection would help confirm POSTN-mediated FZD5 stabilisation.

I proceeded to determine if POSTN was indirectly stabilising FZD5 by targeting RNF43, one of its putative binding partners (Figure 4.2D). Considering that POSTN enhances Wnt signalling, it is conceivable that POSTN may interact with RNF43 to inhibit RNF43-mediated FZD5 degradation and Wnt suppression. POSTN expression led to the reduction of the protein level of RNF43 as observed in whole cell lysate western blot to potentially stabilise FZD5 indirectly (Figure 4.5A). Conversely, gain-of-function TCF-TOPFlash assays failed to show POSTN-mediated rescue of Wnt suppression in RNF43-overexpressing HCT116 cells (Figure 4.5B). HCT116 cells are reported to have frameshift mutations in *RNF43* and exogenous WNT3A no longer has an effect when WT RNF43 is introduced to the cells (Koo et al., 2012). However, it has been reported that RNF43 is capable of interacting with TCF4 and inhibit transcription of Wnt target genes even in the

presence of constitutively active β-catenin mutations (Loregger et al., 2015). Thus, overexpression of RNF43 likely results in Wnt suppression via its dual activity at both cell surface and nuclear membrane, and the latter may be masking the consequence of POSTN-RNF43 interaction at the cell surface that I had anticipated. Relatively weaker POSTN-mediated Wnt potentiation observed in HEK293T cells compared to HCT116 cells may be due to the presence of WT RNF43 in HEK293T cells suppressing Wnt activation at both cell surface and nuclear membrane, and POSTN potentially inhibiting cell surface RNF43 only. In this is the case, however, it is curious how POSTN is enhancing Wnt activity in RNF43-mutant HCT116 cells.

The data showed that RSPO1 treatment promoted Wnt activity in HCT116 cells (Figure 4.1D). It may be the case that RSPO1 suppresses ZNRF3, a close homologue of RNF43, that also promotes Wnt receptor turnover much like RNF43 (Hao et al., 2012, Koo et al., 2012). The functional redundancy of RNF43/ZNRF3 has been demonstrated whereby deletion of both genes is required to cause hyperproliferation of intestinal epithelial cells in animal models (Koo et al., 2012). Since my data suggest that POSTN interacts with both LGR receptors and RNF43, it is possible that POSTN functions similarly or synergistically with RSPO to facilitate the interaction of LGR and RNF43/ZNRF3. Further investigation will be needed to validate the involvement of RNF43 in POSTN-mediated Wnt activation. For instance, flow cytometric analyses may provide a conclusive readout of RNF43 protein level on cell surface in the presence of POSTN. Reconstitution assays using RNF43 mutants such as RNF43/ZNRF3△DIR that can no longer ubiquitinate FZD for internalisation may also help elucidate whether the role of POSTN in Wnt activity is dependent on the RNF43-mediated FZD degradation.

On the other hand, the putative interactions of POSTN with LGRs receptors may hint at alternative Wnt regulatory mechanisms. Interestingly, POSTN showed the strongest binding with LGR6 when co-immunoprecipitated with the three LGRs (Figure 4.2C). LGR6 does not serve as a stem cell marker for the intestine, rather it has been reported to be found in other organs such as lung and skin (Snippert et al., 2010a, Oeztuerk-Winder et al., 2012). However, LGR6 upregulation has been observed in human CRC tissues when compared to adjacent normal counterparts (Wang et al., 2018). It is possible that POSTN selectively potentiates Wnt activity in

LGR6-expressing cancer cells. Gain-of-function TCF-TOPFlash assays may provide insight into whether POSTN-LGR6 interaction indeed has an effect on Wnt activity. Further studies to validate the binding, and whether that interaction could lead to FZD5 stabilisation, either via RNF43 or an unknown alternative pathway regulating FZD receptor(s), will be needed to better understand the implication of the LGR6-POSTN interaction.

Finally, a potential RNF43-independent mechanism underlying POSTN-mediated Wnt potentiation involving LRP6 may be possible. POSTN has previously been described to increase Wnt activity and promote cancer stem cell proliferation in breast cancer (Malanchi et al., 2011). *In vitro* studies have identified the interaction between POSTN and Wnt ligands (Malanchi et al., 2011). Combined with my data showing that POSTN binds to LRP6, it is tempting to speculate that high POSTN availability in tumour stroma facilitates the binding of Wnt ligands with receptors on epithelial cancer cells to indirectly enhance Wnt activity. This could also explain why I only observed an effect of POSTN on Wnt activation in the presence of Wnt ligands, such as with WNT3A treatment of reporter cells.

There are several changes that could be made to improve the analyses. In the absence of exogenous WNT3A, Wnt activity was lower in cells overexpressing both POSTN and RNF43 than in cells overexpressing RNF43 alone, potentially due to overexpression load, in which case a control construct expressing a protein of similar size but not associated with the canonical Wnt pathway will serve as a better control than empty vector. Similar control can be employed to compare the FZD5 level on cell membrane by FACS analyses, as well as the Earth Mover's Distance which can likely be more appropriate than comparing the mean fluorescence intensity as it has been shown to allow quantification of cell population expressing the marker as well as the change in said marker expression (Orlova et al., 2016). Moreover, examination of POSTN in its mechanistic role could further benefit from assays using mutant POSTN that lack specific domains. Higher number of repeats may reveal significance in assays that showed insignificant *p* values in close margin.

My current data suggest several potential mechanisms through which POSTN may potentiate the Wnt pathway (Figure 4.6). Firstly, POSTN may interact with both LGR

and RNF43 similar to or in corroboration with RSPO, which then stabilises FZD5. However, no significant effect on Wnt activity from the interaction between POSTN and RNF43 has been observed. Due to conflicting data, further investigations are needed to determine if RNF43 is indeed targeted by POSTN to achieve FZD5 stabilisation at the cell membrane. Instead, the interaction with LRP6 and/or LGRs may result in Wnt activity enhancement through yet unidentified mechanism(s) that cancer cells exploit within the TME. Further studies will be needed to elucidate the role of POSTN in Wnt activation.

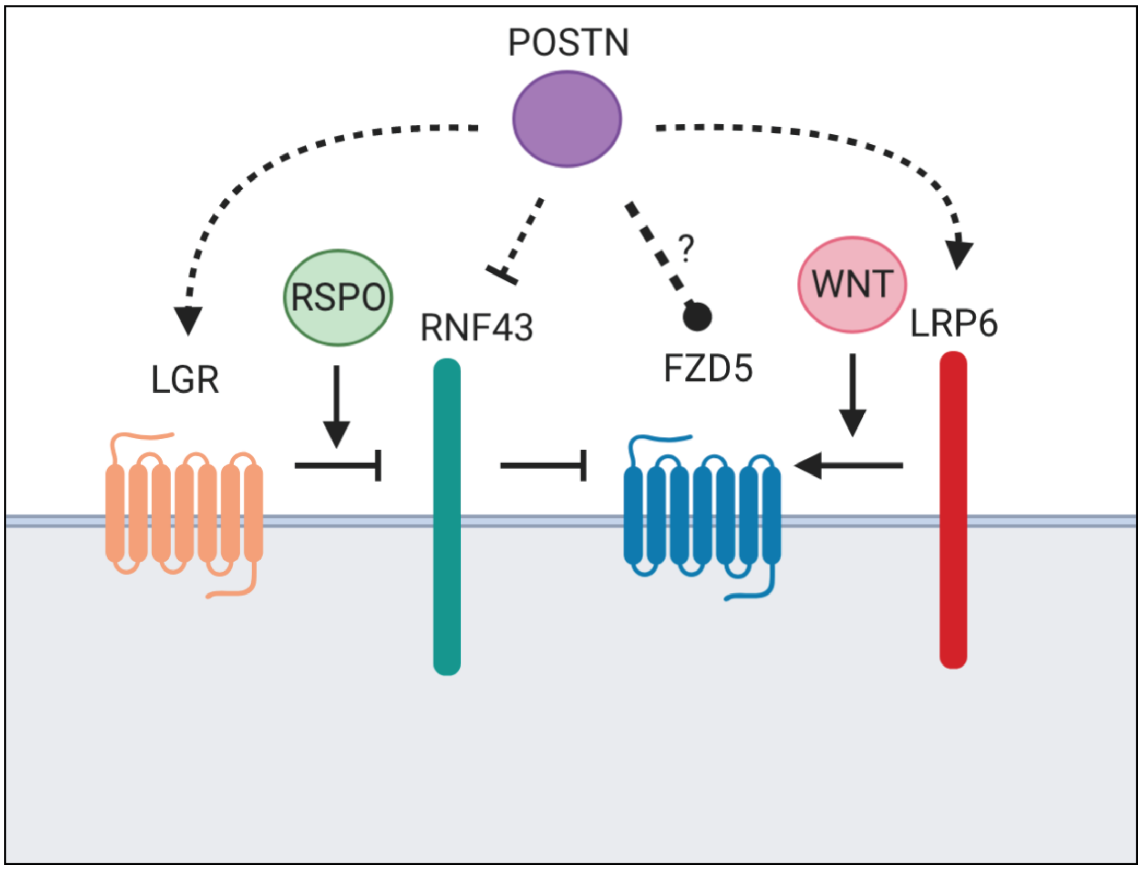

Figure 4.6 Potential molecular mechanism(s) of POSTN-mediated Wnt potentiation.

POSTN is capable of enhancing Wnt signalling dependent on WNT3A and physically interacts with LGRs, RNF43, and LRP6 (indicated here with a dashed line). Based on POSTN-induced FZD5 stabilisation on cell surface (indicated with a dashed line), I hypothesise that the interaction(s) of POSTN with these Wnt components results in a higher Wnt activity. POSTN may interact with LGRs in a similar manner to RSPO1 to inhibit RNF43 from targeting FZD5 from internalisation, or independently of RNF43 to trigger FZD5 stabilisation. POSTN may promote RNF43 degradation to indirectly increase FZD5 level. Alternatively, POSTN-LRP6 interaction, and previously reported POSTN-Wnt ligand binding, may result in a stronger Wnt activity by facilitating the binding of the Wnt ligands to the receptors. Figure created with Biorender.com.

Chapter 5. Tissue engineering a 3D model for stromal-epithelial interaction

This chapter is in collaboration with a postdoctoral researcher in the lab, Isobel Massie.

5.1 Introduction

High-throughput sequencing has facilitated genomic and transcriptomic profiling of cancer patients, and consequently more information regarding tumourigenesis and tumour progression has been gained. However, due to patient-to-patient variation and convoluted crosstalk with the TME, further work is required to fully appreciate the disease and underlying molecular mechanisms. Given that cancer is a multifaceted disease that encompasses alterations both at the cellular level and in the microenvironment, recapitulating such complexity *ex vivo* is required to better understand the disease. Construction of an *in vitro* cancer model that truly recapitulates the *in vivo* environment would be useful towards this aim.

To date, two-dimensional (2D) cancer cell culture or patient-derived xenograft (PDX) models are widely used, but the translation of the research to clinical trials for cancer therapy remains low (Mak et al., 2014, Perel et al., 2007). The establishment of primary cancer cells in 2D culture has proven difficult and often exerts selective pressure on a few fast-growing cells, thus failing to represent the original tumour (Barretina et al., 2012, Lacroix and Leclercq, 2004, Masters, 2000). As described previously, intestinal organoids are self-organising 3D culture of ISCs and progenitors that can be maintained for long term with genetic integrity and can be differentiated into all differentiated cell types found in the intestinal epithelium (Sato et al., 2009, Sato et al., 2011a). As an *ex vivo* 3D culture of cancer patient cells, tumour-derived organoids have the potential to provide a novel approach for disease modelling (Sato et al., 2011a, van de Wetering et al., 2015, Kim et al., 2019, Broutier et al., 2017). Indeed, whole genome sequencing and proteomic analyses revealed that CRC patient-derived organoids conserve the identity and profiles of the primary tumours (van de Wetering et al., 2015, Cristobal et al., 2017). These stable organoid

cultures with unlimited expansion capacity allow high-throughput drug screening to personalise medicine for individual patients once established (Sato et al., 2011a, van de Wetering et al., 2015, Kim et al., 2019, Broutier et al., 2017). However, the abundance of growth factors in the culture media may confound the physiology of cells and fail to truly represent their *in vivo* behaviour.

Moreover, ECM proteins have been shown to provide signals to help regulate cell proliferation and differentiation by acting either as direct ligands or as a reservoir of soluble growth factors (Basson et al., 1996, Wang et al., 2010, Wolpert et al., 1996, Hynes, 2009). Importantly, organoid cultures, although physiological, lack stromal cell-derived contribution to the microenvironment, thus fail to truly recapitulate the TME. For instance, several studies have suggested that stromal cells confer resistance to anticancer drugs, highlighting the importance of the TME for drug screening (Straussman et al., 2012, Wilson et al., 2012, Paraiso and Smalley, 2013). Whilst animal models can be used to test specific pathological processes in a 3D setting, they may not be able to fully replicate human diseases because of physiological differences between human and mouse, and are too costly and time-consuming for high-throughput applications (Perlman, 2016). Therefore, development of an *in vitro* 3D primary CRC model that integrates both the organoids and cancer stromal cells with physiological architecture would provide a more biomimetic cancer model.

Early efforts that have been made to include some aspects of the stromal microenvironment to generate *in vitro* models of cancer involve co-cultures. For example, non-tumourigenic epithelial cells co-cultured with CAFs acquire increased proliferative capacity *in vitro* and enhanced tumour formation when co-injected into the murine renal capsule (Hayward et al., 2001). Functional contributions of fibroblasts were further characterised by breast carcinoma xenograft models whereby CAFs enhanced tumourigenesis than patient-matched NFs by promoting angiogenesis and cancer cell proliferation (Orimo et al., 2005). Direct co-culture of stromal cells and cancer cells has provided insight into the ramifications of the heterotypic crosstalk, but is still largely limited to 2D conditions. On the other hand, with the advent of organoid culture technology, 3D co-culture of tumour organoids and stromal cells has become possible. Different experimental systems in studying

the interaction between cancer cells and stromal components using organoids have been extensively reviewed previously and will not be discussed in detail here (Fiorini et al., 2020). Co-culture of organoids with stromal components has offered novel insights into the disease as it captures heterotypic crosstalk between different cell types. Human tumour organoids co-cultured with immune cells have been shown to provide a viable model for studying therapeutic strategies by allowing immune cell infiltration (Courau et al., 2019). Recently, an exciting approach in studying the mixed population of organoids and stromal cells by mass cytometry has been described enabling analyses of cell type-specific signalling pathway activity resulting from the crosstalk (Qin et al., 2020).

Another notable approach to build a more physiologically relevant 3D system is tissue engineering, which can include the ECM to investigate the multifaceted TME. Tissue engineering combines synthetic or biological scaffolds with cells that aim to reconstitute the tissue *in vitro*. It is essential that the tissue-engineered microenvironment mimics the ECM *in vivo* and provides mechanical and biochemical signals. Artificial cancer masses comprised of CRC cell lines embedded in dense collagen hydrogels were surrounded by a loose collagenous matrix containing fibroblasts and endothelial cells to mimic the TME (Nyga et al., 2013). This system could model invasion of cancer cells from the cancer mass to the surrounding matrix. Moreover, a dynamic microfluidic platform in which cancer cell lines, fibroblasts, and endothelial cells were seeded in fibrinogen/thrombin polymerised gels has been reported to generate 3D microtumours (Sobrino et al., 2016). The efficacy of drug delivery to the vasculature could also be tested with these engineered tumours. Nevertheless, these models fail to incorporate the physical complexity, such as the tissue architecture and mechanical properties, to faithfully recapitulate CRC.

Towards building a more complex system, utilisation of biological scaffolds has been described whereby an organ is decellularised to remove cellular material (Ott et al., 2008, Ott et al., 2010, Totonelli et al., 2012, Song et al., 2013, Mazza et al., 2015). A tissue engineering approach to add complexity to this model system has been reported using decellularised porcine small intestinal scaffolds, and seeding cancer cell lines as well as stromal cells and culturing them under dynamic flow (Moll et al., 2013, Nietzer et al., 2016). Administering chemotherapy drug 5-FU revealed that the

treatment was only effective in the 3D model emphasising the benefit of reconstructing the TME. Another organotypic CRC model was suggested where decellularised human colonic matrix was repopulated with 2D primary colonic epithelial cells, myofibroblasts, and endothelial cells (Chen et al., 2016). Epithelial cells with random driver mutations were introduced and depicted malignant phenotypes in this system, such as invasion into the submucosa. New insights gained from these 3D engineered CRC models confirm that both stromal cells and the ECM play crucial roles in cancer.

While biological scaffolds are touted to retain biological cues similar to the ECM, synthetic scaffolds derived from biocompatible polymers can offer a minimal and tuneable system to dissect mechanisms underlying cell behaviour of interest (Ott et al., 2008, Ott et al., 2010, Totonelli et al., 2012, Song et al., 2013, Mazza et al., 2015, Gjorevski et al., 2016). Here, I aim to establish a novel 3D co-culture system of CRC with associated TME by combining representative primary patient-derived cells (obtained in Chapter 3) and simplicity of synthetic scaffolds. Such a 3D co-culture system would be a beneficial tool in investigating the interaction of two different cell types in an environment closer to that of *in vivo*. The Wnt pathway is examined in particular to explore heterotypic regulation between fibroblasts and epithelial cells. In this chapter I first evaluated the paracrine cues derived from NFs and CAFs in modulating epithelial cell behaviour via transwell co-culture. In addition to paracrine factors, I also explored the effect of fibroblast-mediated ECM remodelling on epithelial cells in tissue engineered 3D co-culture. Finally, CAF-derived POSTN was examined utilising these models.

5.2 Results

5.2.1 Paracrine fibroblast factors modulate epithelial cell behaviour

To study the communication between fibroblasts and epithelial cells via secretory factors, I performed transwell co-culture experiments using patient-derived fibroblasts and human small intestinal organoids (Figure 5.1). Well-differentiated small intestinal organoids exhibit budding morphology and can be distinguished from

cystic organoids which are generally more proliferative (Sato et al., 2009, Sato et al., 2011a). Therefore, small intestinal organoids provided a straightforward readout as key features of interest were proliferation and/or differentiation of epithelial cells after co-culture, and the availability of human colonic organoids was limited. In addition, transwell co-culture was utilised rather than direct co-culture of the two populations to allow separate isolation after co-culture for analyses of cell type-specific changes.

Epithelial organoids were seeded onto transwells and inserted into the well plate where either NF or CAFs were plated (Figure 5.1A). After 7 days of co-culture, organoids were collected for proliferation and transcriptomic analyses. Organoids co-cultured with NFs or CAFs showed differential size and morphology (Figure 5.1B). Quantification of organoid morphology revealed that fewer organoids were budding after co-culture with CAFs, suggesting that differentiation is impaired (Figure 5.1C). EdU staining revealed increased proliferation in organoids co-cultured with CAFs than those with NFs (Figure 5.1D and E). Organoids co-cultured with CAFs also displayed larger size than those co-cultured with NFs (Figure 5.1F). RT-qPCR analyses were carried out to examine Wnt activity of the organoids. Organoids co-cultured with CAFs showed higher expression levels of stem cell marker *LGR5*, and Wnt target genes *CMYC*, and *TCF7*. The expression of *AXIN2* was comparable between the two co-cultured organoid populations (Figure 5.1G). Overall, the transwell data suggest that patient-derived normal fibroblasts secrete paracrine factors to support organoid differentiation, while CAFs promote proliferation and Wnt activation of epithelial cells in transwell co-culture.

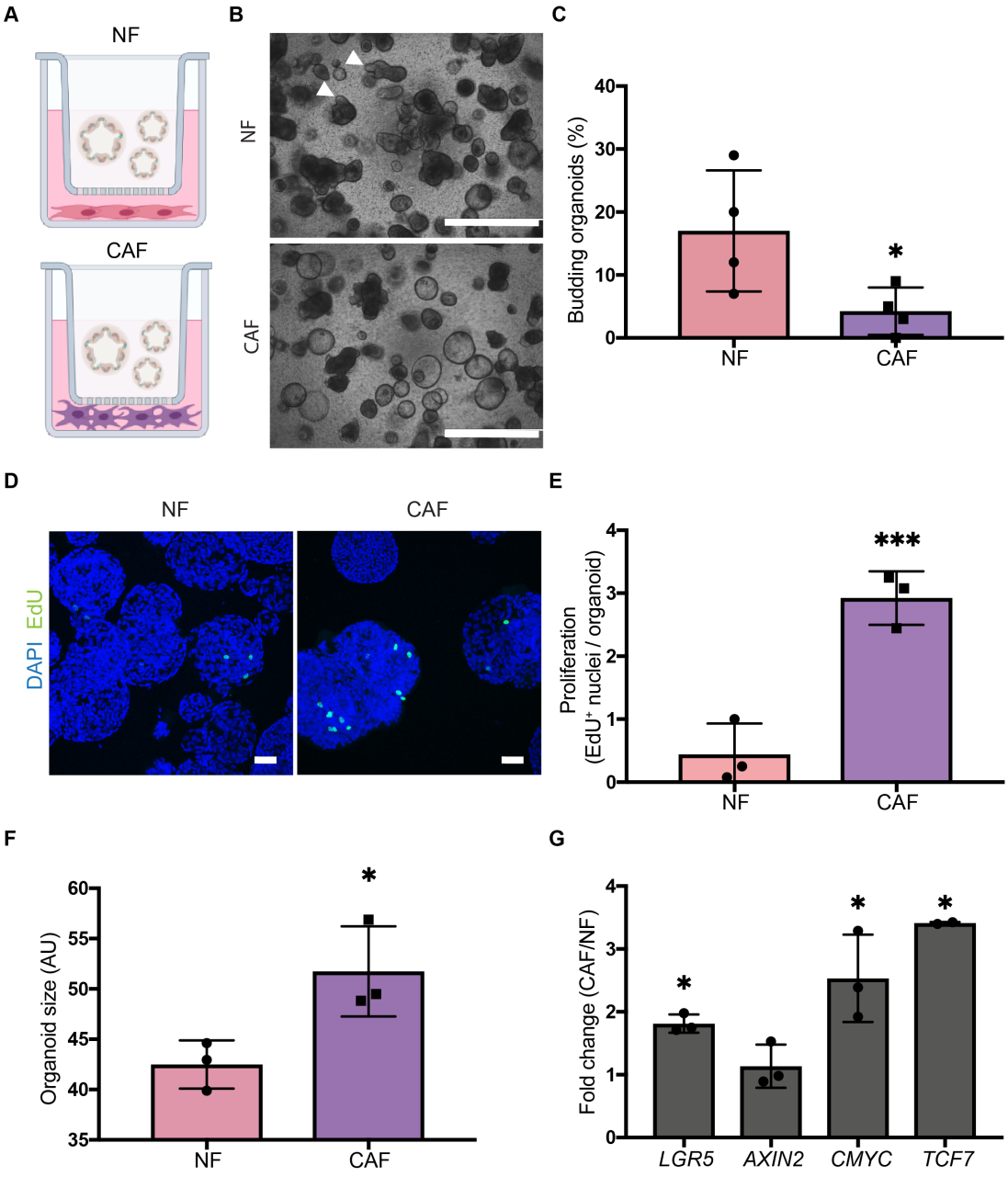

Figure 5.1 Organoid phenotype modified by co-culture with NF or CAF.

(A) Schematic of transwell co-culture of patient-derived primary fibroblasts and human intestinal organoids. Organoids were seeded on transwell inserts and cultured with either NFs or CAFs plated on well plate in media consisting of both organoid growth medium and fibroblast growth medium for seven days. (B) Representative bright field images of organoids after 7 days of co-culture. Scale bars, 1000 µm. Exemplary buds are indicated with white arrowheads. (C) Quantification of budding organoids co-cultured with either NFs or CAFs. Data are shown as percentages. N = 4 biological replicates ± SD, and * p <0.05 compared to organoids co-cultured with NFs. (D) Representative immunofluorescent images of organoids with EdU tracing. Co-cultured organoids were treated with EdU for 2 hours before fixation and nuclei were counterstained with DAPI (blue). Scale bars, 100 µm. (E) Quantification of proliferative cells in co-cultured organoids. N = 3 biological

replicates ± SD, and *** p<0.005. (F) Quantification of organoid size after co-culture with fibroblasts. N = 3 biological replicates ± SD, and * p<0.05. (G) RT-qPCR analyses of stem/Wnt markers in organoids after co-culture with fibroblasts. Data are fold change of mRNA levels compared to NF co-cultured organoids after normalisation to β-ACTIN. N = 3 technical replicates ± SD, and * p<0.05. Data generated in collaboration with Isobel Massie, who contributed in maintaining primary cells and performing experiments.

5.2.2 Synthetic tissue engineering of 3D model system using collagen I scaffold

Tissue engineering of a synthetic scaffold would allow generation of a flexible *in vitro* system to study cell behaviour and communication potentially providing insight into cell physiology that may not be apparent in 2D culture. I aimed to build a physiological 3D co-culture system resembling the *in vivo* structure, with fibroblasts located basally to epithelial cells within a biomimetic matrix. Fibroblasts were first embedded in collagen I matrix, followed by dehydration to stiffen the fibroblast-embedded collagen sheets. Human small intestinal organoids were then dissociated and seeded on top of the collagen sheets (Figure 5.2A).

The duration of the 3D co-culture was first optimised by characterising the tissue engineered system at different timepoints (Figure 5.2B). The scaffolds with epithelial cells on top were air-lifted after two weeks of submerged culture to mimic how epithelial cells are apically facing intestinal lumen *in vivo* (Nossol et al., 2011). Histology of the 3D co-culture showed a remarkable contrast of epithelial cells after long-term co-culture with NFs and CAFs (Figure 5.2C). A monolayer of epithelial cells on top of the fibroblast-embedded collagen I sheet was observed two weeks after airlifting at week 4. Surprisingly, when analysed a week later at week 5, the epithelial cells showed distinct polarisation on NF-embedded collagen I scaffold, with a crypt/villus-like structure. On the other hand, epithelial cells growing on CAF-embedded scaffolds showed a noticeable loss of organisation compared to those on NFs. Interestingly, additional week of culture (six weeks in total) appeared to have negative effect where the epithelial layer lost the organisation they exhibited the

week before. One possible explanation is that the co-culture model may not be able to sustain long-term expansion and the complex structure of the epithelial layer, and prolonged culture could induce metabolic or nutrient stress. This is evident in the CAF co-culture setting, where the upper layer of the epithelial cells appeared to be undergoing necrosis and shedding off. Of note, I did not observe any sign of necrosis in the NF-co-culture condition, suggesting that the loss of structure might be caused by other unknown reason. The data suggest that the best time point to study the fibroblast-mediated effect on epithelial organisation and differentiation would be three weeks post-airlifting at week 5.

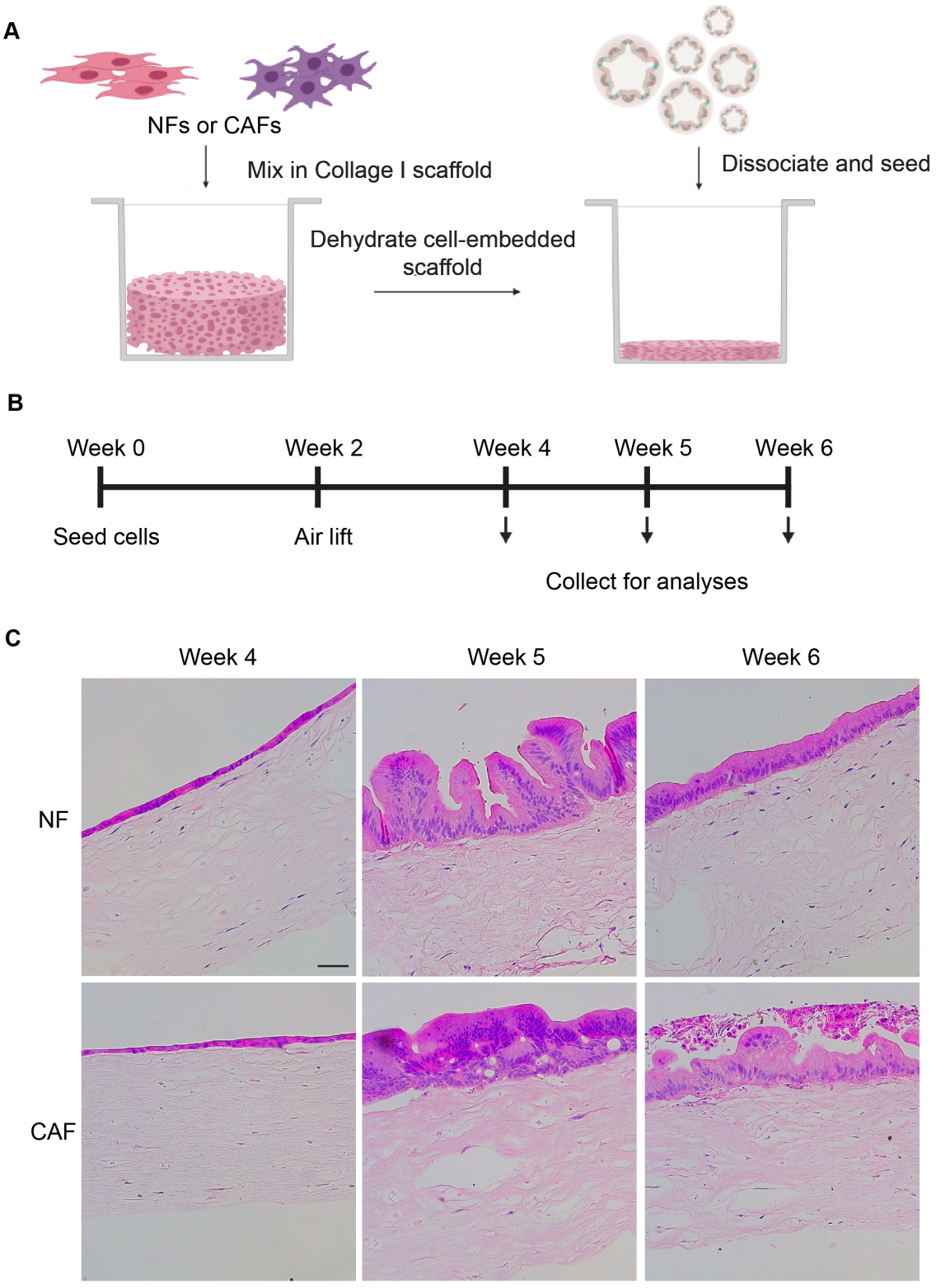

Figure 5.2 Optimisation of tissue-engineering collagen I 3D culture system.

(A) Schematic of collagen I 3D co-culture system. Patient-derived fibroblasts were resuspended in neutralised collagen I and dehydrated to create a collagen sheet embedded with fibroblasts. Human organoids were then seeded as single cells on top of the sheet and cultured in a mix of fibroblast- and organoid growth media. (B)

Timeline of 3D collagen I co-culture for culture duration optimisation. 3D co-cultures were air-lifted two weeks after seeding and were collected every week two weeks thereafter. (C) H&E (eosin-haematoxylin) staining of 3D collagen I co-cultures at three sequential time points, representatives of five images spanning the whole scaffold on average for each time point. Epithelial cell polarisation was best observed when intestinal cells were seeded on NF-embedded collagen I sheet and loss of such organisation was observed the week after. Scale bar, 50 μm. Data generated in collaboration with Isobel Massie, who contributed in maintaining primary cells and performing experiments

5.2.3 NF and CAF co-culture on collagen I scaffold attains differential epithelial cell organisation

Five weeks of culture with three weeks of airlifting yielded discernible differences between epithelial cells seeded on top of NF-embedded scaffolds and those on CAF-embedded scaffolds. Next, I proceeded to further characterise the epithelial cells following 3D co-culture with either NFs or CAFs. Epithelial cells cultured on NF-embedded collagen I sheets showed a well-organised villi-like structure (Figure 5.3A). Conversely, when cultured with CAFs, a disorganised hyperproliferation of epithelial cells was observed on the scaffolds. The epithelial cells on NF-embedded scaffolds appeared to be well anchored to the scaffold and organised into structures akin to crypt-villi of the small intestine, whereas epithelial mass on CAF-embedded scaffolds was reminiscent of hyperproliferative transformed cells with multiple intracytoplasmic lumen, which are characteristic of CRC. Immunohistochemistry showed enrichment of the Wnt targets SOX9 and CYCLIN D1 at the “crypt-like region” in the NF scaffolds, while the expression was increased and randomly distributed in the epithelial cells seeded on CAF scaffolds. Differentiation of the epithelial cells was also evaluated: staining of intestinal alkaline phosphatase (ALPi) and AB-PAS (Alcian blue-periodic acid-Schiff) suggested that the epithelial cells were differentiated in the villi-like structure when grown on NFs, while differentiation was impaired upon co-culture with CAFs.

Proliferation of the epithelial cells was determined via EdU incorporation (Figure 5.3B). Epithelial cells grown on top of NFs showed a few proliferative cells in the lower, crypt-like region. Conversely, epithelial cells grown on CAFs appeared more proliferative and their location had no clear pattern. Instead, proliferating cells were found both in lower and upper part of epithelial mass on the collagen I scaffolds, indicating a clear disorganisation of the epithelium when cultured with CAFs. RT-qPCR was performed to investigate the expression of stem cell markers and differentiation markers. Two time points, two weeks (week 4, Figure 5.3C) and three weeks (week 5, Figure 5.3D) post-airlifting were examined to gain better insight into the potential differentiation of the epithelial cells grown on the collagen I scaffolds. Epithelial cells grown on CAFs at week 4 showed a trend of relatively higher expression levels of stem cell marker *LGR5*, and Wnt target genes *AXIN2*, *TCF4*, and *CMYC* (Figure 5.3C). Interestingly, a pattern of upregulation in *LYZ*, *ALPI*, *MUC2*, and *CDH1* expressions was also observed (Figure 5.3C). On the other hand, epithelial cells cultured on the CAF-embedded scaffold at week 5 had modest upregulation of *LYZ*, *AXIN2*, *TCF4*, *CMYC*, and *CDH1*, but showed a decrease in *LGR5*, *ALPI*, and *MUC2* expression (Figure 5.3D). Relatively lower expression of *ALPI* in particular concurred with little alkaline phosphatase^{+} cells on the CAF scaffold, suggesting loss of enterocyte differentiation mediated by CAFs. Of note, none of the RT-qPCR data was significant, possibly due to the high variability of the culture model and the low number of biological replicates. Further increase in sample size will help improve statistical power.

Remodelling of the scaffold by the patient-derived fibroblasts was also examined. The morphology of collagen I scaffolds was examined after co-culture by immunofluorescent staining of collagen I which appeared to be distinct depending on the fibroblast population. In particular, NF-embedded scaffolds were more isotropic, while CAF-embedded scaffolds appeared more aligned (Figure 5.4A). RT-qPCR analyses showed that CAFs had overall higher expression levels of *ACTA2*, *LOX*, and *FN* compared to NFs (Figure 5.4B). Atomic force microscopy (AFM) was further performed to acquire a quantitative readout of the scaffolds' mechanical properties (Zemła et al., 2018) (Figure 5.4C). In line with the visualised collagen I structure and fibroblast gene expression profile, CAF-embedded collagen I scaffolds were found to be significantly stiffer than NF-embedded scaffold (Figure 5.4D). Distinct gene

expression and scaffold stiffness suggest differential ECM deposition and remodelling activity of the patient-derived CAFs.

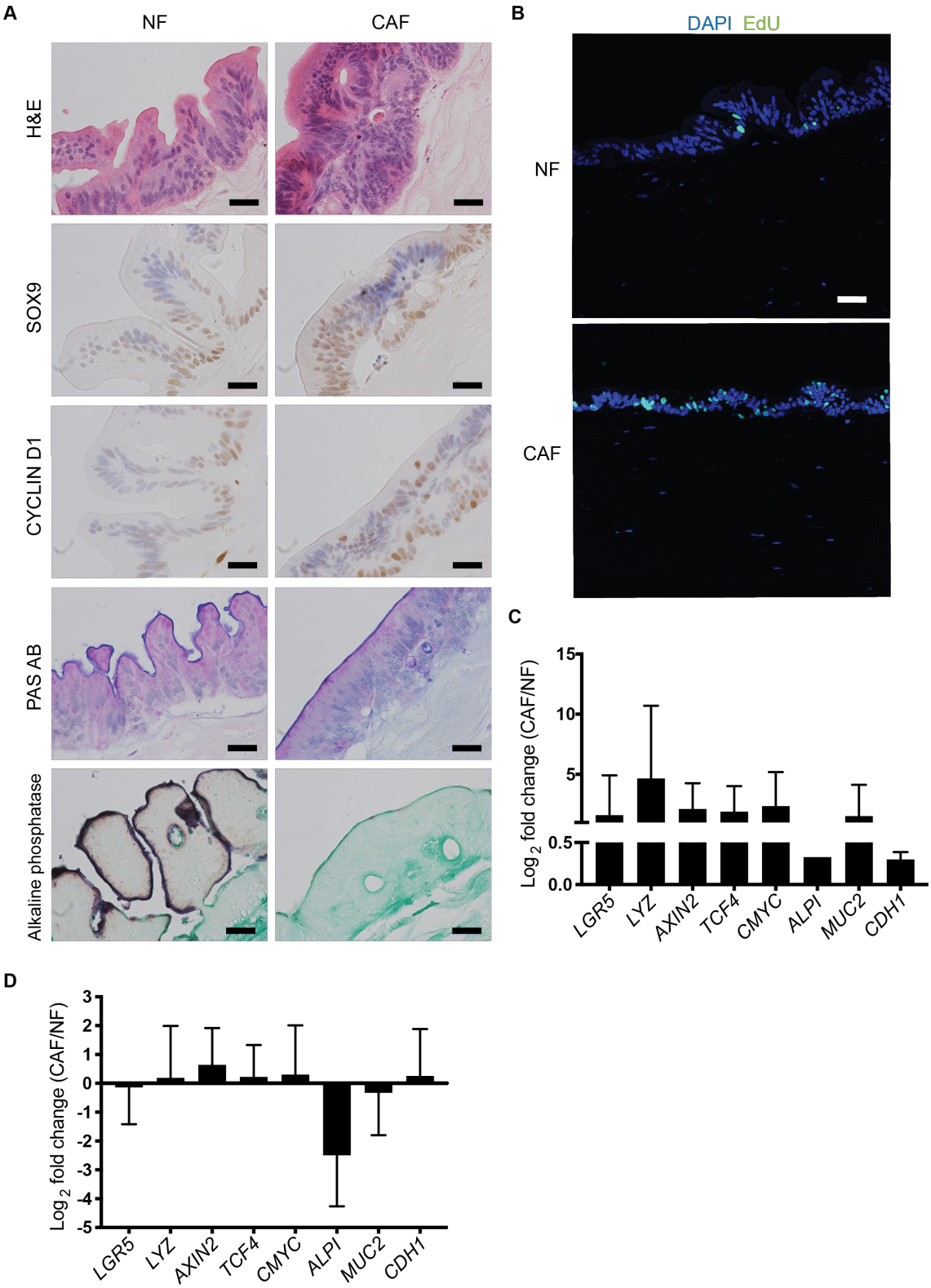

Figure 5.3 Altered epithelial phenotype after co-culture with NF or CAF in biomimetic 3D scaffolds.

(A) Representative images of epithelial cells cultured on fibroblast-embedded collagen I scaffolds stained with H&E, SOX9, CYCLIN D1, PAS AB, or alkaline

phosphatase at week 5 after three weeks of airlifting. Scale bars, 100 μm. (B) Representative immunofluorescent images EdU incorporation (green) in epithelial cells at week 5 after three weeks of airlifting culture on collagen I scaffolds. Nuclei were counterstained with DAPI (blue). Scale bars, 100 μm. (C) RT-qPCR analyses of epithelial cells following two weeks of airlift culture (week 4) with either NFs or CAFs on collagen I scaffolds. Data are shown as fold change of expression in cells cultured with CAFs over those with NFs after normalisation to *β-ACTIN* levels. N = 3 ± SD. (D) RT-qPCR analyses of epithelial cells following three weeks of air-lift culture (week 5) on NF- or CAF-embedded collagen I scaffolds. Data are shown as fold change after normalisation to *β-ACTIN* levels. N = 3 ± SD. Data generated in collaboration with Isobel Massie, who contributed in maintaining primary cells and performing experiments.

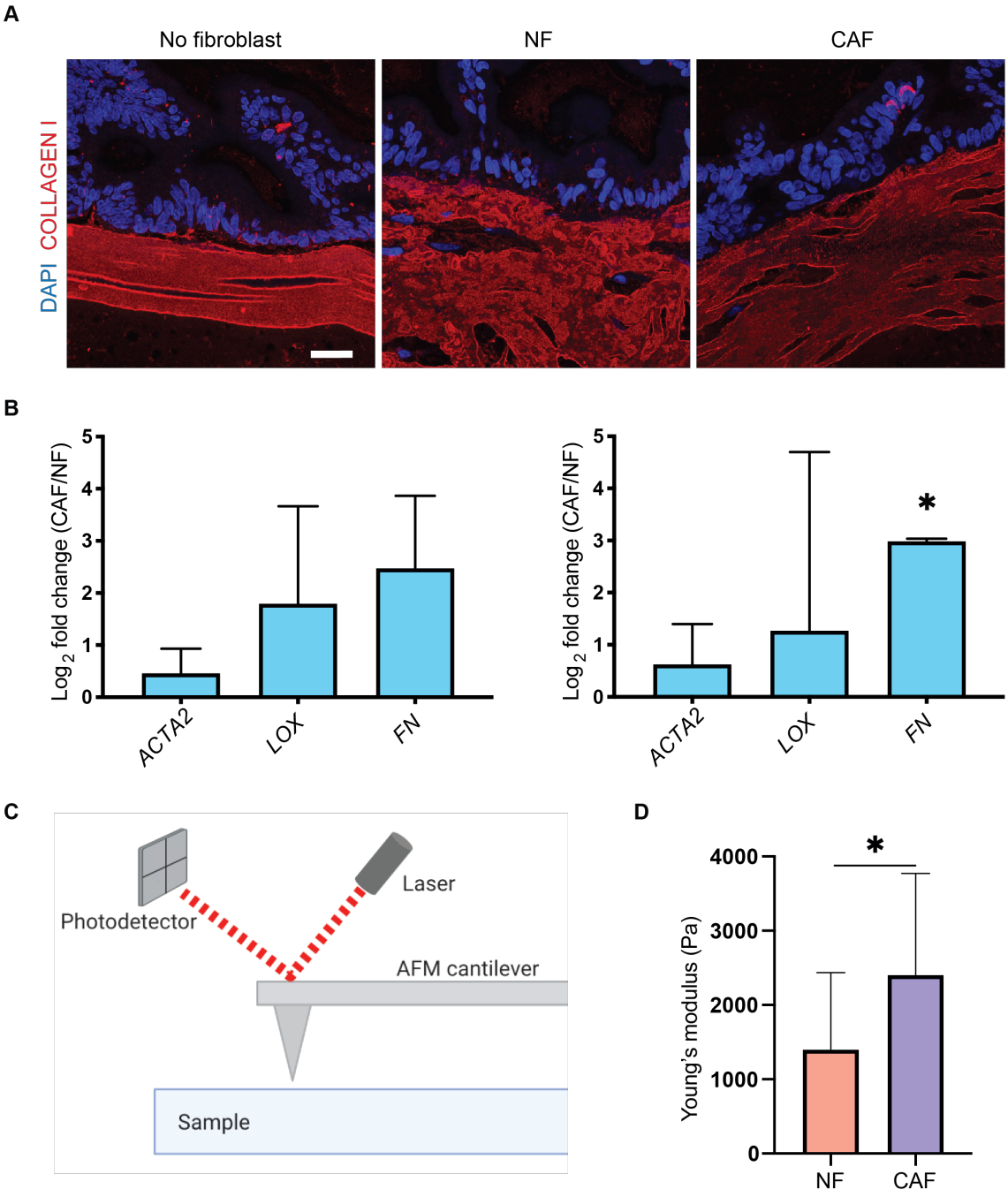

Figure 5.4 Collagen I scaffold remodelling by primary fibroblasts.

(A) Representative immunofluorescent images of collagen I scaffolds stained with COLLAGEN I (red) after three weeks of airlifting culture (week 5) without fibroblasts, or with NFs or CAFs. Nuclei were counterstained with DAPI (blue). Scale bars, 100 µm. (B) RT-qPCR analyses of cells after two weeks (left, week 4) and three weeks (right, week 5) of airlift culture. Data are shown as fold change of relative expression levels normalised to *β-ACTIN* or *PPIB*. N = 3 ± SD, and *p<0.05 compared to NFs. Data generated together with Isobel Massie who contributed in maintaining primary cells and performing experiments. (C) Schematic of AFM measurement acquisition from collagen I scaffolds. A cantilever with a tip was used to create nanoindentations into the sample and measure its stiffness. (D) Young's moduli from AFM analyses of NF- or CAF-embedded collagen I scaffolds with epithelial cells seeded on top after

two weeks of culture. N = 3 ± SD, and *p<0.05. The AFM data generated together with Hamid Mohammadi.

5.2.4 Fibroblast-derived POSTN and epithelial organoids in 3D co-culture models

Next, the co-culture model was applied to study the role of CAF-derived POSTN in epithelial cells. Previously, I observed that POSTN enhanced Wnt activity and stabilised FZD5, potentially by interacting with Wnt components present on cell membrane. To examine whether CAF-derived POSTN indeed has such paracrine effect on epithelial cells, I first generated POSTN-deficient CAFs (Figure 5.5A). Patient 17 CAFs with high POSTN expression was chosen for KO study. Three guide RNAs (gRNAs) targeting exon 4 were delivered all together with Cas9 protein into primary fibroblasts by nucleofection and the editing was confirmed by western blot (Figure 5.5B). Wnt TCF-TOPFlash assay was carried out to measure and compare the Wnt activity mediated by the CAFs with or without POSTN. The Wnt reporter cells were either co-cultured with the CAFs or treated with the CAF conditioned media. Unexpectedly, POSTN KO did not show any significant effect on Wnt pathway activity in either CAF conditioned media or co-cultured conditions (Figure 5.5C).

To further examine the ability of CAF-derived POSTN in modulating epithelial cell behaviour, small intestinal organoids were cultured with either POSTN-producing CAFs or POSTN KO CAFs in transwell co-culture. The epithelial organoids showed little proliferative capacity in both conditions (Figure 5.5D). However, the morphology and size of the organoids showed modest yet significant differences between cells cultured with WT and POSTN KO CAFs. Organoids cultured with POSTN KO CAFs were smaller in size with increased budding in comparison to those cultured with WT CAFs (Figure 5.5E), suggesting that POSTN promotes organoid growth and suppresses differentiation. However, the RT-qPCR analyses of the two organoid populations showed negligible differences in stem cell or differentiation markers (Figure 5.5F).

Moreover, the effect of POSTN KO CAFs on the matrix was evaluated using the collagen I scaffolds optimised earlier where epithelial cells were cultured on top. The tissue engineered scaffolds were collected three weeks post-airlifting at week 5 for RT-qPCR analysis to study the effect on epithelial cells with or without POSTN. Overall, expression of stem cell markers *LGR5* and *OLFM4* seemed to be reduced upon POSTN KO albeit insignificant (Figure 5.5G). In addition, little difference was observed in differentiation markers, while activated fibroblast marker *ACTA2* was moderately reduced in the POSTN KO CAF scaffolds. Importantly, AFM assay showed that stiffness was significantly reduced in the POSTN KO CAF collagen scaffolds, suggesting that the CAF-mediated ECM remodelling is POSTN-dependent (Figure 5.5H).

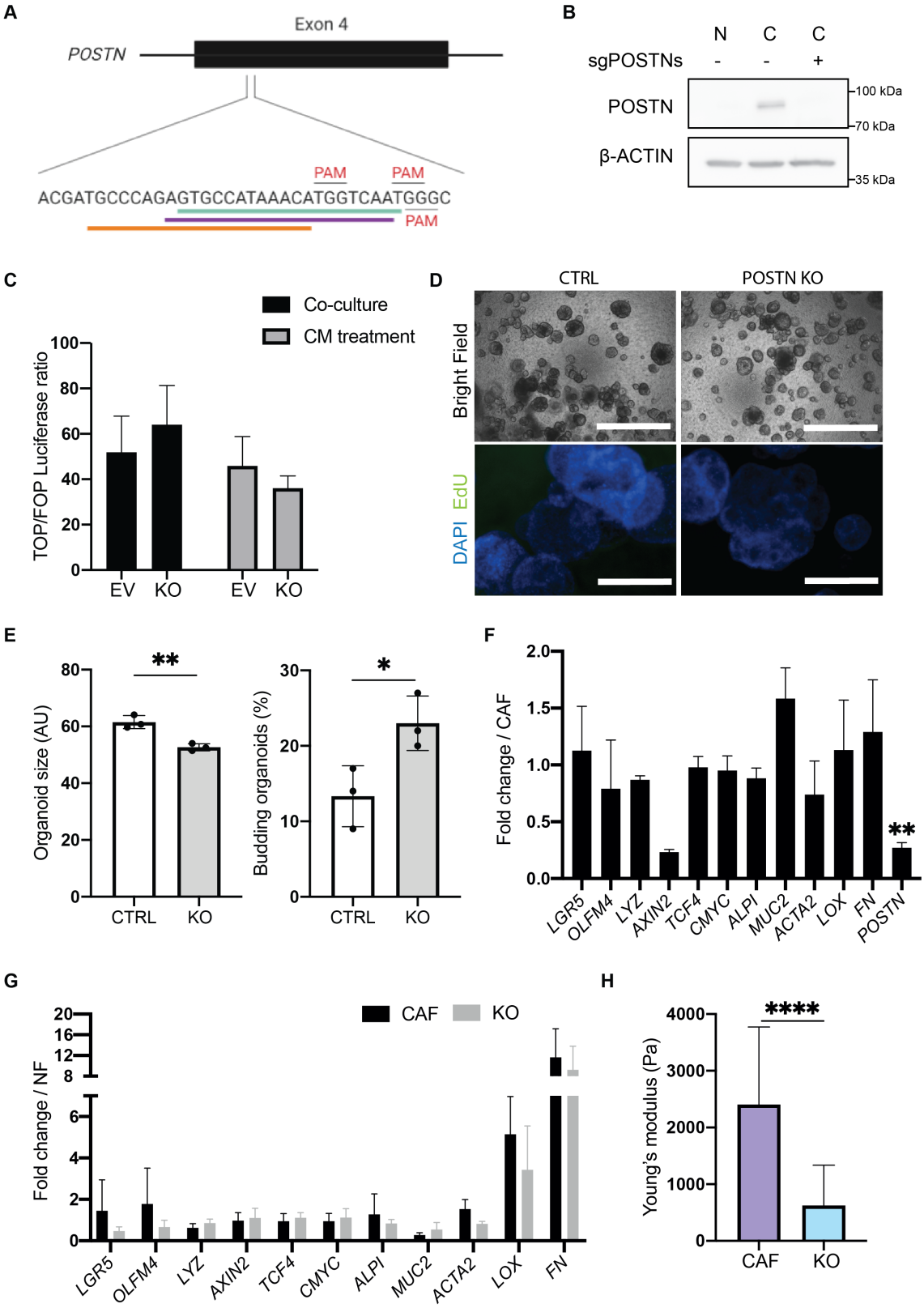

Figure 5.5 POSTN KO CAF-mediated modulation on epithelial cells.

(A) Schematics of CRISPR/Cas9 targeting for generation of POSTN KO CAFs. (B) Western blot showing loss of POSTN in nucleofected CAFs. N, NFs, C, CAFs. (C) TCF-TOPFlash assay with Wnt reporter cells after co-culture with control or POSTN

KO CAFs or after conditioned media treatment. N = 3 ± SD. (D) Representative bright field images and immunofluorescent EdU staining of organoids after co-culture with control or POSTN KO CAFs. Scale bars, 1000 μm (bright field) and 200 μm (EdU). (E) Quantification of organoid size and percentage of budding organoids after co-culture with fibroblasts. N = 3 technical replicates ± SD, * $p<0.05$, and ** $p<0.01$. (F) RT-qPCR analyses of organoids after transwell co-culture. Data are shown as fold change of relative expression levels normalised to *β-ACTIN*. N = 3 ± SD and ** $p<0.01$. (G) RT-qPCR of cells after co-culture on collagen I scaffolds. Data are shown as fold change of relative expression levels normalised to *β-ACTIN*. N = 3 ± SD. Data generated with Isobel Massie, who performed RT-qPCR. (H) Young's moduli from AFM analyses of control CAF- or POSTN KO CAF-embedded collagen I scaffolds. N = 3 ± SD, and **** $p<0.0001$. Data generated together with Hamid Mohammadi.

In the reverse study, POSTN-overexpressing colonic fibroblasts were generated by inserting a POSTN overexpression construct into CCD-18Co cells, a commercially available human colonic fibroblasts regarded as normal colonic fibroblasts (Henriksson et al., 2011, Paduch and Kandefer-Szerszen, 2011) (Figure 5.6). Overexpression of the protein was confirmed by western blot showing a stronger POSTN detection in the colonic fibroblasts integrated with POSTN construct compared to control (Figure 5.6A). TCF-TOPFlash Wnt reporter assay, either by co-cultured with the colonic fibroblasts or treated with conditioned media, showed a consistently higher Wnt activity in the POSTN-overexpressing CCD-18Co cells (Figure 5.6B), supporting the notion that POSTN promotes paracrine Wnt activity.

Next, I examined the POSTN-mediated paracrine effect by transwell co-culture of POSTN-positive colonic fibroblasts and epithelial organoids. Similar to what was observed with POSTN KO CAFs, expression of POSTN in fibroblasts did not affect the proliferative capacity of the organoids, both epithelial populations showing little EdU incorporation (Figure 5.6C). Nevertheless, organoids co-cultured with POSTN-expressing fibroblasts were significantly bigger, and showed marginally reduced budding morphology compared to control (Figure 5.6D). On the other hand, analyses of stem cell marker, Wnt target genes, and differentiation markers revealed that the two populations did not diverge during co-culture (Figure 5.6E). Altogether, the data suggest that the CAF-derived POSTN regulates ECM remodelling, and enhances organoid growth, possibly via paracrine Wnt modulation.

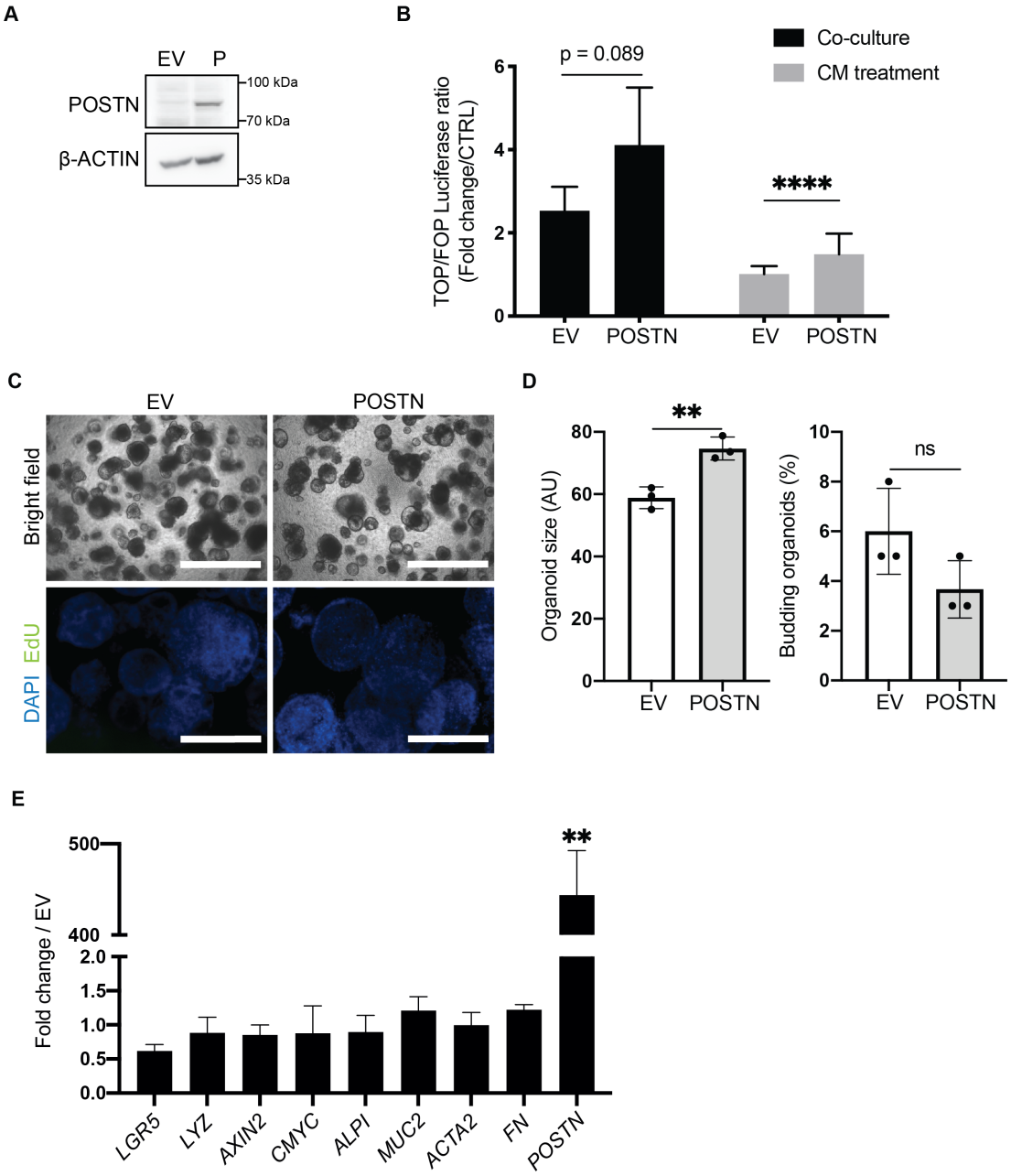

Figure 5.6 POSTN overexpressing colonic fibroblasts and epithelial cells.

(A) Western blot of cell lysate of fibroblasts overexpressing POSTN (P) and control construct (EV). (B) TOPFlash assay with Wnt reporter cells after co-culture with control or POSTN-overexpressing colonic fibroblasts, or after conditioned media treatment. N = 3 ± SD, and **** p<0.0001. (C) Representative bright field images and immunofluorescent EdU staining of intestinal organoids after transwell co-culture with control or POSTN-overexpressing colonic fibroblasts. Scale bars, 1000 µm (bright field) and 200 µm (EdU). (D) Quantification of organoid size and proportion of budding organoids after co-culture with fibroblasts. N = 3 ± SD, and ** p<0.01. (E) RT-qPCR analyses of cells after transwell co-culture. Data are shown as fold change of relative expression levels normalised to *β-ACTIN*. N = 3 ± SD, and ** p<0.01.

5.2.5 CAF-derived POSTN modulates tumour development *in vivo*

Finally, I investigated whether CAF-derived POSTN indeed plays a role in tumourigenesis and tumour progression *in vivo* utilising xenograft models. Patient-derived NFs, CAFs, and POSTN KO CAFs were co-injected with patient-derived CRC tumour organoids subcutaneously into SCID mice (Figure 5.7A). Tumours were collected after 20 days for weight measurement and histology comparison. The weight and size of tumour bulk derived from tumour organoids mixed with either NFs or CAFs were comparable, while tumours co-injected with POSTN KO CAFs were significantly lighter (Figure 5.7B, C).

Immunohistochemistry of SOX9 and CYCLIN D1, which serve as indirect readout for proliferation of intestinal epithelial cells in part resulting from Wnt activation, showed that their expression levels were similar in all three tumours co-injected with different fibroblast populations, indicating high Wnt activity in all xenografts (Figure 5.8). On the other hand, tumours co-injected with CAFs showed a tendency of high protein expression of FN and ACTA2 compared to xenografts with NFs and POSTN KO CAFs. Tumours with NFs and POSTN KO CAFs had comparable ACTA2 expression levels whilst FN expression level was relatively weaker in NF tumours. Reflective of high Wnt activity observed across xenografts, similar distribution of proliferative cells was observed (Figure 5.8). The data suggest that POSTN contributes to CAF activation during tumour development and progression.

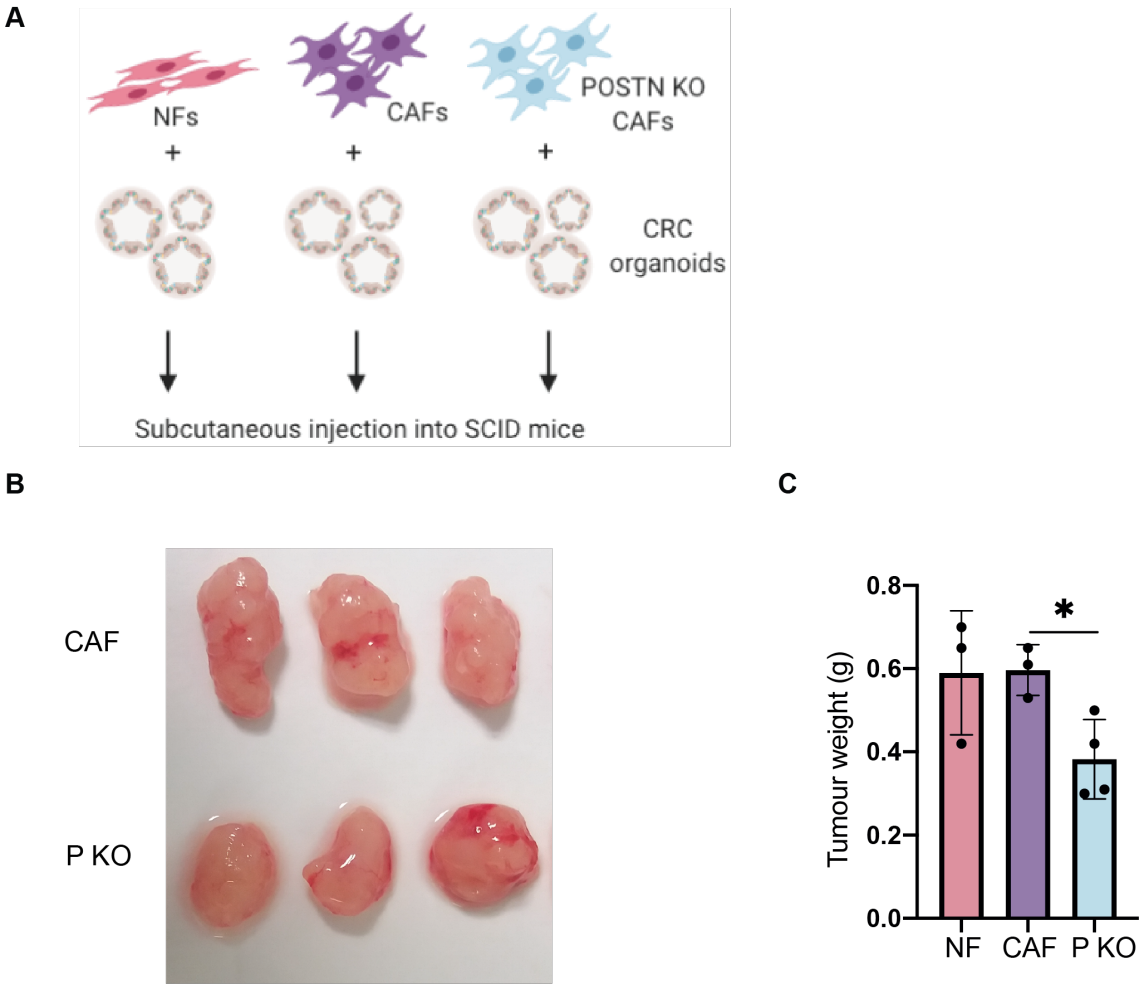

Figure 5.7 POSTN in xenograft tumour growth *in vivo*.

(A) Schematic representation of xenografts established from patient-derived CRC organoids and fibroblasts. Patient-matched (Patient 17) NFs, CAFs, or POSTN KO CAFs were mixed with CRC organoids (Patient 22) and subcutaneously injected into SCID mice. (B) Tumour weights in three groups after tumour removal. N = 3 (NF, CAF) or 4 (P KO) ± SD, and * $p<0.05$ by two-tailed t-test between CAF and P KO. Data was generated together with Laura Novellasdemunt who injected the cells into mice for xenograft formation.

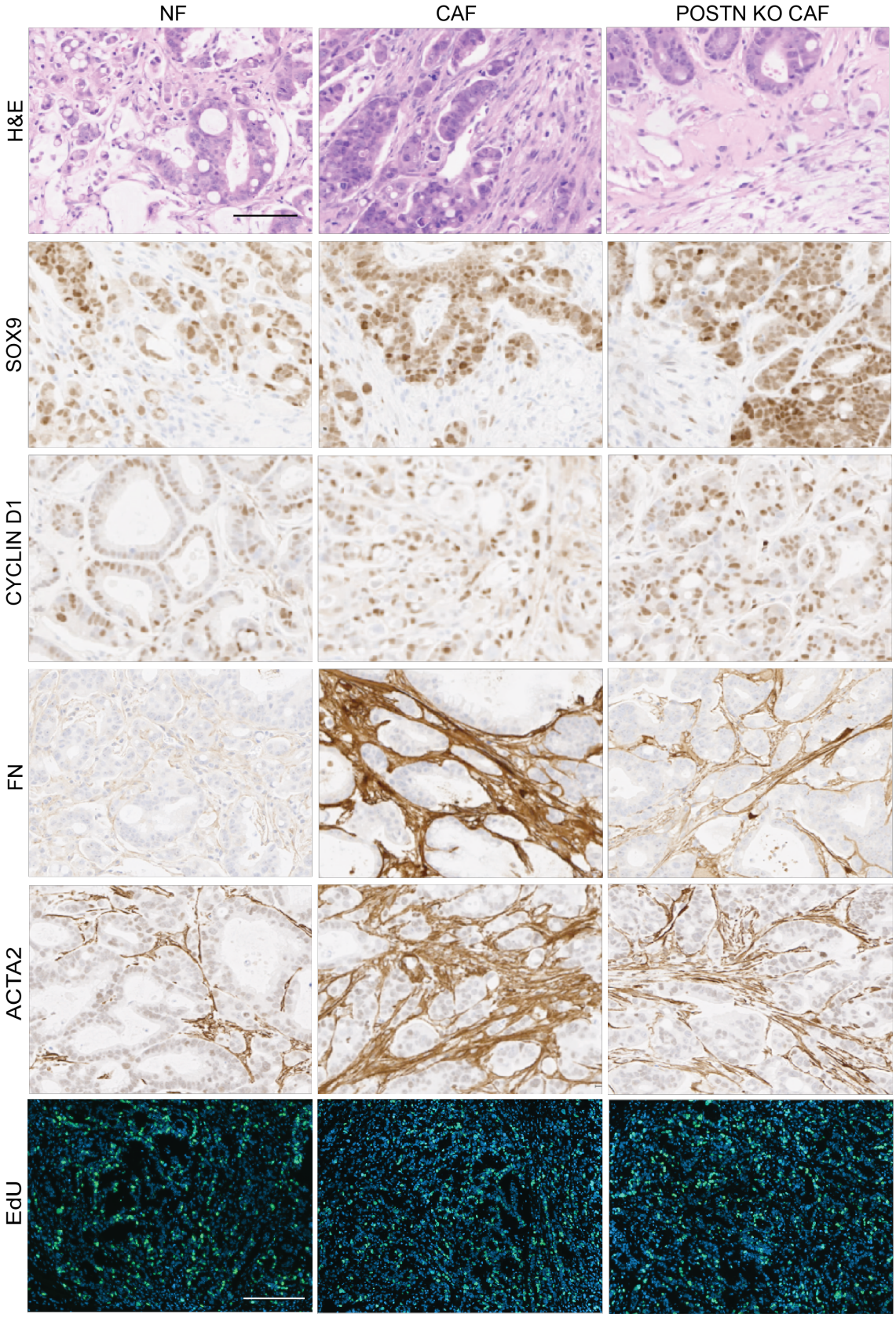

Figure 5.8 Protein expression profile and proliferation in xenografts.

Representative H&E stainings and immunohistochemistry of SOX9, CYCLIN D1, FN, and ACTA2 as indicated. Scale bar, 100 μm. Last row, representative immunofluorescent images of EdU incorporation in xenograft tumours. Scale bar, 200 μm. Data was generated together with Laura Novellasdemunt.

5.3 Discussion

It is becoming increasingly clear that the Wnt pathway is complex, and that crosstalk between multiple cell populations occurs. I hypothesise that such crosstalk between cancer cells and stromal cells contributes to the aberrant Wnt activation observed in CRC, even those without activating mutations in the Wnt pathway. Therefore, I aimed to reconstruct these interactions between epithelial cells and fibroblasts *ex vivo* to better study pathological Wnt activation in CRC.

To examine potential paracrine Wnt regulation by fibroblasts, transwell co-culture of intestinal organoids and patient-derived fibroblasts was performed. Contrasting epithelial cell fate decisions after transwell co-culture with NFs and CAFs were observed in the organoid morphology, EdU incorporation, and the gene expression pattern. Moreover, additional analyses such as nuclear β-catenin staining and LGR5 RNA scope will allow a more direct readout of canonical Wnt activation. Examination of differentiation markers would help determine whether organoids indeed terminally differentiated when cultured with NFs in transwell, although it may be the case that the culture duration needs to be extended to achieve terminal differentiation of the organoids. Mixing the two cell types for co-culture and sorting them by flow cytometry followed by further analyses such as single cell RNA-sequencing would ideally capture both fibroblast-derived effect on epithelial cells via secretory factors and heterotypic communication. Nevertheless, the transwell co-culture provided a valuable insight into the Wnt activity in epithelial organoids in response to fibroblast-derived secretory factors.

Transwell data indicated that fibroblasts were able to contribute to the regulation of epithelial cell proliferation and differentiation. However, paracrine factors are unlikely

to be the only way fibroblasts communicate with epithelial cells. As described previously, studies have reported that fibroblasts remodel the ECM, providing both altered biomechanical and biochemical signals to the cells (Bonnans et al., 2014, Kalluri, 2016). Therefore, it is important to generate and implement a 3D co-culture system that could model the communication between fibroblasts and epithelial cells to better recapitulate the interaction between these two cell types taking place *in vivo*. For this, I established a fibroblast-embedded collagen sheet on which intestinal organoids could be seeded to form an epithelial layer.

A stark contrast in epithelial organisation was observed after 3D co-culture with collagen I scaffolds mixed with fibroblasts depending on NFs and CAFs. On NF-embedded scaffolds, Wnt target protein expression was found in epithelial cells located in the lower region resembling crypt-villi organisation, whilst Wnt-active cells were distributed randomly when grown on CAF-embedded scaffolds. To our knowledge, this is the first *in vitro* tissue engineered model with the ability to form organised crypt-villus like structure using primary human organoids. The epithelial cell mass was bigger and disorganised when cultured with CAFs in the 3D system, suggestive of CAF-mediated transformation of epithelial cells. It is important to highlight that any changes in epithelial cells were a consequence of the crosstalk with fibroblasts as the epithelial cells were derived from the same normal organoid culture and were maintained in the same human organoid culture media condition. Such contrasting epithelial cell behaviour depending on fibroblast population also likely reflects the fibroblast-mediated enhancement of Wnt activity observed whereby CAFs confer higher Wnt activity by either direct co-culture or indirectly via secreted factors. Lower Wnt activity induced by NFs may be coupled with additional stromal factors to promote differentiation of epithelial cells cultured on NF-embedded collagen I scaffolds (Roulis and Flavell, 2016). In particular, air-liquid interface facilitating oxygen supply has been described to be essential for derivation of cells that show morphological traits of differentiation, polarisation on basement membrane and columnar cell shape with apical microvilli (Nossol et al., 2011). The airlifting might have created growth factor gradient for the epithelial cells to achieve vilification. It is worth noting that the constructs were maintained in the organoid media containing WNT3A and RSPO1, which may alleviate the epithelial Wnt-dependence on the fibroblasts. Moreover, the change in epithelial morphology at different time

points may be less of genuine epithelium transformation and more of culture variation on scaffolds. Further biological repeats utilising more primary cells may help elucidate the optimal time point in observing epithelial organisation. Nevertheless, the transcriptomic changes were in accordance with the epithelial morphologies observed, strongly supporting distinct fibroblast-mediated effect on the epithelial behaviour. Together, the data indicate that the CAF-derived factors promote epithelial proliferation and impair differentiation, possibly via paracrine Wnt regulation.

Furthermore, it should be noted that unlike transwell co-culture, technical limitation existed whereby separate RNA extraction from either epithelial cells or fibroblasts was not possible from collagen I 3D co-culture samples. Bulk RNA was extracted from both epithelial cells and fibroblasts for subsequent RT-qPCR analysis. It may be the case that cell type-specific changes in certain gene expression were in fact masked by the mixed RNA input. In addition to alkaline phosphatase staining, it would be of interest to check terminal differentiation markers for enterocytes such as IFABP (intestinal fatty acid-binding protein) as well as functional competency of the putative differentiated cells cultured on NF-embedded scaffolds (Storch and Corsico, 2008).

My data further showed that CAFs remodel ECM and increase matrix stiffness. Stiffening of a tumour is considered to be one of the deciding factors of tumour growth as *in silico* modelling has shown that tumours less than 1.5 times stiffer in comparison to the surrounding host tissue fail to expand (Voutouri et al., 2014). Biomechanically, stiffness provides tumours with a key advantage to overcome restraint imposed by the host tissue (Jain et al., 2014, Stylianopoulos, 2017). Increasing ECM stiffness confers malignancy and preclinical studies have demonstrated that targeting tumour ECM integrity can hinder metastasis (Oudin and Weaver, 2016, Kai et al., 2016, Venning et al., 2015). Moreover, AFM examination of CRC tumour tissues has reported highly diverse degree of stiffness between patients showing mean Young's modulus of 900 to 4400 Pa, although still stiffer than normal tissue controls (Brauchle et al., 2018). The constructed 3D model with CAFs exhibited stiffnesses comparable to CRC tumour tissues and the difference from NF scaffold stiffness was analogous to what was observed between colon carcinoma tissues and respective control

tissues from the same patient (Brauchle et al., 2018). Taken together, the collagen I 3D co-culture system permits examination of the impact of fibroblasts at multiple scales: capturing fibroblast-mediated ECM remodelling activities; as well as alterations to epithelial behaviour. Use of patient-derived fibroblasts and intestinal organoids affords the added benefit of investigating the pathways in faithful human *ex vivo* model systems.

I subsequently employed the transwell and collagen I co-culture system to examine the CAF-derived POSTN in paracrine Wnt regulation. However, POSTN KO CAFs did not show significant reduction in Wnt potentiation when TCF-TOPFlash assays were carried out using Wnt reporter cells. Collagen scaffolds were also generated with POSTN KO CAFs to explore POSTN-mediated Wnt potentiation by CAFs in 3D system. As use of primary fibroblasts was constrained due to their limited capacity for expansion, I elected to downscale the size of the collagen I scaffolds to reduce the number of cells required. However, an unexpected technical problem emerged where the epithelial cell layers were lost during processing for histological analyses. Nevertheless, direct harvest of the scaffolds allowed examination of the smaller scaffolds with POSTN KO or WT CAFs via RT-qPCR analyses. The magnitude of the transcriptomic changes was revealed to be marginal, likely reflecting the current limitation of the system whereby independent analysis of the two cell populations is not possible. The effect of POSTN KO CAFs on epithelial cell behaviour is likely reminiscent of what was observed in their Wnt potentiation using TCF-TOPFlash assays. It should be noted that the small intestinal epithelial cells appeared to be unhappy with low proliferation rate, as indicated by little EdU incorporation. It is possible this stemmed from batch to batch variation in conditioned media that affect organoid growth.

Surprisingly, the scaffolds in which POSTN KO CAFs were cultured showed a significant decrease in its stiffness compared to CAF scaffolds. The stiffness of scaffolds with POSTN KO CAFs was similar to that of scaffolds with NFs, the Young's modulus of each comparable to previously published control tissues from CRC patients (Brauchle et al., 2018). POSTN has been shown to promote expression and activity of LOX and LOXLs responsible for collagen crosslinking (Kumar et al., 2018, Liu et al., 2016). As mentioned previously, POSTN has also been described to bind

to both collagen and BMP-1, which cleaves LOX to its active form, to facilitate collagen crosslinking (Maruhashi et al., 2010). In conjunction with a lower expression level of *LOX* in POSTN KO CAFs, it may be the case that reduced covalent crosslinking of collagen fibres resulted in relatively softer scaffolds with POSTN KO CAFs.

Contrary to my hypothesis that POSTN confers paracrine Wnt activity, loss of POSTN showed an insignificant decrease in Wnt activity, and long-term intestinal epithelial cells on POSTN KO CAF-embedded collagen I scaffolds showed little change in their behaviour despite differential ECM remodelling of the scaffold by CAFs and POSTN KO CAFs. Conceivably, organoid media condition with high WNT3A and RSPO1 may have compromised the sensitivity of the assays. Alternatively, POSTN may not be the only stromal factor that contributes to Wnt regulation rendering the loss of this one putative regulator insufficient to modulate Wnt activity. Functional redundancy in Wnt pathway has been demonstrated to reflect the importance of tight Wnt regulation with β-catenin being the unique canonical Wnt effector (Ikeya et al., 1997, Grigoryan et al., 2008). It may be possible that POSTN is functionally redundant with other secreted factor(s) to regulate paracrine Wnt activation in CRC.

In contrast to the KO experiment, POSTN-overexpressing colonic fibroblasts showed significant increase in Wnt activity in Wnt reporter cells, supporting the Wnt-activating role of POSTN. Although POSTN expression promoted organoid growth, little discernible change was observed in expressions of stem cell and differentiation markers. Expansion of the colonic fibroblasts was limited hindering generation of collagen I 3D co-culture system. The promoter used for overexpressing POSTN in fibroblasts is comprised of Elongation factor-I alpha (EF-1α) core promoter alone, whilst that of transfection construct primarily used in the previous chapter contains EF-1α followed by the R segment and part of the U5 sequence (R-U5') of the Human T-cell Leukaemia Virus (HTLV) type 1 Long Terminal Repeat that increases the RNA stability (Kim et al., 1990, Takebe et al., 1988). It may be possible that overexpression in colonic fibroblasts were in fact less efficient than that of *in vitro* assays described in Chapter 4, thus failed to similarly enhance Wnt activity in the tissue-engineered model. Utilisation of an expression system with a stronger

promoter may help clarify the effect of fibroblast POSTN expression, in colonic fibroblast cell line or patient-derived NFs, on epithelial cell behaviour.

As a more direct approach to investigate the role of POSTN in tumour progression, patient-derived fibroblasts and patient-derived CRC organoids were subcutaneously injected into SCID mice. CRC organoids, and not normal organoids, were utilised to examine the role of fibroblasts on tumour progression rather than potential (and slow) transformation and tumour initiation of normal organoids. Utilisation of patient-matched organoids and fibroblasts were not possible due to variable success in establishing CRC organoid cultures. Surprisingly, tumour formation of CRC organoids with NFs was comparable to that of organoids injected with CAFs and Wnt activity was observed to be high in all tumour types regardless of fibroblast population. A more direct approach of Wnt activity assessment by examining nuclear localisation of β-catenin will help determine Wnt activation in the cells. CRC organoids most likely had already high Wnt activation and rendered comparison of any fibroblast-mediated effect on Wnt activity difficult. Notably, NF xenografts exhibited high levels of FN and ACTA2, although still relatively weaker than CAF xenografts. FN is not a defined CAF marker, but has been described to be a marker for CAFs from tumour tissue whose expression profile could be linked to patient survival (Calon et al., 2015). In addition, transformed epithelial cells have been suggested to enhance tumourigenesis by inducing fibroblast activation into CAFs which in turn promote epithelial cell proliferation (Liotta and Kohn, 2001). It may be possible that NFs were passively activated to resemble their patient-matched CAFs once in contact with CRC organoids, leading to limited difference in their effect on tumour formation and progression when compared to CAFs.

Interestingly, despite the similar degree of Wnt activity observed, the weight of the xenografts with POSTN KO CAFs was significantly lower than those with CAFs. POSTN KO xenografts had relatively lower expressions of FN and ACTA2 than tumours with CAFs suggestive of altered ECM composition. Taken together with the lower *LOX* expression in tissue-engineered models with POSTN KO CAFs, it would be of interest to examine the expression of the collagen crosslinkers and collagen deposition by POSTN KO CAFs in the xenografts to determine whether weight difference between xenografts could be partly attributed to the ECM remodelling by

fibroblasts. Also, it is possible that POSTN KO CAFs provided weaker pro-tumourigenic signal to CRC organoids, however the impact remained modest most likely due to the collection of cues CAFs already provided CRC cells with, and the mutational background of CRC organoids. More appropriate and ideal approach in examining fibroblast-mediated effect on CRC tumour formation would involve an autotopic graft generation rather than by subcutaneous injection, use of labelled organoids for better tracking and immune competent hosts for genuine readout of tumour formation *in vivo*, as well as POSTN and HCNA staining. Also, it may be informative to utilise normal organoids and examine whether CAFs or POSTN KO CAFs are capable of promoting xenograft formation originating from the normal epithelial cells.

Nevertheless, taken together with findings of the previous chapter, POSTN may depend on specific receptor profile of cancer cells to exert Wnt potentiating effect, such as LGR6 upregulation. Lack of such responsiveness in cells used in the experiments may be responsible for marginal effect of POSTN deletion in CAFs or overexpression in fibroblasts, but significant reduction observed in CRC organoid-derived xenografts with POSTN KO CAFs. Using CRC patient-matched organoids and fibroblasts to study the impact of POSTN loss or gain may provide insights into such potential context-dependence of POSTN. Moreover, it would be interesting to generate colonic organoid line of suitable receptor profile, for example by upregulating LGR6, to investigate potential sensitivity to POSTN-mediated Wnt potentiation. Preliminary work of tissue-engineering 3D model with POSTN KO CAFs and CRC organoids may provide early insights into whether indeed the responsiveness of epithelial cells is key in POSTN-mediated Wnt potentiation. Nevertheless, elucidation of the molecular mechanism of POSTN would help design further works applying the tissue-engineered 3D co-culture to recapitulate paracrine Wnt regulation.

In this chapter, transwell co-culture revealed that exposure to CAF-derived factors increased proliferation of the epithelial organoids whilst factors from NFs promoted differentiation. Also, a collagen I 3D model in which primary fibroblasts are embedded in collagen scaffold seems to be able to reflect differential behaviour of epithelial cells modulated by fibroblasts and can be potentially utilised to study the

paracrine communication between the two cell types. Even though *in vitro* loss-of-function studies did not show significant reduction in Wnt activity in either Wnt reporter cells or co-cultured intestinal organoids, loss of POSTN still conferred significant change in the collagen I scaffold and tumour formation *in vivo*. This most likely advocates the benefit of the tissue-engineered model in recapitulating fibroblast-mediated ECM remodelling that in turn alter epithelial cell behaviour. POSTN may require specific receptor repertoire in epithelial cells to be able to potentiate Wnt activity according to their interaction described in the previous chapter. Also, it would be informative to further tissue-engineer models with primary fibroblasts and patient-derived colonic organoids instead to recapitulate the CRC tissue and obtain a more physiologically relevant readout on the fibroblast-derived Wnt regulation in the epithelial cells.

Chapter 6. General discussion

Canonical Wnt signalling is one of the crucial regulatory pathways involved in ISC homeostasis and is frequently found to be abnormally activated in CRC. Technological advances in high-throughput sequencing have provided insights into molecular mechanisms underlying tumourigenesis and tumour progression of CRC (Guinney et al., 2015, Network, 2012). Consequently, understanding of the disease has broadened to include more effectors that regulate the Wnt signalling pathway. It has been reported that Wnt activation within tumours is heterogeneous, showing relatively high nuclear β-catenin at the edge of the tumour where cancer cells are located close to stromal cells (Guinney et al., 2015). CAFs have been previously shown to contribute to tumour progression (Calon et al., 2015). In addition to Wnt ligands, fibroblasts secrete Wnt regulators such as DKKs and SFRPs, providing both positive and negative signals. Fibroblasts adopt a CAF phenotype with differential secretome upon activation and are considered to play a dynamic role in driving tumour progression by providing a pro-tumourigenic microenvironment (Gascard and Tlsty, 2016, Paunescu et al., 2011). The aim of my PhD was to explore potential paracrine Wnt regulators secreted by CAFs that could contribute to aberrant Wnt activation in CRCs. Following analyses of available data on upregulated genes in the CAF-enriched cluster, POSTN was selected as a putative paracrine Wnt regulator promoting Wnt activity and tumour progression.

I first established CRC patient-derived primary fibroblasts and organoids. They are a viable *ex vivo* model system in which to study fibroblast-epithelial crosstalk. The data suggests that CAFs enhance Wnt activity and that POSTN is likely a ligand-dependent Wnt potentiator that stabilises FZD5. Additionally, I constructed a 3D co-culture system to more closely recapitulate the interaction between fibroblasts and epithelial cells, in which, the role of CAFs and CAF-derived POSTN on the Wnt pathway and the subsequent effect on epithelial cell phenotype could be studied. The system has successfully captured some NF- and CAF-mediated changes in epithelial cell behaviour and morphology in 3D. However, further work is required to fully elucidate the molecular mechanism of POSTN and appreciate the impact of CAF-derived POSTN on Wnt activity.

A pattern of Wnt enhancement was observed when Wnt reporter cells were co-cultured with CAFs or treated with CAF conditioned media. Wnt activation enhancement was similarly induced by CAFs when intestinal organoids were cultured together with the fibroblasts in transwell. As a potential Wnt regulator contributing to such Wnt enhancement, POSTN showed an increased expression pattern in CAFs. This was consistent with its upregulation revealed in the "CAF-enriched cluster" which included a number of ECM components like collagens and CAF markers (such as *FAP* and *CALD1)*. Upregulation of such genes have been associated with poor patient outcome (Calon et al., 2015). It has been proposed that CAFs can polarise resulting in two functionally distinct subtypes in CRC (Madar et al., 2013, Li et al., 2012a, Augsten, 2014). Notably, POSTN upregulation, in conjunction with increased expression of ECM remodelling enzymes and ACTA2, is described as one of the markers for the pro-tumourigenic CAF subtype as opposed to Slit-high anti-tumourigenic CAFs (Augsten, 2014, Chang et al., 2012). This is in accordance with my data as patient-derived CAFs showed tumourigenic effect on normal intestinal organoids after co-culture in transwell or in 3D scaffolds, in part via Wnt activation.

However, the transwell co-culture system likely does not fully recapitulate the crosstalk between the two cell types in the current experimental setting. The media condition that combines both organoid expansion media and fibroblast growth media may in fact hinder detection of changes in cell signalling or behaviour induced by the co-culture. Fresh supply of growth factors and inhibitors with regular media changes may remove, inhibit, and/or dilute secretory factors. This may also confound *in vitro* assays with POSTN-deleted CAFs and POSTN-overexpressing fibroblasts which showed little effect on intestinal organoids after transwell co-culture. Additional study into the fibroblast-mediated changes in the epithelial cells by using more conservative media conditions for culture, as well as optimising cell type-specific harvest for analyses after mixed co-culture, will help overcome the limitations of the current set-up. A more ideal experimental design would involve mixing the two cell types and sorting them after co-culture by FACS for separate transcriptomic analyses which could allow investigation of the comprehensive changes in both populations resulting from the crosstalk.

Mechanistic data suggests POSTN can amplify Wnt activity in the presence of Wnt ligands and can stabilise FZD5 levels at the cell surface. I have demonstrated the interaction of POSTN with LRP6, LGRs, and RNF43. Pursuing putative interactions detected in this project and what is known of Wnt components at the cell surface would allow better understanding of the Wnt regulatory network that cancer cells potentially exploit via CAFs. POSTN has been previously suggested to interact with Wnt ligands and potentiate canonical Wnt activity in breast cancer (Malanchi et al., 2011). Confirmation of POSTN-Wnt ligand interaction could help explain the underlying molecular mechanism of how POSTN confers Wnt enhancement. In *Drosophila*, the secreted molecule Swim interacts with Wnt proteins, acting as a chaperone, but the mammalian equivalent has not been identified (Mulligan et al., 2012). It will be important to determine if POSTN functioned to facilitate the travel of Wnt ligands from source cells to receiver cells by interacting with them and eventually promoting Wnt ligand binding to LRP6. Alternatively, POSTN may represent another mode of canonical Wnt activation involving LRP6. In head and neck cancer, POSTN has been shown to promote invasion and proliferation by binding to protein tyrosine kinase 7 (PTK7) and LRP6, resulting in β-catenin nuclearisation (Yu et al., 2018). PTK7 is one of the receptors involved in the non-canonical Wnt/PCP pathway, suggesting a crosstalk between β-catenin-independent and -dependent Wnt pathways that may be at play in cancer (Jung et al., 2004, Lu et al., 2004). Studies have reported PTK7 upregulation in CRC but contrasting data suggest its association with both poor and favourable prognosis perhaps due to yet unidentified functional diversity (Lhoumeau et al., 2015, Tian et al., 2016). It would be of interest to examine whether the POSTN-LRP6 interaction that I detected is a part of an alternative canonical Wnt activating mechanism with PTK7 that is conserved in CRC.

On the other hand, a direct interaction of LGR5 and Wnt receptors has been described (Carmon et al., 2012). LGR5 is found to increase LRP6 and FZD5 internalisation thus suggesting a negative feedback loop via rapid receptor degradation upon over-activation of the pathway by both Wnt and RSPO1. POSTN may possibly be involved in such a negative feedback mechanism by inhibiting receptor internalisation to promote FZD5 stabilisation on cell surface and enhance Wnt activity. In the intestine, Wnt proteins are found to bind directly to FZD on the cell membrane creating a gradient of Wnt activation as dividing cells distribute

membrane-bound Wnt ligands since ISCs do not internalise the ligands (Farin et al., 2016). FZD stabilisation by POSTN may help enhance Wnt activation and prolong the active signalling by allowing dilution of more Wnt molecules on receiver cells. Interestingly, a reduction in RNF43 level was also observed when cells co-overexpressed tagged RNF43 and POSTN. However, POSTN did not rescue cells from RNF43-mediated Wnt suppression. It needs to be further confirmed whether the alleviation of RNF43-mediated Wnt suppression by POSTN was indeed masked by a dual mode of RNF43 at both cell membrane and the nucleus (Loregger et al., 2015).

It is possible that POSTN-induced Wnt enhancement requires specific context, such as differential receptor availability like that of LGR6, one of its putative binding partners, which is upregulated in CRC. Modelling such a scenario, whereby one cell type provides specific context for a factor derived from another cell type, may benefit from generating a biomimetic culture system. In order to further investigate the role of CAF-derived secretory factor(s), I have established a 3D co-culture system of epithelial cells and fibroblasts using collagen I scaffold. Marked polarisation and organisation of the epithelium was observed on NF-embedded scaffolds whilst a disorganised epithelial mass was found on CAF-embedded scaffolds. Analyses of gene and protein expression profiles of cells cultured in the collagen I system showed that epithelial cells were capable of differentiating on NFs and epithelial cells with high Wnt activity were confined to “crypt-like” regions. To our knowledge, this is the first 3D co-culture model that allows organoids to form such highly-organised crypt-villus-like structures on collagen gel. Conversely, when cultured with CAFs, epithelial cells were highly Wnt active and proliferative. The collagen I 3D co-culture system allows *in vitro* recapitulation of differential mechanistic cues provided by NFs and CAFs to epithelial cells. AFM measurements revealed that CAF-embedded scaffolds were significantly stiffer than those with NFs. Indeed, primary fibroblasts had distinct expression profiles for ECM proteins and remodelling enzymes in 2D that were maintained in the 3D system. This resulted in generation of scaffolds with disparate biomechanical, and likely biochemical, properties.

Primary fibroblasts derived from CRC patients have provided insight into the potential Wnt augmentation by CAFs and epithelial modification. Nevertheless, utilisation of

primary cells confers several caveats. Patient-to-patient variability as well as between patient-matched NFs and CAFs may suggest appropriate recapitulation of the tumour heterogeneity seen *in vivo* (Sugimoto et al., 2006, Calvo et al., 2013). Alternatively, it may reflect the heterogeneity within the primary cultures and/or patient-to-patient variation since the mutational background of these patients has not been profiled (Sugimoto et al., 2006, Calvo et al., 2013, Herrera et al., 2013, Berdiel-Acer et al., 2014c). In addition to patient-specific mutational background, the CAF phenotype is considered to be in part defined by the interactive crosstalk with cancer cells (Augsten, 2014, Green et al., 2013). Depending on the mutational background of the epithelial cancer cells, the same line of CAFs conferred anti- and pro-tumourigenic effects indicating yet unknown mechanisms of how malignant cells programme CAFs (Green et al., 2013). Utilisation of small intestinal organoids in this co-culture assay likely did not allow modelling of such crosstalk. This may explain why the *in vitro* assays had differential outcome of POSTN loss in normal intestinal organoids as compared to xenografts using CRC organoids. For the duration of the project, mutational backgrounds of the patient-derived primary cells have not been profiled. Molecular profiling of patient-derived fibroblasts and CRC organoids would help to clarify whether patient-to-patient variation observed in POSTN upregulation in CAFs, or the degree of differences between patient-matched NFs and CAFs, is associated with CRC molecular subtypes. In addition, it would facilitate examination of the role of POSTN in the presence or absence of mutations in downstream Wnt components such as APC in CRC cells, as well as other factors available in the CAF secretome. Coupled with a better understanding of putative stromal regulation by POSTN, the use of colonic organoids with or without appropriate receptor expression profiles would enable analyses of the mechanism of stromal Wnt regulation. Importantly, future work on the 3D co-culture model by introducing more stromal components, such as immune cells, will allow modelling of a more complex crosstalk taking place in the TME.

Collagen has been identified as the most common ECM component across colon carcinoma tissues (Brauchle et al., 2018). While simplified, the collagen I co-culture system likely provides adequate biomimetic microenvironment for the cells where novel ECM deposition and remodelling can be detected, in addition to alterations in cell proliferation. Nevertheless, it should be noted that the model does not fully

convey ECM diversity present in the *in vivo* microenvironment. This likely indicates the limitation of the system in modelling a range of CRC behaviours, as ECM heterogeneity has been reported to be critical for regulating collective cell invasion as well as the efficiency of metastasis (Zhu et al., 2015). The simple ECM composition of the 3D scaffold that likely hinders complex tumour behaviour may in part underlie relatively insignificant effect of POSTN deletion observed in *in vitro* assays. Further work is required to conclude whether CAF-derived POSTN truly has any considerable impact on tumour progression and Wnt hyperactivation as the *in vitro* data and *in vivo* data from this project are not precisely in accordance on that point. In the long-term, the 3D system may further offer a useful model to dissect the role of CAFs in metastasis as well as primary tumour progression. A recent study has demonstrated that CRC cells cultured on either liver- or lung-derived ECM *in vitro* preferentially formed xenografts in the corresponding organ, thus highlighting the role of the ECM in tissue-specific metastasis (Tian et al., 2018). It would be of interest to examine whether CAFs are implicated in conveying tissue tropism during metastasis and explore the underlying mechanism(s). Understanding how CAFs contribute to formation of different metastatic sites could potentially provide insights into therapeutic interventions to disrupt metastasis where response to treatments may be site-specific (Higashiyama et al., 2012).

The role of CAF-derived factors, such as POSTN, in promoting tumour progression may involve nudging cancer cell behaviour when they are exposed to a myriad of contrasting signals in the TME. Tumour immune surveillance is associated with CRC patient survival outcomes for its suppressive effect on metastasis (Malladi et al., 2016). CMS4 CRCs in particular are often linked with increased immune infiltration (Network, 2012, Becht et al., 2016). A study in breast and lung cancer has demonstrated that disseminated cancer cells that later give rise to metastasis express DKK1 to self-induce quiescence (Malladi et al., 2016). This Wnt inhibition in cancer cells in perivascular niches inundated with proliferative signals such as stromal Wnt proteins seemed to allow them to downregulate ligands that trigger immunologic elimination (Malladi et al., 2016). It is possible that similar Wnt inhibitory immune evasion is present in these CRCs, and that POSTN functions to selectively potentiate Wnt activity in cancer cells close to the stromal source against such autocrine Wnt inhibition. Further studies will be needed to examine the potential link

between POSTN expression and immunomodulation. Moreover, CMS4 CRC tumours have been associated with distinct Wnt receptor-mediated gene signatures rather than those stimulated by intrinsic Wnt activation such as APC loss underscoring Wnt activity driven by cell surface components' actions rather than mutations in downstream Wnt effectors (Michels et al., 2019). It would be of interest to investigate whether POSTN secreted by CAFs plays a role in CAF-derived Wnt potentiation in CRC and contributes to tumour progression in the context of immune evasion.

Aberrant Wnt activation in CRC is largely driven by mutations in the pathway, which is followed by mutations in other pathways such as PI3K and RAS pathways (Cristobal et al., 2017, Bos et al., 1987, Powell et al., 1992). However, Wnt hyperactivation is common across CRCs regardless of Wnt mutations suggestive of a more complex pathway regulation beyond ligands and downstream transducers (Polakis, 2007, Yang et al., 2016). Growing evidence points to the role of TME in tumour progression. This includes the action stromal cells in the TME that secrete paracrine regulators or directly interact with cancer cells to modify their behaviour (Xing et al., 2010, Shiga et al., 2015, Tommelein et al., 2015, LeBleu and Kalluri, 2018). On the other hand, it may also reflect tumourigenic impact of transformed cells that dysregulate stromal cells in the TME to trigger heterotypic communication in cancer (Vermeulen et al., 2010, Malanchi et al., 2011, Green et al., 2013). It is possible that POSTN alone does not confer CAF-derived tumourigenicity. POSTN could be a ligand-dependent contributor, either upregulated by CAF activation independently of cancer cells or by direct induction by cancer cells as seen in breast cancer (Malanchi et al., 2011), in generating a pro-tumour context along with other factors available in the TME (Figure 6.1).

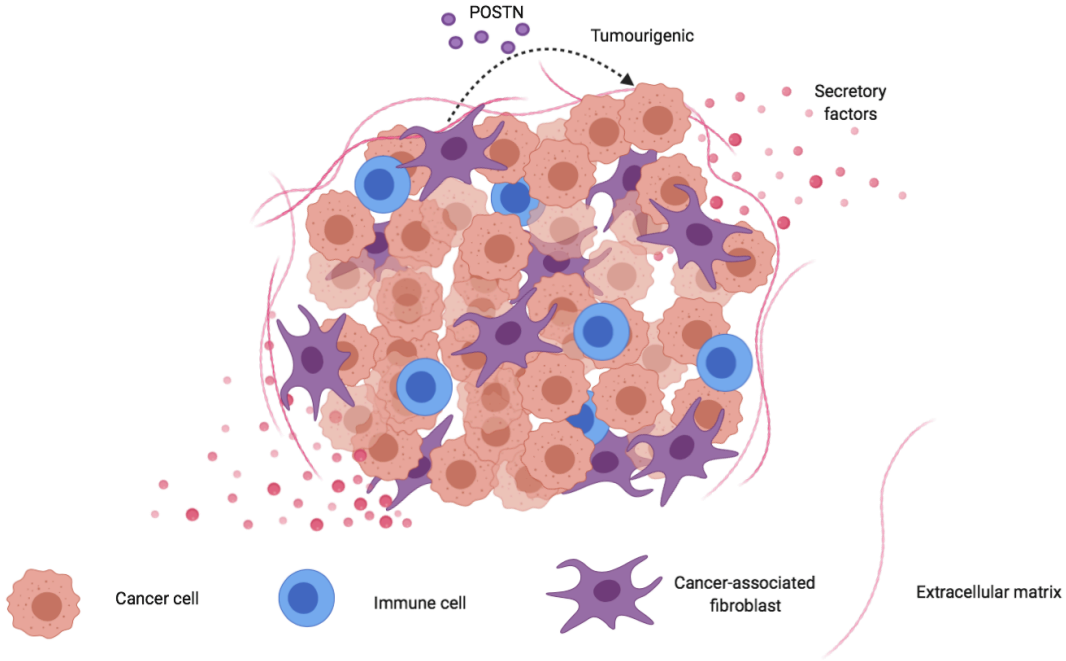

Figure 6.1 POSTN in tumour microenvironment

Cancer cells residing in tumour infiltrated with stromal cells such as CAFs and immune cells are exposed to numerous secretory factors that often provide conflicting cues. One of the paracrine regulators, CAF-derived POSTN promotes tumour progression and metastasis possibly by enhancing canonical Wnt pathway. The specific Wnt mechanism underlying pro-tumorigenic effect of POSTN is yet unclear. The elucidation of its full role in augmenting Wnt pathway will be a valuable addition to our appreciation of pro-tumourigenic CAFs in CRC.

In summary, I have characterised the putative paracrine Wnt regulator POSTN in the CRC microenvironment using a 3D collagen-based model. To better understand the role of CAF-derived POSTN in CRC, it will be important to obtain high-throughput sequencing data of the patient samples in order to correlate POSTN expression levels and Wnt activity in the CAF lines with regard to their genotypes and cancer subtypes. In addition, understanding of POSTN-mediated Wnt enhancement would not only shed light on additional Wnt regulation at cell surface, but also provide valuable insights into Wnt hyperactivation in cancer that exploits abnormal expression of stromal factors regardless of mutation in the Wnt pathway. With the current data, I propose that POSTN contributes to the TME-derived tumourigenic

effect by potentially interacting with Wnt receptors or transmembrane regulators to enhance Wnt activity in a ligand-dependent manner likely resulting from increasing FZD5 availability via yet unknown mechanism. My data in conjunction with what is currently known of POSTN in the Wnt pathway may provide insights into the underlying molecular mechanism of how POSTN, upregulated in CAFs, potentiates Wnt activity in cancer cells.

In particular, investigation of the mechanism of POSTN interaction with the Wnt receptor LRP6 and the transmembrane regulators LGRs and RNF43 can potentially provide several modes of novel Wnt regulation. It would be of interest to find out if POSTN also binds to PTK7 as a part of a conserved mechanism observed in other cancers, or potentiates Wnt activity by suppressing RNF43 and/or LGR/LRP/FZD internalisation. Potential interaction of POSTN with PTK7, reduction of surface RNF43 by POSTN, and the role of POSTN in receptor endocytosis should be examined. Conversely, POSTN may represent an alternative Wnt potentiation by interacting with these Wnt components on cell surface via yet unknown mechanism(s). Unbiased IP-Mass spectrometry will help identify potential binding partners that may contribute to POSTN-mediated Wnt regulation. Further *in vitro* assays are required to fully characterise the molecular mechanism of POSTN. Ultimately, alternative Wnt enhancement by stromal factors will not only shed light on complex Wnt regulatory mechanism but also targetable vulnerabilities in relatively stable CRC stroma to obstruct tumour progression. Although the 3D collagen I system offers a biomimetic model to study epithelial and stromal cells interaction, lack of ECM heterogeneity could possibly limit its ability to recapitulate the full complexity of the TME. Future efforts on constructing a more biomimetic system with additional ECM components may address such a limitation. Further study on the CAF-derived POSTN by incorporating the knowledge into 3D models will be important in reaching a complete understanding of paracrine regulation of the Wnt pathway in CRC microenvironment. Moreover, better understanding of CAFs will help untangle the conundrum whether it is the extrinsic context or intrinsic mutations that bestow them with the CAF identity and function. The TME as a source of tumourigenic context for heterotypic communications between stromal cells and both normal and transformed cells will then be better understood.

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