SIGNALLING TISSUE RENEWAL AND CRYPT SURVIVAL IN THE HUMAN COLONIC EPITHELIUM AND BARRETT'S OESOPHAGUS

A thesis submitted for the degree of

Doctor of Philosophy

by

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January 2013

Acknowledgements

I would like to thank my primary supervisor, Mark Williams, for your constant support and advice throughout my time in the lab. I greatly appreciate the opportunities and experiences I gained during this study. Your expertise and enthusiasm in the world of colonic crypts inspired me to take on this PhD.

My secondary supervisors, Mike Lewis and Mohammed Hajihosseini, thank you for your support and advice in shaping this study.

I am very grateful to Amy Reynolds, Alyson Parris, Loren Bigwood, Esther Mitchell and Ahmed El Hadi for all your contributions to the progress of this work and your support during my study.

Thank you also to all the doctors and nurses at the Norfolk and Norwich University Hospital for constantly providing me with tissue samples. The Humane Research Trust, thank you for proving me with the funding to carry out this study.

This thesis is dedicated to my husband and family. Richard, this study allowed me to meet and fall in love with you, and your constant support, patience and motivation kept me going. Mum, Stas and Nathaniel, thank you for always being there and supporting me.

Abstract

Stem cell driven tissue renewal in the intestinal epithelium is a tightly regulated and controlled process. The colonic epithelium is organised into millions of invaginations called crypts, each of which represents the self-renewing unit of the tissue. In the mouse, renewal of the intestinal epithelium is regulated by signalling cross-talk between the Wnt, Notch, EGF and TGFβ/BMP pathways. The molecular mechanisms that regulate the processes of tissue renewal in the human are of great interest because they are disrupted in colorectal cancer and inflammatory diseases. Barrett's oesophagus is an intestinal metaplasia arising in response to inflammation and ulceration provoked by gastroesophageal reflux. Detailed knowledge of the processes and signalling pathways involved in tissue renewal in Barrett's oesophagus is still lacking and is required to understand more fully the risk and pathogenesis of this metaplasia and oesophageal adenocarcinoma.

Intact human colonic crypts were isolated and placed into 3D tissue culture conditions optimised for steady-state tissue renewal. The role of Wnt and TGFβ/BMP signalling pathways in tissue renewal was investigated.

Native human colonic crypts exhibited distinct activation profiles for canonical Wnt, TGFβ and BMP pathways. A population of intestinal Lgr5/OLFM4^{+} stem cells were found to be interspersed between goblet cells at the base of the crypt. Exogenous Wnt signals maintained Lgr5/OLFM4^{+} stem cells, whilst BMP and TGFβ inhibited and caused complete loss of stem cells. Wnt signals also rescued the inhibitory effects of Dkk1, IWP2 and dnTCF4 on Wnt target gene expression, cell proliferation and crypt length. BMP and TGFβ inhibited Wnt target gene expression, cell proliferation and crypt length.

A near-native human Barrett's oesophagus *ex vivo* culture model was developed similar to the colonic model which was amenable to real-time time-lapse microscopy and imaging techniques. The Wnt and NFκB signalling pathways exhibited distinct activation profiles. A population of OLFM4^{+} stem cells were found to reside in the lower third of the Barrett's crypt.

Steady-state tissue renewal in the human colonic epithelium is dependant on Wnt signals combined with suppressed TGFβ/BMP pathways. The human colonic crypt model and the Barrett's oesophagus crypt model will permit functional interrogation of the mechanisms underlying tissue renewal and risk of inflammatory diseases and adenocarcinoma.

Abbreviations

APC	Adenomatous Polyposis Coli
BMP	Bone Morphogenic Protein
BrdU	5'Bromodeoxyuridine
CBC	Crypt base columnar
COX	Cyclooxygenase
CRC	Colorectal cancer
DIC	Differential interference contrast
Dkk	Dickkopf
EGF	Epidermal growth factor
FACS	Fluorescence-activated cell sorting
FGF	Fibroblast growth factor
Fz	Frizzled
GFP	Green fluorescent protein
GSK3β	Glycogen synthase kinase 3β
Hh	Hedgehog
IBD	Inflammatory bowel disease
IGF	Insulin-like growth factor
ISC	Intestinal stem cell
ISEMFs	Intestinal subepithelial myofibroblasts
JNK	c-Jun N-terminal kinase
MOI	Multiplicity of infection
PBS	Phosphate buffered saline
ROI	Region of interest
SFRP	Secreted frizzled related protein
TGFβ	Transforming growth factor β
TNF-α	Tumour necrosis factor α
TLRs	Toll-like receptors
WIF	Wnt inhibitory factor

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Chapter 1 Introduction

1.1 General Introduction

The colonic epithelium is one of the most dynamic renewing tissues in the body which is tightly regulated and controlled. Approximately 10 billion cells are shed from the gut epithelium each day and these are continuously replaced by intestinal stem cell progeny. The epithelium undergoes continuous replacement of cells through a cycle of cell proliferation, differentiation, migration and shedding at the crypt surface during a 3-8 day transit time. Several key regulatory signals are involved in colonic epithelium stem cell renewal and differentiation, including Wnt, Bone Morphogenic Protein (BMP), Transforming Growth Factor β (TGFβ) and Notch pathways (1).

Wnt signalling plays a major role in maintaining epithelial stem cell fate and progenitor cell proliferation. Adenoviral and transgenic expression of Dkk1, an inhibitor of the canonical Wnt signalling pathway, led to complete loss of proliferation and presence of mouse intestinal crypts (2) (3). The predominance of Wnt signals at the base of the crypt led to the discovery that the Wnt target gene *Lgr5* was enriched in mouse intestinal stem cells (4). Recently it was also found that ligation of R-Spondin-1 to the Wnt ligand-Fz-LRP5/6 receptor complex synergistically activates Wnt signals (5) (6).

BMP and TGFβ signals in contrast predominate at the top of the crypt and are involved in inhibition of intestinal stem cell activation and promote intestinal differentiation (7) (8). Loss of the TGFβ/BMP pathway activation augments the Wnt signalling pathway which disrupts tissue renewal and drives intestinal polyp and tumour formation (7) (9) (10). Notch signalling is involved in cell fate decisions in the colonic epithelium, specifically directing cells towards a secretory lineage (11) (12). Precancerous lesions such as Crohn's and ulcerarive colitis have been found to have up-regulated levels of transcription factors downstream of Notch signalling, which may be responsible for the altered goblet cell differentiation and mucin formation (13) (14).

The hierarchy of tissue renewal in the colonic epithelium is thought to minimise the accumulation of molecular damage by the position of the long-lived stem cells residing at the base of the crypt in a safe environment. The mechanisms that regulate the processes of tissue renewal are of great interest since they are found to be disrupted in inflammatory bowel disease and colorectal cancer.

Barrett's oesophagus is an intestinal metaplasia arising in response to inflammation and ulceration provoked by gastroesophageal reflux. This metaplasia predisposes the oesophageal tissue to adenocarcinoma through a low-to-high grade dysplasia. The Wnt, BMP and TGFβ signalling pathways have been implicated in this neoplastic progression by activating genes that induce a columnar phenotype of the cells. Abnormal activation of β-catenin and increased expression of Wnt target genes cyclin D1, Sox9 and c-Myc are common during neoplastic progression of Barrett's metaplasia (15) (16). The BMP signalling pathway is also activated in Barrett's oesophagus and could play a role in the transformation of normal squamous cells into a columnar phenotype (17) (18). Loss of SMAD4 and TGFβRII has been found in Barrett's oesophagus and adenocarcinoma tissue (19). Detailed knowledge of the processes and signalling pathways involved in tissue renewal in Barrett's oesophagus is still lacking and is required to understand more fully the risk and pathogenesis of this metaplasia and oesophageal adenocarcinoma.

Tissue culture conditions that favour Wnt signalling pathway activation and suppression of TGFβ/BMP pathways have led to the development of human intestinal and Barrett's organoids *ex vivo*. Isolated single crypts from both colon and Barrett's epithelium form multiple budding structures which are composed of immature stem/progenitor cells that can be induced to differentiate (20). We have developed a culture model of near-native human colonic and Barrett's crypts that preserves crypt length, morphology and polarity and demonstrate that the hierarchy of stem-cell driven tissue renewal is recapitulated in this model *ex vivo* (21).

1.2 Colon Physiology

1.2.1 Structure of the colon

The human colon is a dynamic organ whose functions include absorption of water and electrolytes, salvage of unabsorbed nutrients, and transport of luminal contents and formation of faeces. The 1.5m long muscular tube is subdivided into the ascending colon, transverse colon, descending colon and the sigmoid colon which connects to the rectum.

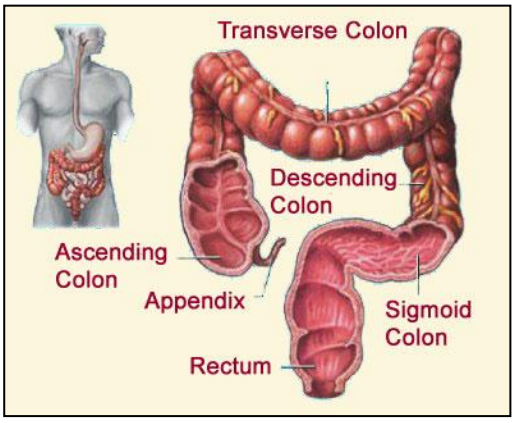


Figure 1.1 Anatomy of the colon. *Figure from* (22).

The wall of the colon is composed of four major tissue layers; the serosa, muscularis externa, submucosa and mucosa (Fig 1.2). The outermost layer of the colon is the serosa which is comprised of a thin layer of connective tissue. Sheets of connective tissue attach the serosa to the abdominal wall, supporting the colon in the abdominal cavity. Below the serosa is the muscularis externa, two layers of smooth muscle tissue. These muscle contractions force the gastrointestinal contents to move along the tract. The inner layer of the muscularis externa is a thick circular muscle that allows contractions that shorten the colon. Between the two muscle layers is a network of nerve cells known as the myenteric plexus. Beneath the muscularis is a connective tissue known as the submucosa that contains a network of nerve cells termed the submucosal plexus, blood and lymphatic vessels. The mucosa comprises of a thin layer of smooth muscle, a thin layer of connective tissue layer comprising of the lamina propria and the epithelium.

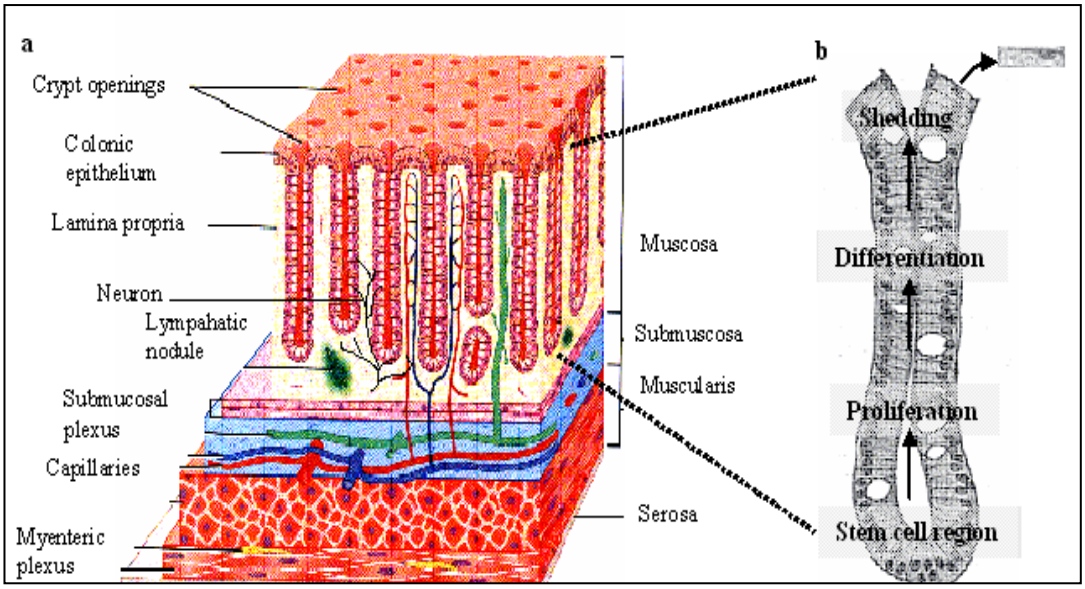


Figure 1.2 Structure of the colonic wall. *A) Colonic wall showing the four layers: serosa, musularis, submucosa and mucosa. b) The epithelium of the mucosa comprises of single unitary structures called crypts of Lieberkühn. Stem cells at the base of the crypt provide the rest of the crypt with all the cell types required for its function as they migrate up along the crypt axis and are eventually shed at the top of the crypt into the lumen. Figure adapted from* (23).

Unlike in the small intestine, the mucosa in the colon lacks the villus projections thereby creating a smooth surface that is continuously renewed. The epithelial layer of the human colon is made up of a single sheet of columnar epithelial cells, which form finger-like invaginations into the underlying connective tissue of the lamina propria to form the basic functional unit of the intestine, the crypts of Lieberkühn or colonic crypts. Within the colon there are millions of crypts and a few stem cells at the base of the crypts are responsible for the renewal of the colon by producing all the functioning cell types of the epithelium. Stem cells are supported by underlying myofibroblasts known as intestinal subepithelial myofibroblasts (ISEMFs) which are in close proximity to the smooth muscle cells of the muscularis mucosa layer. These cells contribute to the stem cell niche and act as regulators of intestinal stem cell self-renewal and differentiation. They are thought to secrete factors that can maintain the stem-like phenotype of stem cells in the niche (24).

Wnt signalling in particular has an important role in the crosstalk between epithelial and mesenchymal cells in maintaining the survival of stem cells. Farin *et al.* (25) have recently demonstrated that deletion of Paneth cell derived Wnt3 in the intestinal epithelium showed no effect *in vivo* but was required for growth and sustainability in organoid *in vitro* culture. Co-culturing of mesenchymal derived Wnt2b ligand restored the growth of organoids, thus demonstrating a compensatory mechanism in the stem cell niche that safeguards against stem cell dysfunction.

1.2.2 Cell types of the intestinal epithelium

The intestinal epithelium is probably the most dynamic renewing tissue in the body which is tightly regulated and controlled. It is renewed by a small number of intestinal stem cells that give rise to a pool of multipotent progenitor cells, also known as transit amplifying cells that are highly proliferative and differentiate into one of several cell lineages: absorptive enterocytes, the mucus-secreting goblet cells, peptide hormone-secreting enteroendocrine cells and Paneth cells (in the small intestine and proximal colon). Enterocytes, goblet and enteroendocrine cells migrate towards to the lumen of the gut whilst Paneth cells move towards the bottom of the crypt (26). Less common cell lineages are also present such as Tuft (caveolated) cells and M-(membranous or microfold) cells (Fig 1.3). Tuft cells are characterised by long and blunt microvilli with prominent rootlets and by a well developed tubulovesicular system in the supranuclear cytoplasm (27). Experiments by Bezencon *et al.* (28) have shown that *Trpm5*-expressing cells are Tuft cells that express the cyclooxygenase 1 and 2 (COX1 and COX2) enzymes and this was also confirmed by Gerbe *et al*. (29). The expression of COX1 and COX2 enzymes is the rate limiting step for the biosynthesis of prostanoids. Tuft cells also express *Hpgds* and represent a likely epithelial source of prostaglandin-D2 (29). Gerbe *et al.* (30) also show that tuft cells are the only intestinal epithelial cells to produce β-endorphin and likely

contribute to the regulation of vasoconstriction, peristaltic movements and pain in the intestine.

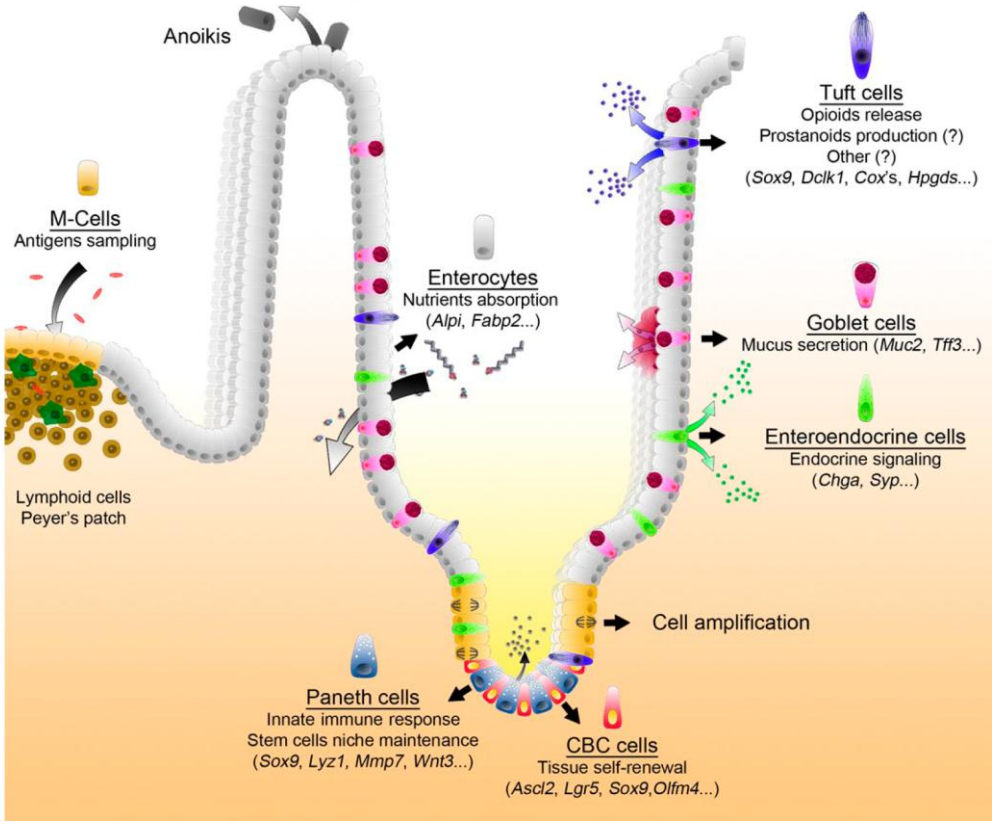


Figure 1.3 Cell types of the intestinal epithelium. *Absorptive cells have a brush border of microvilli on the apical surface. Goblet cells secrete mucous with their apical cytoplasm containing mucous-filled secretory granules. Enteroendocrine cells are smaller and secrete gut hormones such as peptides and catecholimes. Paneth cells secrete antibacterial proteins such as lysozymes and defensins. Tuft cells release opioids and produce prostanoids. Figure from* (29).

The major function of enterocytes include: ion uptake through active transport of sodium, calcium, magnesium and iron, water uptake via an osmotic gradient established by Na^+/K^+ ATPase on the basolateral surface, sugar uptake using the GLUT2 and GLUT5 receptors, peptide, amino acid, lipid and Vitamin B12 uptake, re-absorption of unconjugated bile acids and secretion of immunoglobulins.

Goblet cells synthesize secretory mucin glycoproteins (MUC2) and bioactive molecules such as epithelial membrane-bound mucins (MUC1, MUC3, MUC17), trefoil factor peptides (TFF), resistin-like molecule beta (RELMbeta), and Fc-gamma binding protein (Fcgbp). The MUC2 mucin protein forms trimers by disulfide bonding in cysteine-rich amino terminal von Willebrand factor (vWF) domains, coupled with crosslinking provided by TFF and Fcgbp proteins with MUC2 vWF domains, resulting in a highly viscous extracellular layer. This viscous extracellular layer provides the frontline host defense against endogenous and exogenous irritants and microbial attachment and invasion but allows the transport of nutrients into the cell (31).

Enteroendocrine cells comprise less than 1% of the overall epithelial cell population and their primary function is to secrete hormones. They are characterised by the presence of secretory vesicles that are either large dense-core vesicles (LDCVs) or the smaller synaptic-like microvesicles (SLMVs) that are similar to those found in neurons. Components of the vesicles such as Chromagranin A can be used as markers of enteroendocrine cells in immunohistochemistry since it is a matrix-soluble glycoprotein found in LDCVs and a synaptophysin, a membrane glycoprotein of SLMVs (32). Enteroendocrine cells also express Toll-like receptors (TLRs) that are involved in innate immunity response to pathogens. Palazzo *et al*. (33) have shown that enteroendocrine hormone cholecystokinin (CKK) production in STC-1 cells was increased with stimulation of the TLR agonists LPS, flagellin and CpG oligodeoxynucleotides leading to the release of keratinocyte-derived chemokine and β-defensin 2 which neutralise intestinal bacteria.

Paneth cells provide host defence against pathogens in the small intestine. The defence molecules secreted by Paneth cells include alpha-defensins, also known as cryptdins, defensins, lysozyme and phospholipase A2. Sato *et al.* (34) have recently shown that Paneth cells also provide stem cell niche factors that are essential for the maintenance of stem cells. Gene expression profile on stem cells and Paneth cells found that Paneth cells were highly enriched in the *Wnt3*, *Wnt11*, *Egf*, *Tgfa* and the Notch ligand *Dll4* genes, all

which provide essential support for stem cells. Their co-culture of $Lgr5^+$ cells with Paneth cells *in vitro* increased organoid formation compared to single stem cell organoids. In their *in vivo* model they also showed that stem cells disappeared coincident with Paneth cells and the remaining stem cells crowded around the remaining Paneth cells. Thus they concluded that Paneth cells were essential for the maintenance of crypts and stem cells.

The colonic epithelium however, does not contain Paneth cells. Rothenberg *et al*. (35) investigated the existence of colonic Paneth-like cells that could support $Lgr5^+$ stem cells. Using fluorescence-activated cell sorting (FACS) to isolate different subregions of colon crypts and single-cell gene expression analysis they characterised distinct cell types. They found that some goblet cells contained a distinct cKit/$CD117^+$ crypt base subpopulation that expressed Dll1, Dll4 and epidermal growth factor (EGF), similar to Paneth cells which are also marked by cKit. In the colon the $cKit^+$ goblet cells interdigitate with the $Lgr5^+$ stem cells. *In vivo* they regulated and increased the number of $cKit^+$ cells with the administration of γ-secretase inhibitor. When they isolated these cells from the mouse colon and co-cultured with $Lgr5^+$ stem cells, they found that the $cKit^+$ cells promoted organoid formation and when organoids were depleted of $cKit^+$ cells the organoid formation decreased.

1.2.3 Search for the stem cell

Colonic stem cells have remained elusive for many years due to lack of specific markers that can distinguish them from other progenitor cells in the crypt. About 40 years ago, the unitarian theory proposed that crypts are monoclonal populations that are derived from a single intestinal stem cell (36). Evidence for the Unitarian hypothesis was shown by Novelli *et al*. (37) who studied the colon of a rare XO/XY mosaic patient with familial adenomatous polyposis (FAP) and found that individual colonic crypts were composed entirely of either XO or XY cells, and not a combination of the two. Immunostaining for

the crypt enteroendocrine cells confirmed that they share the karyotype of the other cells in the crypt, suggesting that they were derived from a stem cell precursor.

Taylor *et al*. (38) have also shown that accumulation of defects in cytochrome c oxidase (COX) activity in intestinal stem cell mitochondrial DNA (mtDNA) were passed on to daughter cells that then populated the crypt. They found that a few crypts had ribbons of COX deficient cells reaching from the bottom to the top of the crypt, suggesting that one of the multiple stem cells within the niche had acquired sufficient mtDNA mutations to result in a functional deficit. A study by Bjerknes and Cheng (39) found that the crypt contains both short-lived (lifespan ≤10 days) and long-lived (lifespan ≥100 days) progenitors of cell lineages as well as long-lived multipotent stem cells. The specific progenitor assay was demonstrated by firstly crossing the Dlb $^{-/-}$ SWR mice with Dlb $^{+/+}$ C57BL/6 mice. The resulting F1 chimeric Dlb $^{-/+}$ mice show heterozygous expression of a binding site on intestinal epithelial cells for the *Dolichos biflorus* agglutitin (DBA) lectin, which is abolished when the Dlb locus becomes mutated either spontaneously or by the chemical mutagen ethylnitrosourea (ENU). Induction with ENU results in clones of cells that are Dlb $^{-/-}$ (unstained) and Dlb $^{+/+}$ (stained) along the crypt axis. Over time they found that the stem cells give rise to both short-lived and long-lived progenitor cells. These long-lived progenitor cells or transitory committed progenitor cells, columnar cell progenitors (C_0) and mucous cell progenitors (M_0) that reside in the lower crypt base, evolve from multipotent stem cells and then differentiate further into the epithelial cell types C_1 and M_1 that migrate up the crypt axis. The short-lived cells probably represent the offspring of an extinct mutant M_1 cell.

To determine the position of the intestinal stem cells, studies using DNA-label-retaining assays concluded that stem cells resided above the uppermost Paneth cell, at positions ranging from +2 to +7, on average at position +4 (40). It was observed that the intestinal stem cells retained the template strand during segregation of the DNA strands possibly providing a protective mechanism against accumulation of replication errors and found

about four to eight label-retaining cells per crypt distributed around the fourth position from the bottom of the crypt. However, using electron microscopy studies, Cheng and Leblond (41) revealed the presence of slender, immature cycling cells wedged in between Paneth cells, referred to as crypt columnar cells (CBC) (Fig 1.4). Following exposure to 3H-Thymidine, the surviving CBC cells actively phagocytosed other dying, radiolabelled CBC cells at the crypt base. The resulting radioactive phagosomes, initially restricted to occasional CBC cells, were subsequently observed within more differentiated cells that belonged to the four lineages higher up the crypt. This demonstrated that the CBC cells were the common origin of all four major epithelial cell lineages.

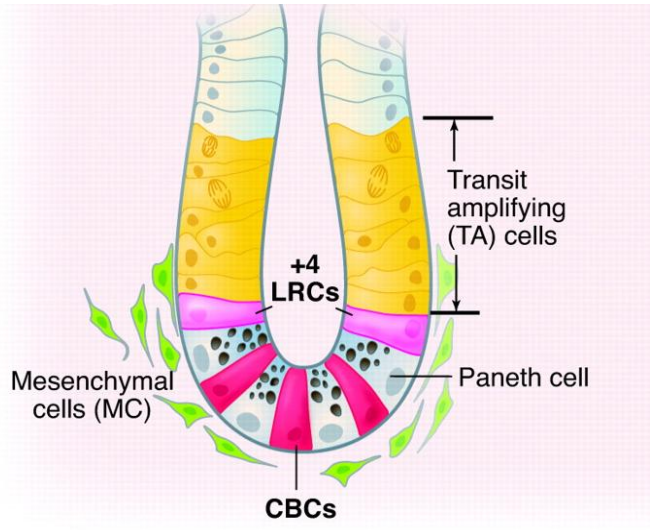


Figure 1.4 The two proposed locations of intestinal stem cells. *Potten et al. demonstrated that intestinal stem cells are located at position +4 whilst Cheng et al. have shown that crypt columnar cells (CBC) are the true stem cells that give rise to all four cell lineages and are located between the Paneth cells at the crypt base. Figure from* (42).

Several strategies have been used to identify intestinal stem cell markers. Musashi-1was first identified as a neuronal stem cells marker and later proposed as an intestinal stem cell marker using immunocytochemistry (43). Bmi1, originally proposed to regulate self-renewal of neural and haematopoietic progenitors was later found to mark rare +4 position cells using lineage tracing experiments (44). Prominin 1 (CD133) was first identified as a

haematopoietic and neuronal stem cell marker and was later shown to mark intestinal stem cells using lineage tracing and experiments (45). Immunohistochemical staining showed that DCAMKL1 may also be an intestinal stem cell marker (46). Lgr5 was identified using a screening of Wnt target genes that had restricted expression at the crypt base and later confirmed using lineage tracing to label intestinal stem cells (47) (4). Found to be highly enriched in human colonic crypt bases, OLFM4 was shown to be an intestinal stem cell maker using *in situ hybridisation* (48). Breult *et al.* (49) propose that since high telomerase levels may be a general feature of adult stem cells, mTert was shown to mark a radiation-resistant stem cell population that generated all differentiated intestinal cell types (50). Hopx, involved in cardiac development (51) and neural stem cells (52) was also found to label intestinal stem cells that gave rise to all lineages (53). The pan-ErbB inhibitor Lrig1, was shown using *in situ hybridisation* to be highly expressed in the stem cell niche and colocalised with other stem cell markers (54).

The musashi-1 (Msi-1) gene encodes a RNA binding protein that is involved in early asymmetric divisions that generate differentiated cells from neural stem cells or progenitor cells. It was first identified in *Drosophila* and plays an essential role in regulating the asymmetric cell division of ectodermal precursor cells known as sensory organ precursor cells through the translational regulation of target mRNA (55). Collaboration between Okano and Potten (56) revealed that there was Msi-1 expression in the small intestine at the +4/ +6 positions of the crypt base and at the very bottom region of the crypt base in the colon. Nishimura *et al*. (43) have also shown Musashi-1 positive cells located in the lower part of the crypt, less than 10 cell positions from the bottom. Sureban *et al.* (57) have shown that Msi-1 expression is upregulated in human colorectal tumours and siRNA-mediated knockdown of Msi-1 in the tumour xenografts resulted in the arrest of tumour growth. Furthermore, inhibition of Msi-1 resulted in decreased cancer cell proliferation, increased caspase-3-mediated apoptosis and enhanced radiation-induced apoptosis,

suggesting a role of Msi-1 in intestinal tumourigenesis as a cell proliferation regulator and inhibitor of mitotic catastrophe.

The *Bmi1* gene is known to be involved in the self-renewal of neuronal (58), hematopoietic (59) and leukemic (60) cells and was first identified in a mouse proviral insertion screen for lymphomagenesis (61). Sangiorgi and Capechhi (44) found that Bmi1 predominantly labelled a discrete cell population above the Paneth cells at the +4 position from the base of the crypt. However, they found that there was a non uniform distribution of Bmi1 expressing stem cells in the small intestine, with more crypts labelled in the duodenum and the first part of the jejunum and progressively fewer to none in the ileum. They suggest that more than one adult stem cell population may be present in the intestine.

CD133, also known as Prominin 1 in the mouse, was first recognised as a marker of human haematopoietic stem cells and later used to mark neuronal and brain tumour stem cells. Zhu *et al*. (45) found that Prominin 1 was expressed in a variety of developing adult tissues and in the small intestine the $Prom1^{+}$ cells were located at the base of the crypts and co-expressed with another intestinal stem cell marker, Lgr5. Using lineage tracing of the $Prom1^{+}$ stem cells, the $Prom1^{+}$ cell progeny could be detected for over 60 days in the small intestine, brain, kidney, lung and pancreas. However, in the colon the Prom1 expression disappeared within 60 days. Zhu *et al*. (45) propose that because the brain, kidney, lung and pancreas have low rates of cell turnover, the $Prom1^{+}$ cells in these tissues and the small intestine are the quiescent adult stem cells rather than the more rapid-cycling $Lgr5^{+}$ CBC cells.

Doublecordin and CaM kinase-like-1 (DCAMKL-1), a microtubule-associated kinase expressed in post mitotic neurons was proposed by May *et al.* (46) to be a putative intestinal stem cell marker. Immunohistochemical staining revealed DCAMKL-1 expression at the +4 position of the crypt base, as well as the occasional crypt columnar cell between the Paneth cells. Co-labelling with Musashi-1 antibody revealed co-

localisation of the DCAMKL-1$^+$ and Musashi-1$^+$ cells in the small intestine. May *et al.* (62) later also co-labelled DCAMKL-1 with Lgr5 and found that they marked two distinct populations of cells. Even though they sometimes observed a DCAMKL-1$^+$ cell in between the Paneth cells where CBC cell normally reside, there was no co-localisation with Lgr5. May *et al*. also isolated DCAMKL-1$^+$ cells from the adult mouse small intestine using fluorescence activated cell sorting (FACS) and observed that these cells self-renewed and ultimately formed spheroids in suspension culture. These spheroids formed glandular epithelial structures in the flanks of athymic mice and expressed multiple gut epithelial lineages. Therefore, although the DCAMKL-1+ cells may not co-localise with Lgr5 and mark the crypt base columnar cells, they may represent the quiescent intestinal stem cells. However, Gerbe *et al*. (30) identified the DCAMKL-1$^+$ cells as terminally differentiated Tuft cells (caveolated cells) that are distributed throughout the crypt-villus epithelium. This was confirmed by Bezencon *et al.* (63) who found *Dcamkl-1* expression in the tuft cells of the mouse.

mTert (mouse telomerase reverse transcriptase) is another very recently identified stem cell marker for the +4 position, or slow-cycling intestinal stem cells. Telomerase is a ribonucleoprotein complex that helps to maintain the telomeric ends of chromosomes that are shortened with each cell division. The loss of telomeric DNA beyond a critical threshold induces senescence so induction of telomerase activity prevents cellular senescence, especially important in the self-renewal of stem cells (50). Montgomery *et al.* (50) generated a *mTert*-GFP transgenic mouse model system in which GFP expression recapitulated endogenous *mTert* expression and telomerase activity. They found that *mTert* expression marks a population of crypt cells distinct from Lgr5$^+$ CBC cells and also a subpopulation of Bmi1$^+$ cells. Their results also show that mTert$^+$ cells are slow cycling cells and are resistant to the effects of radiation, unlike rapid cycling cells that are radiosensitive. So although *mTert*-expressing cells only minimally contribute to the normal intestinal homeostasis, over the life of the organism they progressively contribute

to the crypt/villus unit. This most comes in effect as a regenerative response in which radiation sensitive stem cells might be restored following intestinal injury. This was demonstrated by showing that $Lgr5^+$ stem cells could arise from the *mTert*-expressing stem cells, suggesting a possible mechanism by which the active stem cell population may be renewed throughout the lifetime.

So far the stem cell markers mentioned have been shown to label intestinal stem cells at the +4 position at the crypt base. However, Barker *et al.* (4) identified Lgr5, a gene encoding a G-protein-coupled receptor whose expression was restricted to just crypt base columnar cells (CBC). *Lgr5* (leucine-rich G-protein-coupled receptor 5) was originally identified during a screening of Wnt target genes that had restricted expression at the crypt base. Of approximately 80 Wnt target genes, most were expressed in either Paneth cells or transit-amplifying cells. The *Lgr5* gene however, seemed to mark the cycling CBC cells between the Paneth cells. The stem cell potential of the $Lgr5^+$ cells was assessed by *in vivo* lineage tracing using an *Lgr5-EGFP-ires-CreERT2/Rosa26RlacZ* mouse model. Following induction of Lgr5-CRE activity using tamoxifen, lacZ reporter gene activity was initially observed in isolated CBC cells (Fig 1.5). At later time points the lacZ genetic mark was seen in cells of all lineages throughout the crypt-villus epithelium. This tracing was maintained throughout the lifetime of the mouse, thus identifying the $Lgr5^+$ CBC cells as the self-renewing intestinal stem cells that were multipotent (Fig 1.5). Lgr5 has been shown to mark stem cells in other tissues including hair follicles in the skin (64) and pyloric glands of the stomach (65). Barker *et al.* (66) have also shown that deletion of Apc in the $Lgr5^+$ stem cells leads to their transformation into tumour initiating cells that develop into adenomas.

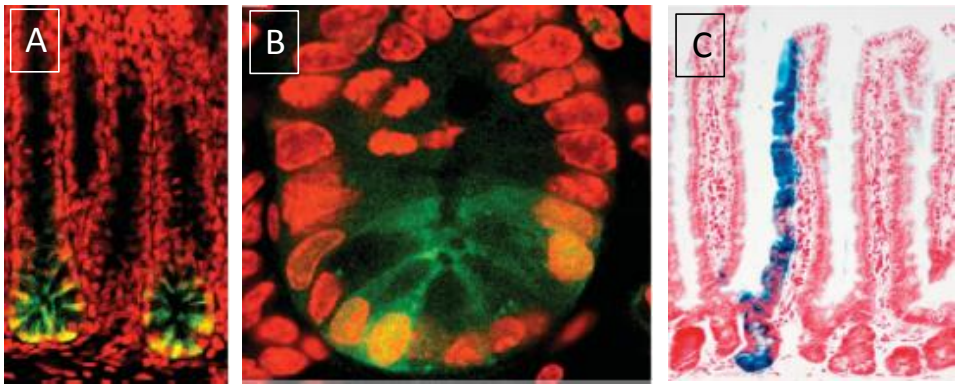


Figure 1.5 A) EGFP expression of Lgr5 *in an Lgr5-EGFP-IRES-creERT2 knock-in mouse along the entire crypt-villus axis and B) enlargement of crypt regions. C) LacZ lineage tracing in the small intestine 60 days after induction. Figure from* (4)

To demonstrate that Lgr5 is a robust intestinal stem cell marker, Sato *et al*. (67) sorted single Lgr5-EGFP^{+} cells using flow cytometry and placed them into culture. Over a period of two weeks these single cells developed into organoids (Fig 1.6) that contained all the cell lineages of an intestinal crypt: Paneth cells, enterocytes, goblet cells and enteroendocrine cells. Remarkably, these Lgr5^{+} stem cells are capable of generating these complex three-dimensional structures in the absence of any mesenchymal components, although R-Spondin 1, Noggin and EGF are essential. R-Spondins have recently been found to be the ligands for Lgr5 as well as its homologues Lgr4 and Lgr6 (5).

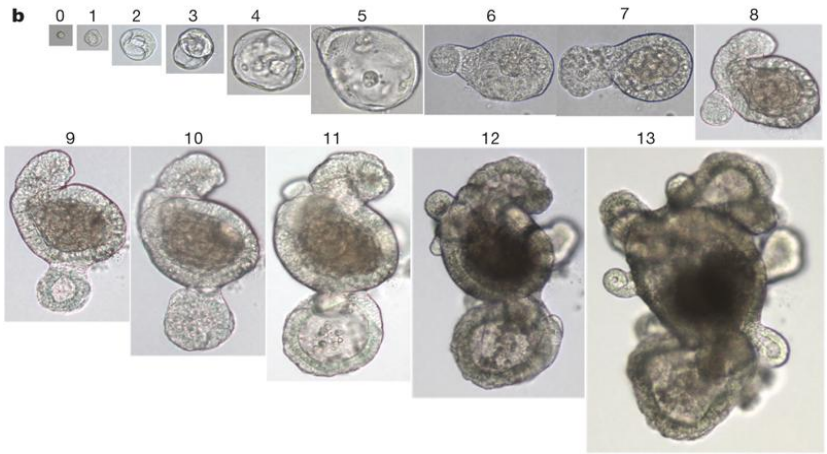


Figure 1.6 Isolated single Lgr5^{+} stem cell growing into a complex three-dimensional organoid structure in a two week period. *Figure from* (67).

Most recently Yui *et al*. (68) have demonstrated that $Lgr5^{+}$ stem cells that grew into organoids *in vitro* could be transplanted into damaged mouse colon and lead to a successful engraftment. They first induced mucosal damage by providing immune-compromised $Rag2^{-/-}$ mice with colitis-inducing dextran sulphate sodium (DSS) so that the mice developed acute colitis. They dissociated the organoids cultured from *Lgr5-EGFP-ires-CreERT2* mice and instilled them by enema into recipient mice. At four weeks after transplantation, tube-like $EGFP^{+}$ crypts appeared in the distal colon and were morphologically indistinguishable from the surrounding $EGFP^{-}$ epithelium. The engrafted crypts were entirely $EGFP^{+}$, indicating the presence of $EGFP^{+}$ stem cells and these crypts contained all the differentiated cell types. This work demonstrates the potential of stem cell therapy in repairing damaged epithelium.

Van der Flier *et al*. (69) carried out a small intestine stem cell signature based on Lgr5 expression. GFP-positive epithelial cells from the *Lgr5-EGFP-ires-CreERT2* mice were isolated using FACS sorting. The FACS analysis distinguished two populations of cells, GFP^{hi} and GFP^{lo} cells corresponding to the Lgr5 stem cells and their transit-amplifying daughters. Gene profile analysis was carried out on the two populations and the *ASCL2* and *OLFM4* genes were found to be enriched in the $Lgr5^{hi}$ population. *ASCL2* is one of the mammalian homologues of the *Drosophila aschaete-scute* complex genes that encodes related bHLH proteins that are powerful regulators of cell fate. Genetic ablation of ASCL2 expression *in vivo* results in silencing of the stem cell signature and rapid stem cell death, suggesting a crucial role for this transcription factor as a master regulator of stemness.

Formeister *et al*. (70) have recently shown that distinct levels of *Sox9* expression mark intestinal stem cells based on enriched levels of *Lgr5* mRNA in discrete cell populations. Sox transcription factors have the capacity to modulate stem/progenitor cell proliferation and differentiation in a dose-dependent manner. Using fluorescence FACS to sort cells of the small intestinal epithelium from a $Sox9^{EGFP}$ reporter gene mouse model, they identified that low levels of $Sox9^{EGFP}$ (Sox^{EGFPlo}) mark cells that are enriched for *Lgr5* and high levels

of *Sox9*^{EGFP} (*Sox9*^{EGFPhi}) mark post-mitotic enteroendocrine cells. These *Sox9*^{EGFPlo} cells were also enriched for *OLFM4* and *ASCL2* which co-localise with the Lgr5 population of cells and had increased expression of Wnt target genes *c-Myc* and *Cyclin D1*. The isolated *Sox*^{EGFPlo} cells were placed into culture condition described by Sato *et al.* (67) and grew into organoid structures within a week. These organoids contained all the differentiated cell lineages, demonstrating that single *Sox*^{EGFPlo} stem cells have the capacity to function as multipotent stem cell in culture (71).

Gene expression and proteome profiling of Lgr5^{+} stem cells has revealed an Lgr5 stem cell signature (72) (73), whose genes contribute to stemness. Genetic ablation or overexpression of *Ascl2* or *Smoc2* results in rapid stem cell death (69) (72). The *OLFM4* gene was also found to be highly enriched in human colon crypt bases (74). OLFM4 is a secreted molecule originally cloned from human myeloblasts that is under the control of Notch signalling and was recently shown that an OLFM4 family member (*Xenopus* ONT1) acts as a BMP antagonist. *In situ* hybridization has revealed OLFM4 as a highly specific and robust marker for Lgr5 stem cells (48). OLFM4 was also highly expressed in subsets of cells within colorectal carcinomas and its expression in these tumour cells was much higher than in normal crypt base columnar cells suggesting that they could be used to mark stem cells that harbour tumour initiating properties. Gersemann *et al.* (75) have found that OLFM4 expression was extensively up-regulated in inflamed inflammatory bowel disease mucosa, expands up to the surface epithelium and is secreted into the mucus. This induction may be mediated by bacteria via the Notch pathway through IL-22, therefore OLFM4 is suggested to have a functional protective role in IBD by binding defensins in the mucus. Oue *et al.* (76) suggest that serum OLFM4 could also be a useful marker for early detection of gastric cancer.

The most recent intestinal stem cell marker to be identified is Lrig1 (54) (77). Lrig1 (Leucine-rich repeats and immunoglobulin-like domains 1) is a transmembrane protein that acts as an inducible, negative feedback inhibitor of ErbB signalling. Lrig1 has also been

suggested to mark a quiescent stem cell population in the mammalian epidermis (78). Using *in situ* hybridisation Wong *et al.* (54) show that Lrig1 is highly expressed in the stem cell niche of the small intestine and colon and using the *Lgr5-EGFP-ires-CreERT2* knock-in mouse to differentiate between the GFP^{hi} ($LGR5^{+}$ stem cells) and GFP^{lo} daughter cells, the levels of *Lrig1* were highest in the GFP^{hi} intestinal stem cells. Immunofluorescence staining and flow cytometric analysis confirmed the overlap of Lrig1 and Lgr5 at the protein level and that Lrig1 is expressed in a gradient with highest levels in intestinal stem cells and is absent in Paneth cells (54). Markers that define stem cells such as *Lgr5, Ascl2* and *Msi1* were found to be enriched in isolated $Lrig1^{+}$ cells. They also observed differential expression of multiple transcripts for Lrig1 interaction partners, in particular epidermal growth factor receptor (EGFR) and that $Lrig^{+}$ cells are proliferative. Loss of Lrig1 caused a marked increase in crypt size along the entire length of the small intestine. This is explained by the expansion of the stem cell compartment by affecting ErbB signalling within the stem cell niche (Fig 1.7). Lrig1 interacts with the ErbB family, cRet and cMet and reduces signalling strength by negatively regulating both protein levels and the activity of the growth factor receptors. Loss of Lrig1 causes an increase in the protein levels of the interaction partners which then leads to a rapid expansion of the stem cell compartment. Treatment with the ErbB inhibitor Gefitinib in the Lrig1-knockout mice restored crypt proliferation, stem cell and Paneth cell numbers to normal levels. Powell *et al*. (77) have also confirmed that Lrig1 is an intestinal stem cell marker, but show that they are relatively quiescent under normal homeostatic conditions and are only mobilised to repopulate the colonic crypt upon tissue damage. Whole transcriptome analysis of $Lrig1^{+}$ and $Lgr5^{+}$ cells revealed that there are differences in the molecular programming of the two cell population (77). Immunofluorescent analysis showed that co-localisation of the two markers rarely occurred in the same cell. Ki67 and BrdU analysis showed that the majority of Lrig1+ cells are infrequently cycling, and the cell cycle inhibitor *Cdkn1a* (p21) was highly expressed in these cells. $Lrig1^{+}$ cells also express genes involved in oxidative stress responses, suggesting their role in damage response. Loss of *Apc* in $Lrig1^{+}$ cells

results in multiple intestinal adenomas and *Lrig1* null mice develop duodenal adenomas, suggesting that Lrig1 functions as a tumour suppressor.

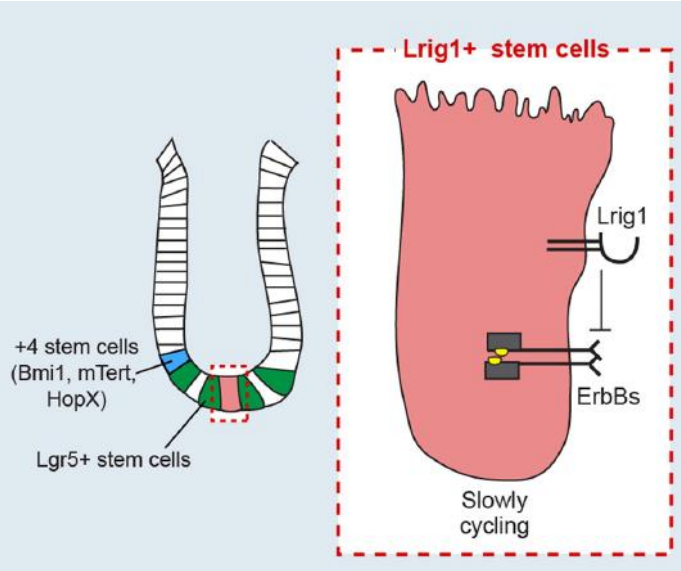


Figure 1.7 *The intestinal stem cell marker Lrig1 is a transmembrane protein that acts as an inducible, negative feedback inhibitor of ErbB signalling. Powell et al. show that Lrig1^{+} cells are slow-cycling and are a distinct population from Lgr5^{+} stem cells. Figure from* (77).

The location of the intestinal stem cells seems to be of debate, with several stem cell markers such as Musashi-1, Bmi1, Prominin 1, DCAMKL1, mTert and Lrig1 labelling the slow-cycling and label-retaining cells at position +4 and Lgr5 and OLFM4 marking rapid-cycling CBC cells. The relationship between them is not well understood, although both give rise to all intestinal epithelial lineages. Using organoid culture from *Hopx*$^{Lacz/+}$; *Lgr5*$^{EGFP\text{-}ERCre/+}$ mice, Takeda *et al.* (53) have identified that quiescent +4 intestinal stem cells express the atypical homeobox gene *Hopx*, and give rise to Lgr5 expressing CBC cells. Conversely, rapid cycling CBC cells expressing Lgr5 give rise to +4 cells expressing *Hopx*. This demonstrates that a bi-directional lineage relationship exists between active and quiescent stem cells in the stem cell niche. A proposed model for this relationship is demonstrated in Fig 1.8 by Powell *et al.* (77). They propose that Lrig1+ stem cells are

downstream of the more quiescent Bmi1^{+} , Hopx^{+} or mTert^{+} stem cells and serve two roles in homeostasis: to protect the niche from stress and to give rise to transit amplifying cells directly and/or the highly proliferative Lgr5^{+} stem cells when needed.

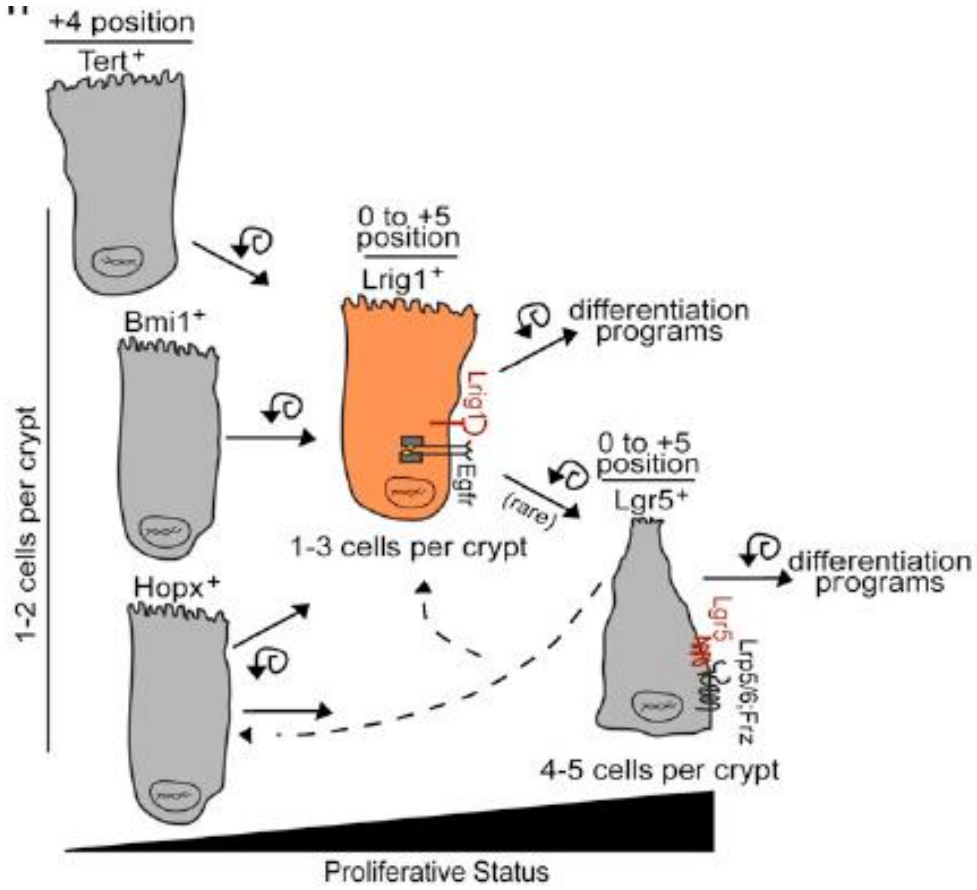


Figure 1.8 Proposed model for the relationship between the slow cycling and rapid cycling intestinal stem cells. *The Lrig1^{+} stem cell may be downstream from the quiescent Bmi1^{+}, mTert^{+} or Hopx^{+} stem cells and may have two roles in homeostasis: to protect the niche from stress and to give rise to transit-amplifying cells directly and/or to Lgr5^{+} stem cells when needed. Figure from* (77).

Whilst the intestinal stem cells have been identified and demonstrated by lineage tracing, culture and transplantation experiments in mouse models, the relevance of these stem cell markers to human intestinal stem cell biology and tissue renewal requires further investigation.

1.3 Tissue Renewal

1.3.1 The stem cell niche

Stem cells have the ability to undergo both self-renewal and give rise to progeny that can differentiate into all the cell lineages. This ability is dependent on the microenvironment or niche in which the stem cell resides. The first germline stem cell niches to be defined were in *Drosophila* and *Caenorhabditis elegans* and subsequently adult stem cells and their niches have been identified in the hematopoietic system, epithelial system, neural system and intestinal system (79). There appear to be several types of stem cell niche, a cellular and non-cellular niche as well as a cancer stem cell niche (Fig 1.9). The cellular and non-cellular components interact with each other in the niche environment and are responsible for the maintenance of intestinal stem cell 'stemness' properties as well as the regulation of symmetric and asymmetric cell division. The cancer stem cell niche comprises of stem cells that have acquired mutations which allow these self-renewing, multi-potent, tumour initiating cells to escape the niche regulation.

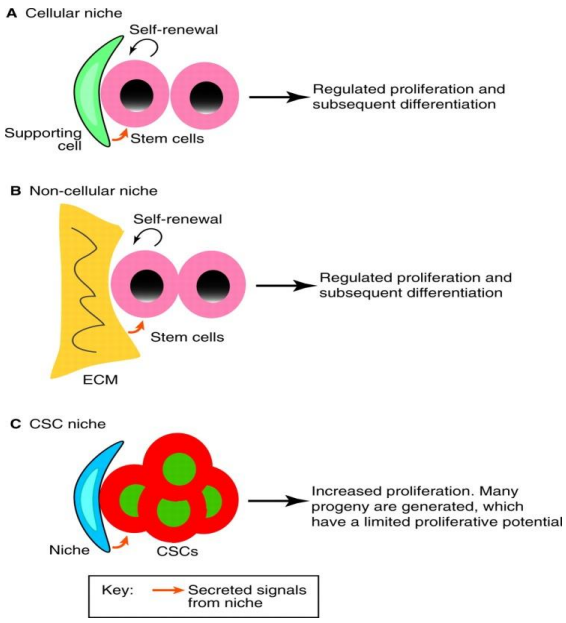


Figure 1.9 Stem cells and their niches. *A) The cellular niche is composed of differentiated cell types that provide cell-cell contact and secreted factors that support and maintain stem cells. B) In the non-cellular niche stem cells reside in a basement*

membrane and the signals that promote self-renewal come from the extracellular matrix. C) Cancer stem cells are the normal stem cells that acquire mutations which allows them the ability to escape the niche regulation. Figure from (79).

The intestinal stem cell niche contains all the cellular and non-cellular components that regulate fate of the stem cells including adjacent epithelial cells, subepithelial myofibroblasts, enteric neurons, endothelial cells, intraepithelial lymphocytes and the basement membrane (26). The subepithelial myofibroblasts are believed to be the most influential mesenchymal cells in the niche since they secrete factors such as Wnt ligands that are important in maintaining the survival of stem cells. The subepithelial myofibroblasts exist as a syncytium, which extends throughout the lamina propria and merges with the pericytes of the blood vessels (80). In the region of the crypts, the myofibroblasts are oval and scaphoid in appearance and appear to overlap like shingles on a roof and are attached one to the other with both gap junctions and adherens junctions. In the upper regions of the colonic crypts and in the small intestinal villi, the myofibroblasts take on a stellate morphology (81). They also secrete Hepatocyte growth factor (HGF), Transforming growth factor β (TGFβ) and Keratinocyte growth factor (KGF), with their receptor located on the epithelial cells and may play a role in colonic tumourigenesis and metastasis (81).

Sato *et al*. (67) show that intestinal stem cells require additional signals in order to grow *in vitro* which may not originate from the epithelium itself. They show that single $Lgr5^+$ cells only grow into organoids in the presence of additional factors such as R-Spondin 1, EGF and Noggin. Noggin has been shown to be derived from stromal tissue suggesting that the factors required for organoid growth originate from the mesenchymal niche (8). Sato *et al*. (34) also demonstrate that co-culture of $Lgr5^+$ stem cells with Paneth cells increased the survival and number of organoids. Paneth cells express EGF, TGFα, Wnt3 and the Notch ligand Dll4 which are all essential signals for stem cell maintenance in culture. The genetic removal of Paneth cells *in vivo* resulted in the loss of $Lgr5^+$ stem cells. Since

Paneth cells are only found in the intestine and proximal colon, it raises the question of what cells take on this role in the distal colon. Rothenberg *et al*. (35) demonstrated that a population of goblet cells that are $cKit^+$ and express Dll1, Dll4 and EGF similar to Paneth cells, support the $Lgr5^+$ stem cells in the colon and promote organoid formation.

The number of stem cells in the niche remains constant and the process regulated possibly through negative feedback. If this homeostasis of stem cell division is altered through the accumulation of genetic mutations, then the stem cells produced through symmetrical division are prevented from exiting the niche or they lose the ability to respond to negative feedback and cause crypt expansion and neoplastic transformation. Stem cells may divide asymmetrically with one stem cell remaining in the niche whilst the daughter cell differentiates into one of the lineages or the stem cells may divide symmetrically and forming two daughter stem cells or two daughter non-stem progenitor cells.

Quyn *et al*. (82) used high-resolution mutiphoton microscopy to determine spindle orientation of dividing cells in the small intestine and colon and compared divisions in the stem cells and transit-amplifying cells of wild-type and *Apc* heterozygous and homozygous mutant human and mouse tissue. They found that in mouse and humans, the cells near the base of the crypts tended to orient their spindles perpendicular to the apical surface of the epithelium, whereas they orientated more parallel above position +7. This orientation correlated with the asymmetric retention of label-retaining DNA. They also found that the preference for perpendicular spindle alignment and asymmetric label retention was lost in precancerous tissue heterozygous for *Apc*, thus suggesting that the loss of asymmetric division in stem cells may contribute to the oncogenic effect of *Apc* mutations in gut epithelium. This perpendicular orientation of stem cell division may cause the generation of unequal daughter cells due to finding themselves in slightly different environments after division, as seen in the *Drosophila* testis where the germ stem cell divides perpendicular to the niche structure called the hub. This ensures that one cell continues as a stem cell attached to the hub, while the other differentiates into a gonial blast (83). However, in the

human and mouse gut epithelium the stem cells span from the basal lamina to the apical lumen and uniformly touch the Paneth cells (84). Therefore, even though the spindle is orientated perpendicular to the epithelial sheet, the daughter cells do not end up in divergent locations. Snippert *et al*. (84) propose that spindle orientation in these stem cells results from special constraints in these flattened polarised cells. By carrying out a fate map of individual stem cell using a generated multicolor Cre-reporter, they found that most $Lgr5^{hi}$ cell divisions occur symmetrically and do not support the model that the two daughter cells adopt divergent fates. Instead their model shows that the stem cells double their numbers each day and stochastically adopt stem or transit-amplifying fates. It could be that following symmetrical division, $Lgr5^{hi}$ cells undergo neutral competition for contact with Paneth cell surface. Once detached, cells lose access to short-range signals that maintain stem cell 'stemness' and progressively differentiate.

Snippert *et al.* (84) have also identified using their model that there are 14 $Lgr5^{+}$ stem cells in the mouse small intestine and Barker *et al*. (4) had previously estimated that their cell cycle time is approximately 1 day. Schepers *et al.* (85) have found that $Lgr5^{+}$ stem cells divide on average every 21.5 hours and contain significant telomerase activity which is gradually lost in their progeny. However, despite the presence of telomere activity in these cells, telomere shortening still occurs, as seen by the average telomere length of $Lgr5^{lo}$ cells being substantially lower than in $Lgr5^{hi}$ cells at all ages of the mouse. They also found that the $Lgr5^{+}$ stem cells randomly segregated newly synthesised DNA strands, which opposes the 'immortal strand' hypothesis in which the stem cells retain the 'old' template DNA strand and transfer the newly synthesised strand to their daughter cells.

1.3.2 Stem cell renewal and progeny lineage specification

Crypt homeostasis is maintained by stem cell proliferation and differentiation which is regulated by several signalling pathways which include Wnt, bone morphogenic protein

(BMP), transforming growth factor β (TGFβ), Notch and Hedgehog (Hh) pathways (Fig 1.10). Wnt signalling plays a major role in maintaining epithelial stem cell fate and progenitor cell proliferation (86). Transgenic and adenoviral expression of Dkk1, an inhibitor of Wnt signalling, causes loss of proliferation and loss of crypts (2) (3). BMP and TGFβ signalling in contrast are involved in inhibition of intestinal stem cell activation and promote intestinal differentiation (8). Notch signalling is involved in cell fate decisions in the colonic epithelium, specifically directing cells towards a secretory lineage (87).

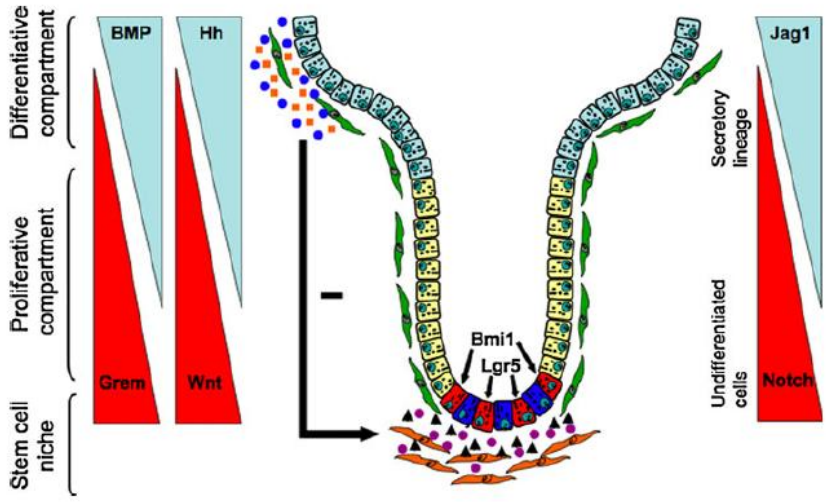


Figure 1.10 Regulatory signals involved in colonic epithelium stem cell renewal and differentiation. *The Wnt protein family and BMP antagonists are expressed in a reciprocal gradient to the BMP and Hh protein families along the crypt axis. The Notch ligand Jagged-1 is expressed in a reciprocal manner to the Notch receptors. Figure from* (26).

Wnt signalling is important in both stem cell self-renewal and effect cell fate determination. It drives cell proliferation through up-regulation of β-catenin target genes such as *c-Myc* and *CyclinD1*. Wnt signals also directly regulate the expression of the transcription factor CDX1 (Caudal-type homeobox protein 1) which plays an important role in enterocyte differentiation. CDX1 induces the expression of alkaline phosphatase, a

marker of intestinal enterocyte differentiation, and regulates the expression of ApoB mRNA editing protein which is located in the villi (26). CDX1 also regulates the expression of the differentiation marker cytokeratin 20 (CK20) by binding to its promoter region (88). Lickert *et al*. (89) show that *Cdx1* was induced in mouse embryonic stem cells by Wnt stimulation and that TCF4-deficient mouse embryos no longer expressed CDX1 protein in the small intestinal epithelium. Beland *et al*. (90) have also found that CDX1 and LEF1, a nuclear effector of Wnt signalling, synergise to induce expression from the CDX1 promoter through LEF/T-cell factor response elements and the physical interaction between the homeodomain of CDX1 and the B box of LEF1 could be the basis of this synergy. Ikeya *et al.* (91) show that among the Wnt family members, Wnt3a appears to regulate CDX1 expression, as the Wnt3a mutants seem to have reduced expression of CDX1. Prinos *et al.* (92) also show that Wnt3a synergises with Retinoic Acid to initiate CDX1 expression during murine development.

Wnt signalling has also been shown the play a role in Paneth cell development. Van Es *et al*. (93) show that the expression of a Paneth gene programme is critically dependent on TCF4, as conditional deletion of the Wnt receptor Frizzled-5 abrogates expression of these genes in Paneth cells. This result is explained by Wnt-dependent expression of EphB3, MMP7 and Cryptdin which are crucial in Paneth cell localisation and maturation.

Sox9, a Wnt-dependent gene is also crucial in Paneth cell maturation as Mori-Akiyama *et al*. (94) found that in the *Sox9* conditional knockout mice no differentiated Paneth cells were present at the bottom of the crypts. The absence of characteristic Paneth cell vesicles in the cell of the crypt bottoms and the lack of expression of Paneth cell markers such as lysozyme, MMP-7, Cryptin-1 and Crypdin-6 verified this result. Sox9 had no role in the differentiation of goblet or enteroendocrine cells. Bastide *et al*. (95) have found that as well as Paneth cell absence, Sox9 mutant mice also had an increase in crypt cell proliferation as well as an increased number of cells expressing c-Myc and CyclinD1,

suggesting that Sox9 may also play a role in negative feedback of the Wnt signalling pathway.

A study by Gregorieff *et al.* (96) found a Wnt responsive gene, termed *Spdef,* is a transcription factor of the Ets family that is involved in the maturation of goblet and Paneth cells. They detected Spdef at around the +4 position just above the Paneth cell compartment, possibly immediate descendents of either $Bmi1^{+}$ or $Lgr5^{+}$ stem cells and that deletion of the *Spdef* gene resulted in major defects in the maturation of both goblet and Paneth cells. At the transcriptional level, they demonstrated that Spdef controls the expression of a subset of Paneth and goblet cell markers such as *Creb314, Ccl6, Hgfac* and *Cryptdins.* Spdef may also be involved in repressing certain Paneth cell genes such as *Mmp7* and *Ang4* in the goblet cell lineage.

The Notch signalling pathway regulates the secretory lineage through its inhibition. Deletion of the downstream component of the Notch pathway transcription factor Math1 (mouse atonal homologue 1), causes depletion of the goblet, Paneth and enteroendocrine cell lineages in the small intestine. This suggests that Math1 is essential for progenitor cell commitment to one of three epithelial adult cell types and that Math1 negative progenitors become enterocytes (97) (Fig 1.11). High levels of Notch switch on the Hes1 (hairy and enhancer of split 1) transcription repressor, which blocks the expression of Math1, leads cells to remain as progenitors and ultimately become enterocytes. Conversely, low Notch expression increases levels of its ligand Delta, which induces Math1 expression by blocking Hes1, leading cells to become goblet, Paneth or enteroendocrine cells (98). Jensen *et al* (99) show that Hes1 null mice have elevated Math1 expression with increased enteroendocrine and goblet cells and fewer enterocytes demonstrating that Math1 regulates the determination of cell fate though Notch/Delta signalling. Inactivation of the Wnt signalling pathway is required for Hath1 (human homologue to Math1) stabilisation through proteosomal destruction by GSK3β. Aragaki *et al*. (100) found that both

constitutive expression of mutant Hath1 and stabilization of Hath1 protein by a GSK3 inhibitor increased the expression of MUC2 , a marker of differentiated goblet cells.

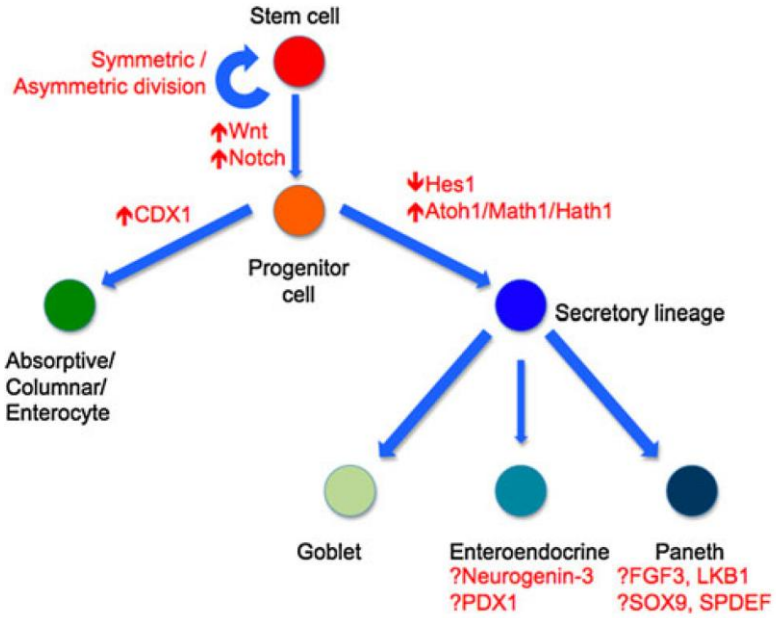


Figure 1.11 Lineage specification of an intestinal stem cell. *Stem cells may divide symmetrically or asymmetrically. Wnt signals maintain the stem-like phenotype of the stem cells, whilst Notch maintains the proliferation of progenitor cells. The intestinal stem cell gives rise to progenitor cells, which can either form enterocytes or the secretory lineage, via inhibition of the Notch pathway, down-regulation of Hes1 and up-regulation of Math1. Candidate genes that determine specification of enteroendocrine and Paneth cells are neurogenin-1 and PDX1 (enteroendocrine) and SOX9 and Spdef (Paneth cells). Figure from* (26)*.*

Both Neurogenin 3 and pancreatic and duodenal homeobox 1 (PDX1) have been implicated in the differentiation of enteroendocrine cells. Lopez-Diaz *et al*. (101) used the mouse villin promoter to drive Neurogenin 3 expression throughout the developing epithelium to measure the affect on cell fate. They found that the Neurogenin 3 expressing transgenics had decreased numbers of goblet cells with an increase in enteroendocrine cells. Ootani *et al*. (102) also show that Neurogenin 3 over-expression leads to enteroendocrine cell differentiation in their murine intestinal spheroids long-term culture in

vitro. PDX1 is a transcription factor involved in pancreatic development and islet cell function and appropriate gene expression in enteroendocrine cells. Yamada *et al*. (103) show that over-expression of PDX1 causes immature rat intestinal epithelial cells to differentiate into enteroendocrine cells.

Tuft cells have a unique signature marker that includes co-expression of SOX9, COX1, COX2, hematopoietic prostaglandin-D2 synthase (HPGDS) and doublecordin-like kinase 1 protein (DCAMKL1). Gerbe *et al.* (29) have found that in contrast to the enteroendocrine cell lineage which depends on Neurog3 function, tuft cells are still produced in the absence of *Neurog3*. $COX1^{low}$ tuft cells that are still in the process of terminal maturation express high levels of SOX9 and never express Neurog3, whereas SOX9 is barely detectable in Neurog3-expressing enderoendocrine precursor cells (104) thus distinguishing them as different cell types. Spdef and Gfi1 are also not essential for tuft cell differentiation (like Paneth cells). However, Atoh1 is essential, as tuft cells are absent in *Atoh1*-defficient mice, thus characterising them as a secretory type (29).

The maintenance of stem cells and progenitor cell lineage specification seems to be mainly driven by Wnt and Notch signalling and Crosnier *et al*. (86) have suggested a model in which these two signalling pathways interact (Fig 1.12). The Wnt signalling pathway drives the expression of Notch pathway components that mediate lateral inhibition within the Wnt-activated (Wnt+) population. Some cells express Delta and escape Notch activation (Notch-) while others fail to express Delta and have Notch activation (Notch+) imposed on them. The (Wnt+, Notch-) cells become committed to a secretory fate and eventually stop dividing whilst the (Wnt+, Notch+) cells continue to divide without differentiating. This generates daughters like themselves that interact with Notch and diversify further. Due to short and long range special signals, some cells have to move further up the crypt axis and lose Wnt activation which leads to their differentiation as enterocytes if Notch was still activated in them at the time of their exit, or as secretory cells if Notch was inactive.

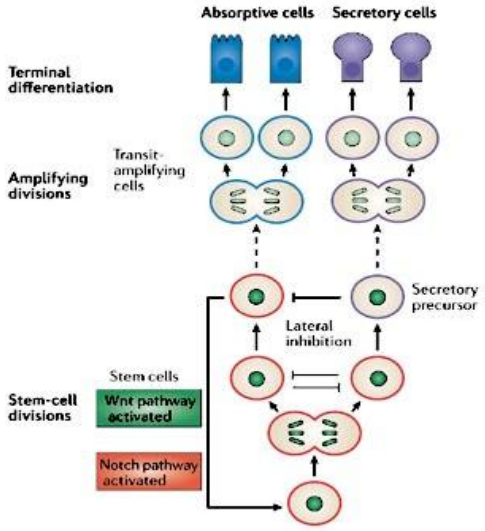


Figure 1.12 Wnt and Notch signalling cooperate to maintain stem cells. *Figure from* (86).

Interactions between Wnt and Hedgehog (Hh) signalling pathways also appear to play an important role in regulating the intestinal stem cell niche. Indian hedgehog (Ihh) is a member of the mammalian hedgehog ligand family and is expressed by differentiated enterocytes. Hedgehog signalling limits the expression of Wnt targets to the base of the crypt and Van Den Brink *et al.* (105) demonstrated that inhibition of Hh signalling using cyclopamine leads to aberrations in epithelial cell differentiation. They also found that transfection of Ihh into colon cancer cells leads to a down-regulation of both components of the nuclear TCF4-β-catenin complex and abrogates endogenous Wnt signalling *in vitro*. Von Dop *et al*. (106) found that constitutive activation of Hh signalling resulted in accumulation of myofibroblasts and colonic crypt hypoplasia. A reduction in the number of epithelial precursor cells was observed with premature development into the enterocyte lineage and inhibition of Wnt signalling. Activation of Hh signalling also resulted in induction of the expression of bone morphogenic proteins and increased BMP signalling in the epithelium. Kosinski *et al*. (107) demonstrated that deletion of Ihh disrupted the intestinal mesenchymal architecture through the loss of the muscularis mucosae,

deterioration of the extracellular matrix, and reduction of crypt myofibroblasts. The epithelial compartment however, had increased Wnt signalling, disturbed crypt polarity and architecture, defective enterocyte differentiation, and increased and ectopic proliferation that was accompanied by increased numbers of intestinal stem cells. Their mechanistic studies revealed that Hh inhibition deregulates BMP signalling, increases matrix metalloproteinase levels, and disrupts extracellular matrix proteins, thereby providing a proliferative environment for intestinal stem cells and progenitor cells.

In addition to Wnt and Notch signalling, BMP signalling has been shown to be important in stem cell self-renewal and maturation of goblet, enteroendocrine and Paneth cells. BMPs are active at the top of the crypts where differentiation occurs (Fig 1.10) and although there is some activity at the crypt base, BMP inhibitors such as noggin and gremlin regulate its levels. He *et al*. (8) have suggested that BMP signalling inhibits intestinal stem cell self-renewal through activation of PTEN, leading to suppression of Wnt/β-Catenin signalling. Overcoming this suppression with transient expression of noggin leads to Akt activation that enhances nuclear β-Catenin activity thus promotes stem cell self-renewal and proliferation. Auclair *et al*. (108) have shown that there was increased cell proliferation and altered intestinal epithelial morphology in *BMPRIA* mutant mice as well as impaired terminal differentiation of cells from the secretory lineage but BMP signalling is not involved with cell fate determination.

TGFβ signalling regulates many aspects of stem cell biology such as stem cell conversion to progenitor/transitional cells and migration of differentiated cells. At the villus tips, TGFβ signalling may be required for apoptosis, thus maintaining the normal size, shape and function of the polarised gut epithelium (109). The linear migration, differentiation and compartmentalisation along the crypt-villus axis is controlled by TGFβ and Wnt gradients, with TGFβ controlling cell polarisation proteins and Wnt controlling the expression of EphB sorting receptors. The presence of TGFβ signalling and the absence of Wnt signalling at the villus compartment results in rapid cell cycle arrest and

differentiation with SMAD 4 and TCF4 being the dominant switch between the proliferative progenitor and the differentiated epithelial cell. In colorectal cancer this switch is permanently reversed, because TGFβ signalling is inactivated whilst TCF4 is constitutively activated by mutations in the Wnt cascade (109).

1.3.3 Migration

Differentiation in the intestinal crypt is a very ordered process. As new daughter cells are produced by the stem cells at the base of the crypt they migrate along the crypt axis to the top of the crypt and differentiate along the way. Eph/Ephrin molecules are involved in maintaining cellular boundaries and establishing migratory paths by segregating cells along the crypt axis (110). Both Ephrins and Eph receptors are membrane bound proteins, restricting their interactions to sites of direct cell-cell contact. The Ephrin/Eph receptor interaction allows bidirectional communication with the signal being conveyed in both the receptor-expressing as well as in the ligand-expressing cell (111). These Eph/Ephrin molecules have been identified as Wnt target genes and the EphB receptors and Ephrin-B ligands are regulated via the β-Catenin/TCF transcription complex (Fig 1.13).

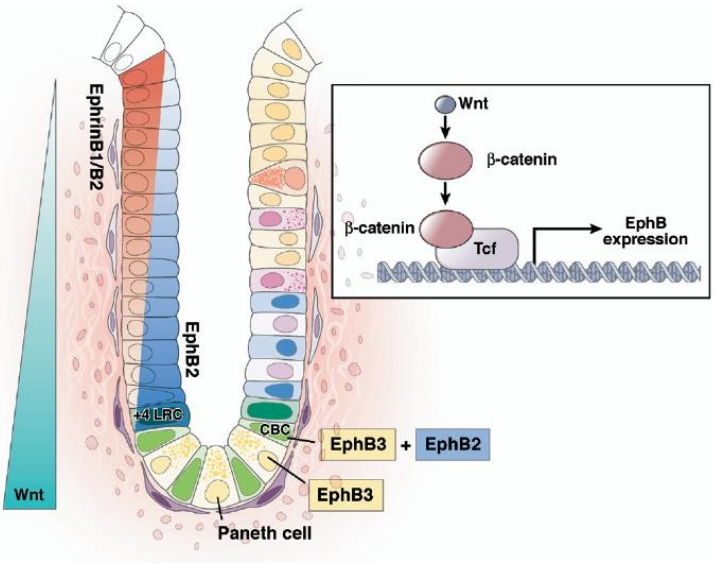


Figure 1.13 Migration within the intestinal crypt is regulated by the interaction of EphB receptors and Ephrin-B ligands. *A gradient of EphrinB1/B2 ligands exists along the crypt axis with high levels being expressed at the top of the crypt and an opposing gradient of EphB2 expression at the bottom of the crypt. Figure from* (112).

A gradient of EphB receptors and Ephrin-B ligands exists along the crypt axis which mirrors the Wnt gradient. The level of Ephrin-B ligand and Wnt-induced EphB expression determines cell location. EphB2 is not found on Paneth cells but is expressed on crypt base columnar cells and decreases in expression toward the top of the crypt. EphB3 however, is expressed on both the crypt base columnar cells and Paneth cells. Ephrin-B1 and Ephrin-B2 are expressed on differentiated cells at the top of the crypt and decrease toward the base of the crypt with Paneth cells having a complete absence of Ephrin-B ligand expression (110). This differential expression specifies the position of the crypt cells because of the repellent effects of EphB/Ephrin-B interaction. Downward migration is prevented due to decrease of EphB expression and increase of Ephrin-B ligand expression as cells move away further from the crypt base and the Wnt source. Also, since Paneth cells do not express Ephrin-B ligands and only EphB3 receptor, their upward migration is prevented (110).

Holmberg *et al*. (111) found by gain- and loss-of-function experiments that Ephrin-B ligands and Eph receptors, independently of their influence on cell positioning, also promote proliferation in the crypts of the small intestine and colon and account for about 50% of the mitogenic activity. EphB2 and EphB3 kinase-dependent signalling promoted cell cycle re-entry of intestinal progenitor cells, thus establishing that Ephrins and Eph receptors are key coordinators of migration and proliferation in the intestinal stem cell niche. Batlle *et al*. (113) show that most human colorectal cancers lose expression of EphB at the adenoma-carcinoma transition and the loss of EphB expression strongly correlates with degree of malignancy. Furthermore, reduction of EphB activity accelerates tumorigenesis in the colon and rectum of Apc(Min/+) mice, and results in the formation of

aggressive adenocarcinomas, demonstrating that loss of EphB expression represents a critical step in colorectal cancer progression. Cortina *et al*. (114) also show that EphB receptor compartmentalise the expansion of colorectal cancer cells through a mechanism dependent of E-cadherin-mediated adhesion. They demonstrate that EphB-mediated compartmentalisation restricts the spreading of EphB-expressing cancer cells into Ephrin-B1 positive areas possibly through the silencing of EphB expression by the cancer cells to avoid repulsive interactions. Batlle *et al*. (113) observed that the *EphB2*, *EphB3*, and *EphB4* genes were coordinately silenced in the majority of colorectal cancer samples.

1.3.4 Shedding

Homeostasis of cell renewal in the intestinal crypt is maintained by matching the number of dividing cells with the number of cells shed at the top of the crypt. The epithelium of the crypt acts as a barrier which needs to be maintained even when cells are shed at the top. When apoptosis is triggered, dying cells are extruded from the epithelium in order to preserve this functional barrier. Extrusion occurs by apoptotic cells signalling to the surrounding epithelial cells to contract and form an actomyosin ring that squeezes the dying cell out of the epithelium (115).

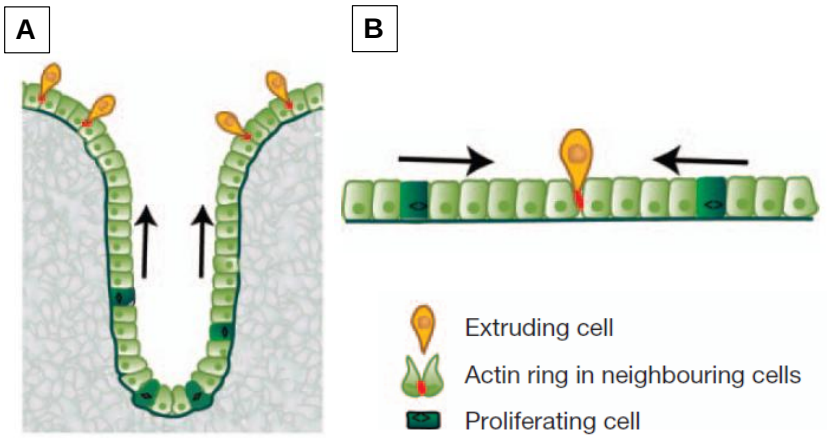


Figure 1.14 Cell shedding at regions of high crowding during homeostasis and development in A) human colonic epithelium, B) confluent MDCK monolayers. *Cells*

destined to die signal to surrounding cells to contract an actomyosin ring that squeezes the dying cell out. Arrows indicate direction of force from mitosis and migration. Figure from (115).

The signal produced by dying cells to initiate this process is sphingosine-1-phosphate (S1P) which triggers Rho-mediated contraction to squeeze the dying cell out. Decreasing S1P synthesis by inhibiting sphingosine kinase activity or by blocking extracellular S1P access to its receptor prevents apoptotic cell extrusion. Extracellular S1P activates extrusion by binding the S1P(2) receptor in the cells neighbouring a dying cell, as S1P(2) knockdown in these cells disrupted cell extrusion (116). Gu *et al*. (116) suggest that the S1P pathway may also be important for driving delamination of stem cells during differentiation or invasion of cancer cells. Eisenhoffer *et al*. (115) show that overcrowding due to proliferation and migration induces extrusion of live cells to control epithelial cell numbers. They simulated overcrowding of cells by growing MDCK cells to confluence on a silicone membrane stretched to 28% of it original length and then released it from stretch. By 6hrs after crowding the number of cells equilibrated to pre-release levels, showing that the MDCK epithelia eliminated cells to achieve homeostatic cell numbers. To investigate signals that might regulate live cell extrusion after overcrowding they tested carboxy-terminal JUN kinase (JNK) and stretch-activated channels which are both activated by stress. They found that JNK inhibitor blocked apoptotic extrusion in response to ultraviolet-C but not live cell extrusion after overcrowding or homeostatic cell turnover. Inhibiting stretch-activated ion channels however, significantly reduced the percentage of both apoptotic and non-apoptotic extrusion events after overcrowding or during epithelial homeostasis. Therefore they suggest that JNK controls apoptosis-induced extrusion, stretch-activated signalling controls live cell extrusion during homeostasis that is induced by overcrowding, possibly upstream of S1P signalling.

Tight junctions, known as *zonula occludens* must be disturbed in order for a cell to be shed. Madara *et al*. (117) propose that lamellipodia from neighbouring cells extend

beneath the shedding cell and form a tight junction which then rises behind the shedding cell. Tight junction elements proliferate between extruding cells and their neighbours and appear to move down the lateral margin of the extruding cell as it extends into the lumen. These observations suggest that newly formed junctional elements "zipper" the epithelium closed as extrusion proceeds therefore preventing epithelial discontinuities from occurring. The tight junction is a complex and multifunctional structure consisting of integral membrane molecules, occludin, claudins and junction adhesion molecule. Occludin and the claudins are tetraspanin proteins with two extracellular loops and are considered to form the variable permeability barrier between the luminal and interstitial spaces separated by the epithelium. Tight junction plaque proteins such as ZO1, ZO2, and ZO3 link the integral proteins to the actin cytoskeleton. They also interact with a diverse group of signalling molecules that connect tight junction function to paracellular permeability, cell division, cell polarity and tumorigenesis (118).

The control of cell death is an important regulator of cell number and susceptibility to neoplastic transformation. Apoptosis following the loss of cell anchorage (anoikis) is a form of programmed cell death that is central to homeostasis in the intestinal epithelium. Cellular inability to undergo apoptosis by damaged and mutated cells may prevent cells from entering the apoptotic pathway and instead survive, divide and expand, increasing the risk of colorectal cancer. An apoptotic cell has distinct morphology during the death process, starting with the cell cytoplasm shrinking and the cell detaching from its neighbours. The nucleolus then disappears and the chromatin becomes condensed around the nuclear membrane, with the cell membrane taking on “blebbed” appearance. The cell and its nucleus fragment into smaller membrane-bound vesicles which become phagocytosed by neighbouring cells (119). Apoptosis is initiated through activation of intrinsic pathways in response to intracellular signals or by an extrinsic pathway responsive to extracellular events. Intrinsic apoptosis occurs following DNA damage or toxic stress, in which mitochondria release cytochrome C from their intramembrane space.

Cytochrome C binds to apoptotic protease-activating factor (Apaf-1), promoting the activation of cysteine-activated proteases (caspases), which target several proteins essential for cellular functions. Mitochondrial membrane permeability is controlled by both pro-apoptotic (*bax, bad, Bclx$_s$, bak, bid*) and anti-apoptotic (*Bcl-2, Bclx$_L$, mcl1, bag-1, bfl-1, brag-1, Bcl-w*) members of the Bcl-2 family of proteins, all of which contain at least one conserved Bcl-2 domain (BH). Bax and Bad oligomerise with additional pro-apoptotic BH-3-motif-containing proteins to stimulate cytochrome C release. On the outer mitochondrial membrane Bcl-2 and Bclx$_L$ can bind to these BH-3 motif-containing proteins and inhibit apoptosis. The formation of stable Bcl-2 heterodimers prevents apoptosis by blocking proteins release from the mitochondria. Apoptosis is also regulated by inhibitors of apoptosis proteins through selective inhibition of specific caspases, allowing the inhibition of both intrinsic and extrinsic pathways. Extrinsic apoptotic signalling occurs after activation of death receptors such as tumour necrosis factor receptor -1 (TNFR-1) and Fas. Stimulation of receptors signalling pathways triggers caspase activation and subsequent steps in regulating mitochondrial permeability (120).

Potten *et al*. (119) have found that spontaneous cell apoptosis also occurs in the lower regions of the crypt. When measured on a positional basis, these cells appeared to be restricted to the stem cell region with 10% of these being apoptotic cells. They suggest that this spontaneous apoptosis may be an inherent part of the regulatory mechanism determining stem cell numbers in normal adult epithelium. The tight control of stem cell number is fundamental in maintaining a stable crypt size. Unlike in the small intestine, spontaneous apoptosis in the proliferative region of the colon is a rare event and rather than just being restricted to the stem cell region, these apoptotic cells are scattered throughout the crypt. This difference in spontaneous apoptosis between the small intestine and colon may explain the difference in colon and small intestinal cancer incidence. If the small intestine is able to remove excess stem cells, whereas the colon is not, the increased

number of (mutated) stem cells may result in hyperplastic crypts susceptible to transformation (119).

1.3 Wnt signalling

1.3.1 Role in tissue renewal

Wnt signals play an important role in the development and renewal of the colonic epithelium. The Wnt pathway is involved in several different processes such as maintaining stem/progenitor cells via cell cycle control and inhibition of differentiation, controlling migration and localization of epithelial cells along the crypt axis and directing early secretory lineage development as well as terminal differentiation of Paneth cells (121). The term 'Wnt' was introduced 20 years ago and fused the names of two orthologous genes: *Wingless (Wg)*, a *Drosophila* segment polarity gene and *Int-1*, a mouse protooncogene (122). Wnt ligands are defined by amino acid sequence rather than by their functional properties and as many as 19 mammalian Wnt homologues are expressed in temporal-spacial patterns (123). All Wnts include a signal sequence for secretion, several highly charged amino acid residues and many glycosylation sites. Wnt ligands are hydrophobic and are mostly found associated with cell membranes and the extracellular matrix (124). Wnt ligands are lipid modified by the attachment of a palmitate on the first conserved cysteine residue and on a serine in the middle of the protein. Palmitoylation of Wnts by Porcupine is necessary for their glycosylation which aids in Wnt transport between cells, as glycosylation increases Wnt interaction with heparin sulphate proteoglycans that are present on the surface of Wnt responding cells. Wnt ligands are secreted by the subepithelial myofibroblasts that are located adjacent to the stem cells and genetic screens have identified the multipass transmembrane protein Wntless (Wls)/Eveness interrupted (Evi) to be required in the secretory pathway to promote the release of Wnts from these cells (125). Sato *et al.* (34) have also shown that Paneth cells are a source of Wnt3 and restrict stem cells to the base of the crypt, and that deletion of the Paneth cells decreased the number of stem cells in the crypt.

B-catenin is an essential cytoplasmic signal transducer of the canonical Wnt signalling pathway (Fig 1.15). The levels of cytoplasmic β-catenin are normally controlled by a destruction complex that targets β-catenin for degradation in proteasomes. This complex is assembled over the scaffold component axin or its homologue conductin, and contains the tumour suppressor adenomatous polyposis coli (APC), the glycogen synthase kinase 3β (GSK3β) and casein kinase 1α/ε (CK1α/ε) which are the binding domains for β-catenin (126). Upon β-catenin binding to the destruction complex, CK1α/ε phosphorylation of Ser 45 at the N-terminus of β-catenin and the subsequent Ser/Thr phosphorylations by GSK3β result in its ubiquitination and proteosomal degradation by β-TrcP, an F-box containing E3 ubiquitin ligase. Within the nucleus, lymphoid enhancer factor /T cell factor (LEF/TCF) transcription factors remain bound to co-repressors such as Groucho and suppress Wnt pathway target genes (112). Wnts are glycoproteins whose secretion is controlled specifically by the transmembrane protein Wntless/evenness interrupted (125). The production of active Wnt requires also a functioning retrometer, a multiprotein complex involved in intracellular protein trafficking. Wnt ligands initiate Wnt signals through the engagement of the Wnt receptor complex composed of a Frizzled receptor (Fz1-10) and a low-density lipoprotein-related protein co-receptor (LRP5 or LRP6) which results in the inactivation of the β-catenin destruction complex. Dishevelled, a protein that can bind the cytoplasmic tail of Fz receptors and axin facilitates the recruitment of axin to the LRP co-receptor, thereby removing the scaffold and destabilising the β-catenin destruction complex. The interaction of Wnts with Frizzled receptors can be modulated by a number of secreted factors that act as antagonists by binding to either Wnts or the co-receptor LRP5/6. The stabilised β-catenin translocates to the nucleus where it binds LEF-TCF transcription factors and displaces the co-repressor Groucho. The β-catenin-LEF/TCF complex then drives the transcription of Wnt target genes and is tightly regulated by factors such as Bcl9, Pygopus, mixed-lineage leukaemia histone methyltransferases, APC, Chibby, inhibitor of β-catenin (iCAT), C-terminal-binding protein (CtBP) and components of chromatin-remodelling complexes (126). Targets of Wnt/TCF/LEF regulated

transcription include the proto-oncogene myc, cyclooxygenase 2 (COX2), matrilysin/MMP7, Cyclin D1 and TCF1. Activation of the Wnt signalling pathway also affects the expression of BMP6, a member of the TGFβ superfamily (127).

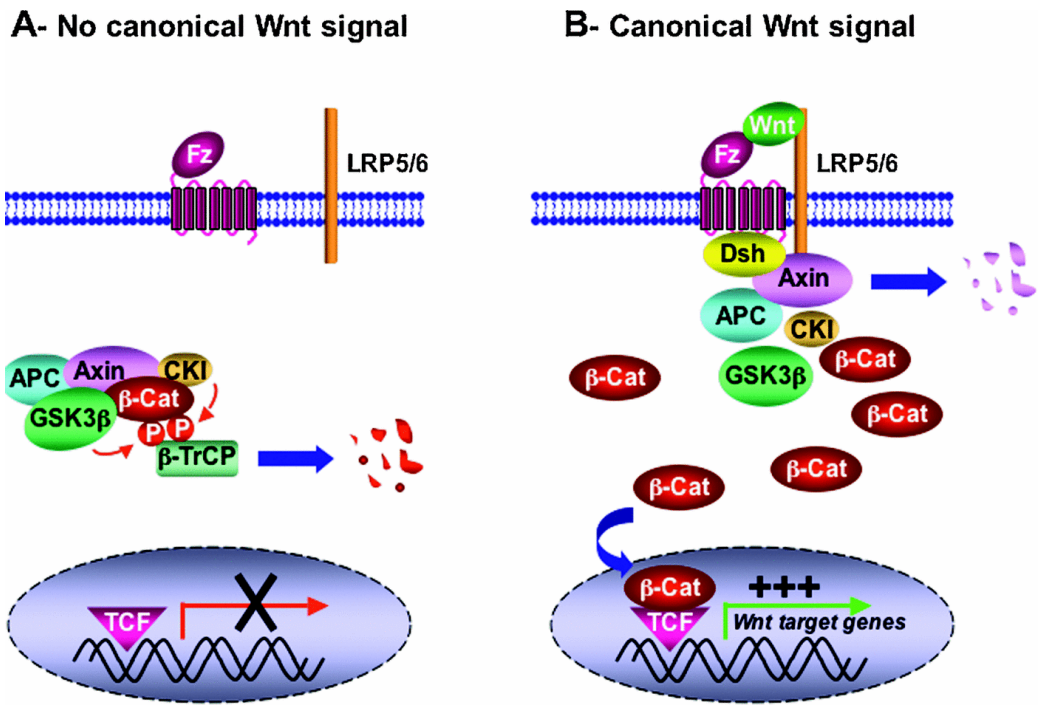


Figure 1.15 Canonical Wnt signalling pathway. *In the absence of Wnt ligands β-catenin is phosphorylated and targeted for degradation by the degradation complex containing Axin, APC, GSK3β and CK1. Upon Wnt ligand binding to Frizzled and LRP transmembrane receptors, the cytoplasmic protein Dishevelled is activated and blocks the action of the degradation complex. β-catenin is then able to accumulate and translocate to the nucleus, where it drives the transcription of Wnt target genes aided by TCF/LEF transcription factors. Figure from* (128).

APC is a large protein (312kDa) known to interact with at least 10 protein partners including β-catenin, axin, EB-1 and DLG. APC has multiple diverse functions in cell migration and adhesion, cell cycle regulation and chromosome stability (122). Although *APC* is mutated in 85% of familial and sporadic colorectal cancers, it is not absolutely necessary for the proper functioning of the β-catenin degradation complex. Nakamura *et al*. (129) show that over-expression of axin can compensate for the absence of functioning

APC. In addition to its structural role in the β-catenin degradation complex, APC also captures and escorts nuclear β-catenin to the cytoplasmic destruction machinery (130). Inclusion of APC in the β-catenin degradation complex likely results in an improved presentation of β-catenin to GSK3β, leading to more efficient phosphorylation and subsequent destruction (122).

The ubiquitious protein phophatase 1 (PP1) has been identified by Luo *et al.* (131) as a conserved positive component in the Wnt/β-catenin signalling pathway. PP1 controls Wnt signalling through interaction and regulated dephosphorylation of axin. Inhibition of PP1 leads to enhanced phosphorylation of specific sites on axin by CK1. Axin phosphorylation enhances the binding of GSK3β, leading to a more active β-catenin destruction complex. Wnt-regulated changes in axin phosphorylation, mediated by PP1, may therefore determine β-catenin transcriptional activity (131).

The tumour suppressor Wilms Tumour gene on the X chromosome (WTX) has been identified by Major *et al.* (132) as another component of the β-catenin degradation complex. WTX is in the complex with axin, APC, β-catenin and β-Trcp, with WTX binding directly to β-catenin and β-Trcp which then promotes β-catenin ubiquitination and degradation. Grohmann *et al.* (133) have also identified WTX as an APC-interacting protein. It recruits APC to the plasma membrane away from microtubules through the binding of WTX to phosphatidylinositol (4, 5)-biphosphate (PIP2). Depletion of WTX via siRNA reduces APC protein level in the cell and promotes APC distribution to microtubule ends.

β-catenin is the mammalian orthologue of the *Drosophila* Armadillo (Arm) protein and was originally identified as a component of the adherens junctions, where it links E-cadherin to α-catenin and the actin microfilament network of the cytoskeleton (134). A large part of the β-catenin protein is taken up by 12 tandemly arranged imperfect residue repeats called Arm repeats that mediate protein-protein interactions with cadherins, APC,

axin and TCF (135). β-catenin's Arm repeats facilitate its docking at the nuclear envelope and nuclear accumulation of the protein (134) (122). In the nucleus, β-catenin does not bind DNA itself but is an essential cofactor of the TCF/LEF transcription factors. The acetyltransferase CBP (CREB-binding protein) acetylates β-catenin which improves transctivation at the *c-Myc* locus and allows better access to promoter sequences (136). Fevr *et al*. (137) generated a mouse model using a tamoxifen-inducible variant of the cre recombinase (creERT2) expressed under the control of the intestinal villin promoter and found that inactivation of β-catenin leads to a rapid loss of intestinal epithelial cells and crypt structures. Ireland *et al*. (138) found that deletion of β-catenin in the small intestine caused crypt ablation, increased apoptosis, depleted numbers of goblet cells, and detachment of villus absorptive cells from the villus core as intact sheets. Activation of mutations in β-catenin and inactivation of mutations in *Apc* leads to intestinal hyperplasia (139). Wetering *et al*. (140) show that disruption of β-catenin/TCF4 activity in CRC cell lines using dnTCF4 induces a rapid G1 arrest and blocks a genetic program that is physiologically active in the proliferative compartment of colonic crypts and a differentiation program is induced instead. The TCF4 target gene c-Myc plays a central role in this switch by direct repression of the $p21^{CIP1/WAF1}$ promoter. Following disruption of β-catenin/TCF4 activity, the decreased expression of c-Myc releases $p21^{CIP1/WAF1}$ transcription, which then mediates G1 arrest and differentiation. Muncan *et al.* (141) have found that deletion of the transcription factor c-Myc resulted in rapid loss of crypts and decreased cell numbers in crypts that remained.

Gregorieff *et al.* (142) examined the expression patterns of all Wnts, Frizzleds, LRPs, Wnt antagonists and TCFs in the mouse small intestine and colon and adenomas using *in situ* hybridisation. Figure 1.16 shows a summary of their findings. Of the 19 Wnt genes tested, 7 Wnts were readily detected in the intestine, although *Wnt2b* and *Wnt3* were not found in the colon. In the small intestine, *Wnt3* was restricted to the very bottom of the crypts where Paneth cells reside as was *Wnt9b* (also termed *Wnt14b*). *Wnt9b* was also

detected in the epithelial progenitor cells above the Paneth cell compartment but in the colon *Wnt9b* was localised throughout the colonic epithelium. *Wnt6* was expressed throughout the crypts of both the small intestine and colon and strongly expressed in adenomas. Several Wnts were also found in specific compartments of the mesenchyme, with *Wnt5a* found abundant in the villus tips and weaker towards the crypt-villus junction of the small intestine and in the colon *Wnt5a* and *Wnt5b* were restricted to the mesenchyme beneath the surface epithelium. *Wnt4* was uniformly expressed along the villus mesenchyme and in the colon was also restricted to the mesenchyme beneath the surface epithelium. *Wnt2b* was strongly expressed in the mesenchymal layer of the villi but in the colon *Wnt2b* seemed to mark endothelial or smooth muscle cells.

The expression of Frizzled receptors 5 and 7 was found in the epithelial cells at the bottom of the crypts whilst *Fz4* was restricted to the differentiated epithelial cells of the villi. *Fz6* was uniformly expressed throughout the epithelium of the small intestine and colon. The co-receptors *LRP5* and *LRP6* were found to be expressed in the proliferative epithelial cells of the crypts.

The functional counterparts of the Frizzled receptors are a family of secreted factors called sFRPs. Both Fzs and sFRPs share an equivalent Wnt-interacting cysteine-rich domain which allows sFRPs to compete with Fzs and antagonise Wnt signalling (142). In the small intestine and colon Gregorieff *et al*. (142) found abundant levels of *sFRP1* in the mesenchymal cells immediately adjacent to the crypts. Expression of *sFRP5* was found in cells located immediately above the Paneth cell compartment.

Another secreted factor that can inhibit Wnt signalling by binding directly to Wnts is WIF (Wnt-interacting factor) which was only detected in adenomas by Gregorieff *et al.* (142) and *Dkk2* which binds to the LRP co-receptors was also detected in adenomas. *Dkk3* was weakly expressed in the villus mesenchyme and up-regulated in adenomas, whilst *Dkk1* and *Dkk4* were not detected at all in the intestine.

The expression of TCF family members was also examined by Gregorieff *et al*. (142) who found that *TCF4* was abundant in differentiated cells of the surface epithelium and the expression diminished in the lower half of the crypts. *TCF1* expression was strongly up-regulated in adenomas and transcripts were found in infiltrating gut lymphocytes, gut-associated lymphoid tissue and Payer patches, suggesting a role of *TCF1* in thymocyte development. *TCF3* on the other hand was expressed in the proliferative compartment of the colon only, whilst *LEF* (lymphoid enhancer factor) was detected in intestinal polyps but absent in normal epithelium.

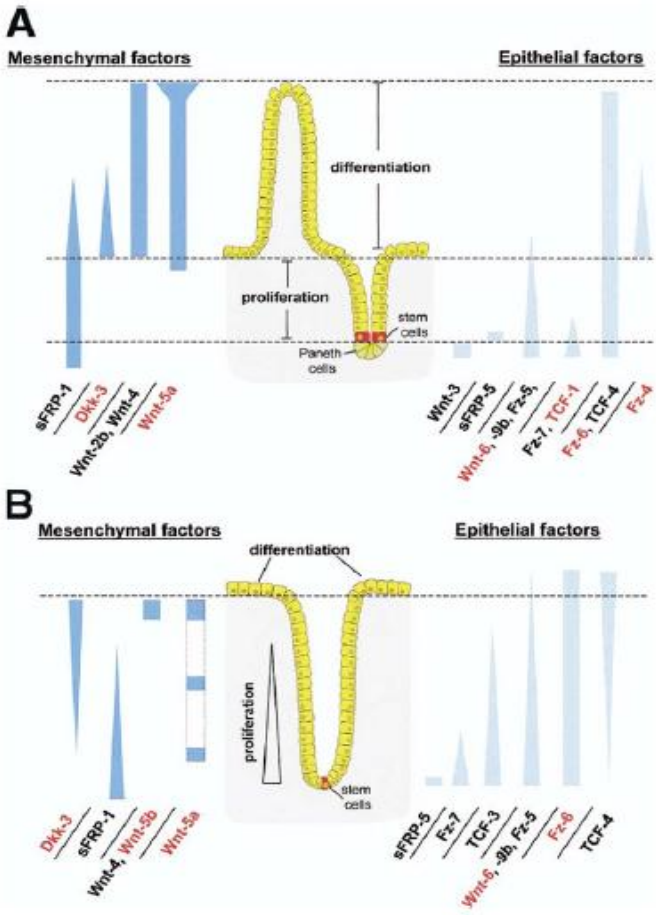


Figure 1.16 Expression pattern of Wnt signalling components in mouse A) small intestine and B) colon. *Figure from* (142).

Holcombe *et al.* (127) also assessed the expression of Wnt ligands and Frizzled receptors in the colonic mucosa, but in the humans not mouse. They found abundant expression of

Wnt1, Wnt4, Wnt5b, Wnt6, Wnt7b and *Wnt10b* in both normal and malignant tissue. As also seen in mouse, *Wnt2* was absent in normal colonic crypts and villi but was expressed in malignant tissue. *Wnt5a,* as in the mouse was also detected in the normal colon with slightly higher expression at the base of the crypts. The expression of *Fz1* and *Fz2* was also assessed but none could be detected in normal colonic mucosa, in contrast to the cancer tissue, where their expression was readily detectable especially at the invasion front. A few years later, Holcombe *et al*. (143) also looked at the expression of Wnt antagonists in human normal and malignant colonic tissue. They found that *Wif1* was expressed in most normal and malignant tissue samples with higher expression at the base of the crypts. *Dkk2* and *Dkk3* exhibited similar expression patters as *Wif1* and *Dkk1* was not expressed in either the normal or malignant tissue mirroring the results by Gregorieff *et al*. Both of these studies by Gregorieff *et al.* (142) and Holcombe *et al*. (127) demonstrate the localization of Wnts and their downstream effectors and inhibitors and suggest that Wnt signalling has a wide role in gut development and homeostasis.

The extracellular antagonists of the Wnt signalling pathway can be divided into two broad classes which prevent ligand-receptor interaction. The sFRPs and WIF primarily bind to Wnt proteins whilst members of the Dkk family bind to one unit of the Wnt receptor complex (144). There are presently 8 known members of the sFRP family (sFRP 1-5, Sizzled, Sizzled2 and Crescent). The cysteine-rich domains of the sFRPs which lie in the N-terminal half of the protein share 30-50% sequence similarity with those of Fz proteins may be the mechanism by which by SFRPs antagonise Wnt signalling. Hypermethylation of the *SFRP* (1,2,4,5) promoters occurs at a high frequency in colorectal carcinomas possibly due to the tumour cells shutting down the expression of sFRPs because these proteins may promote apoptosis (145) (144). Wif1 was first identified as an expressed sequence tag from the human retina (146). Although Wif1 does not share any similarities with the cysteine-rich domains of Frizzled or sFRPs, Wif1 binds to *Drosophila* Wingless and *Xenopus* Wnt8 in the extracellular space and inhibit Wnt8-Fz2 interactions (146). He

et al. (147) and Taniguchi *et al*. (148) have shown that *Wif1* is silenced by promoter hypermethylation in gastrointestinal cancers. The Dkk family comprises of 4 members (Dkk 1-4) and a unique Dkk3-related protein called Soggy (Sgy). Dkk prevents activation of the Wnt signalling pathway by binding to LRP5/6 rather than Wnt proteins. In addition to LRP5/6, Dkk1 interacts with the single-pass transmemrane proteins Kremen 1 (Krm1) and Kremen 2 (Krm2). Krm, Dkk1 and LRP6 form a ternary complex that disrupts Wnt/LRP6 signalling by promoting endocytosis and removal of the Wnt receptor from the plasma membrane (149). Koch *et al*. (150) found that reduced expression of Dkk1 increased proliferation of epithelial cells and lengthened crypts in the colon and was associated with increased transcriptional activity of β-catenin. Crypt extension was particularly striking when Dkk1 was inhibited during acute colitis. This suggests that depletion of Dkk1 induces a strong proliferative response that promotes wound repair after colitis. Pinto *et al*. (3) find that transgenic expression of the Wnt inhibitor Dkk1 results in greatly reduced epithelial proliferation and absence of secretory cell lineages. Disrupted intestinal homeostasis was reflected by an absence of nuclear β-catenin, inhibition of c-Myc expression, and subsequent up-regulation of $p21^{CIP1/WAF1}$. Adenoviral expression of Dkk1 markedly inhibited proliferation in small intestine and colon, accompanied by progressive architectural degeneration with the loss of crypts, villi, and glandular structure (2).

As well as Wnt ligands, The Wnt signalling pathway can also be activated by the R-Spondin family of secreted ligands. Nam *et al.* (151) demonstrated that R-Spondins are novel ligands for the Frizzled 8 and LRP6 receptors. To gain insight into their biological functions, the RNA expression pattern of the mouse R-Spondin family genes was analyzed during mouse development and showed that R-Spondin gene transcripts were widely expressed with distinct patterns in mouse at different developmental stages. Lau *et al*. (5) found that R-Spondins are also the ligands for the Lgr5 receptor as well as its homologues Lgr4 and Lgr6. Each of the four R-Spondins can bind to Lgr4, Lgr5 and Lgr6. The

removal of Lgr4 did not affect Wnt3a signalling, but abrogated the R-Spondin 1 mediated signal enhancement which can be rescued by the re-expression of Lgr4, Lgr5 or Lgr6. No rescue was seen with Lgr1, Lgr7 and Lgr8. It also reduced the proliferation in intestinal crypts and led to crypts being disconnected from the epithelium. The combined loss of Lgr4 and Lgr5 aggravated this phenotype, severely disrupting crypts and halting villus repopulation, leading to the eventual death of the mouse (152). Carmon *et al*. (6) have also demonstrated that Lgr4 and Lgr5 bind the R-Spondins with high affinity and mediate the potentiation of Wnt signalling by enhancing Wnt-induced LRP6 phosphorylation (Fig 1.17). This could be through the R-Spondin-Lgr complex enhancing the internalisation of the frizzled-Wnt-LRP6 signalosome into multivesicular endosomes, leading to enhanced LRP6 phosphorylation.

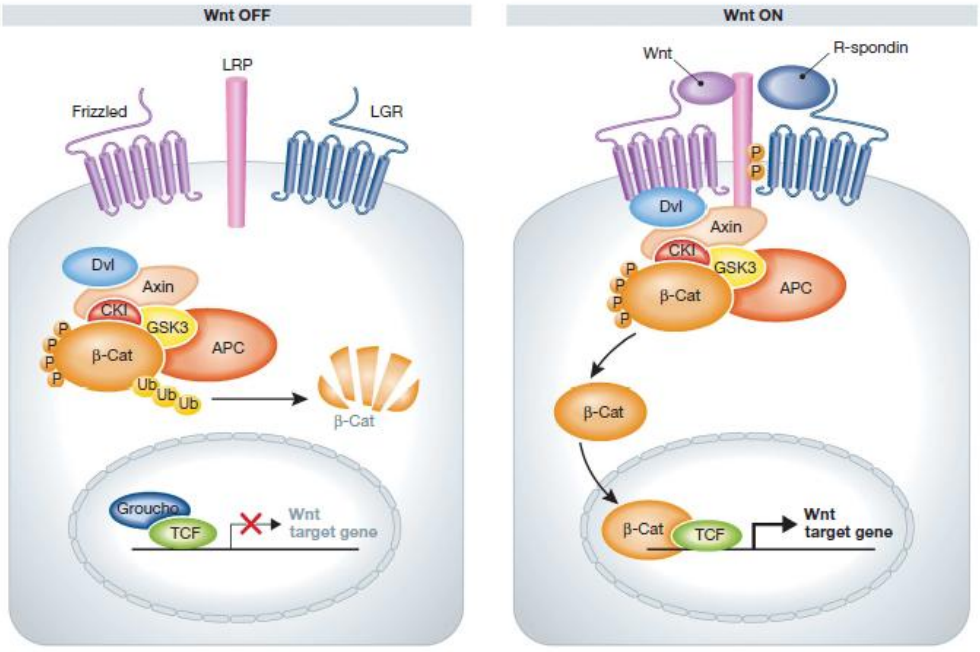


Figure 1.17 R-Spondin-LGR interaction at the cell surface. *LGR homologues interact with Frizzled/LRP5/6 receptors and bind with R-Spondins to enhance Wnt signalling. Figure from* (152).

Non Canonical Wnt signalling

Non canonical Wnt signalling is involved in cell movement and tissue polarity which overlaps with the planar cell polarity pathway described in *Drosophila*. Non canonical Wnt signals are transduced through the Frizzled family receptors but do not involve β-catenin, LRP or TCF molecules (153) (Fig 1.18). Instead it leads to the activation of the small GTPases RHOA (RAS homologue gene-family member A) and RAC1, which activate the stress kinase JNK (Jun N-terminal kinase) and ROCK (RHO-associated coiled-coil-containing protein kinase 1) and leads to remodelling of the cytoskeleton and changes in cell adhesion and motility. Canonical β-catenin signalling can be also be inhibited by the planar cell polarity pathway. Wnt-Ca^{2+} signalling is mediated through G proteins and phospholipases which leads to transient increases in cytoplasmic free calcium that subsequently activate the kinases PKC (protein kinase C) and CAMKII (calcium calmodulin mediated kinase II) and the phosphatase calcineurin. The activation of PLC (phospholipase C) by Dishevelled leads to the cleavage of PtdIns(4,5)P_2 (phosphatidylinositol-4,5-bisphosphate) into $InsP_3$ (inositol trisphosphate) and DAG (diacylglycerol). DAG, together with calcium, activates PKC, whereas $InsP_3$ binding to receptors on the membranes of intracellular calcium stores leads to a transient increase in cytoplasmic free calcium, often also triggering an increase from extracellular stores (154).

Wnt5a is one of the most investigated non-canonical Wnt ligands and its role in cancer development is emerging. It is found to be down-regulated in colorectal cancer, neuroblastoma, breast cancer and leukaemia. Since Wnt5a can inhibit the effects of canonical Wnt signalling, its down-regulation would be an advantage to cancers driven by canonical Wnt signalling. However, Wnt5a over-expression has also been identified in other cancers such as gastric, pancreatic and prostate suggesting that its role depends on the stage of cancer progression and type of cancer (155).

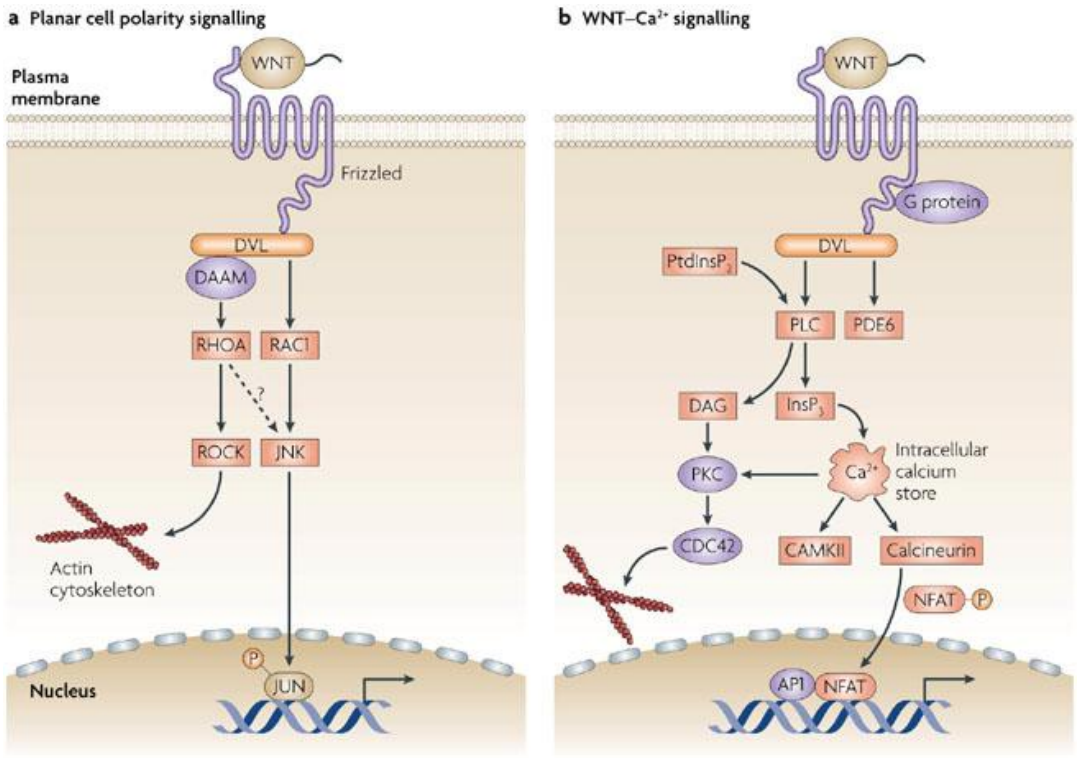


Figure 1.18 Non-canonical Wnt signalling pathways. *A) Non canonical Wnt signals are transduced through the Frizzled family receptors which lead to the activation of RHOA and RAC1 which activate the stress kinase JNK and ROCK and leads to remodelling of the cytoskeleton and changes in cell adhesion and motility. B) Ca^{2+} signalling is mediated through G proteins and phospholipases and leads to transient increases in cytoplasmic free calcium that subsequently activate the PKC and CAMKII and calcineurin. Activation of PLC by DVL leads to the cleavage of PtdIns(4,5)P_2 into $InsP_3$ and DAG. $InsP_3$ binding to receptors on the membranes of intracellular calcium stores leads to a transient increase in cytoplasmic free calcium which often also triggers an increase from extracellular stores. Figure from* (154).

1.3.2 Wnt signalling and cancer

About 90% of all colorectal cancers will have an activating mutation of the canonical Wnt signalling pathway, ultimately leading to the stabilisation and accumulation of β-catenin in the nucleus of the cell. These mutations lead to early premalignant lesions in the intestine,

such as aberrant crypt foci and small polyps (122). Mutations of APC were first identified in patients with familial adenomatous polyposis (FAP). These patients develop hundreds of polyps in the colon after inactivation of the remaining wild-type allele. Although FAP is quite rare, mutations of APC account for up to 85% of all sporadic colorectal cancers. Mutations in the gene encoding β-catenin (*CTNNB1*) mostly account for the remaining 10% of colorectal cancers. The majority of these mutations are due to insertions, deletions and nonsense mutations that lead to frameshifts and/or premature stop codons in the resulting transcript of the gene (122). Hypermethylation of the wild-type APC allele is also found in some sporadic colorectal cancers which may be an alternative mechanism for APC gene inactivation (126). The secreted Wnt signalling antagonist WIF1 has also been shown to be silenced by promoter hypermethylation and the down-regulation of *WIF1* expression is an early event in colorectal cancers (148). Mutations in other Wnt signalling components are uncommon, although mutations in axin/ conductin have been found in DNA-mismatch-repair deficient colon tumours with intact APC. The loss of axin/ conductin results in the nuclear accumulation of β-catenin and formation of β-catenin-TCF complexes (156). The *TCF4* gene contains an oligonucleotide repeat tract in the 3' region of the gene that frequently undergoes slippage in microsatellite-unstable colorectal cancers. This change possibly creates a truncated, more active form of the transcription factor (157).

Several mouse models have been generated to study the role of Wnt signalling in intestinal cancer development. In most of these models, tumourigenesis occurs mostly in the small intestine, whereas in humans, cancers develop mostly in the colon. The mouse model for FAP carrying a nonsense mutation at codon 850 and stably expressing truncated APC was produced by chemical mutagenesis. Mice heterozygous for this mutation developed multiple intestinal neoplasis (Min) and rarely live longer than 3 months (158). Mice homozygous for this mutation died in utero, 8 days postcoitus (159). Oshima *et al.* (160) used homologous recombination to generate mice expressing APC truncated at residue

716. Like the Min mice, the $APC^{\Delta 716}$ heterozygotes developed numerous adenomas throughout the intestinal tract and genotyping the tumours revealed that all had lost their wild-type *APC* allele. Shibata *et al.* (161) created a conditional APC model in which *APC* exon 14 is deleted upon Cre recombinase expression in the colon, resulting in APC truncated at codon 580. These mice developed adenomas within 4 weeks, implying that inactivation of *APC* is sufficient to drive polyp formation (122). Transgenic mouse models inducibly expressing constitutively active β-catenin suffer intestinal tumours similar to the Min mice (162). β-catenin knock-out mice also suffer severe gastrulation defects and die 7 days postcoitus (163). Angus-Hill *et al*. (164) show that TCF4 haploinsufficiency results in colon tumour formation in a mouse tumour model that normally only develops small intestinal tumours. Loss of TCF4 early in development and in adult colon also results in increased cell proliferation and leads to colon tumourigenesis.

1.4 BMP Signalling Pathway

1.4.1 Role in tissue renewal

Besides regulating bone and cartilage formation, bone morphogenic proteins (BMPs) are also involved in the development, morphogenesis, cell proliferation and apoptosis of a variety of tissues and cells. Dysregulation of BMP signalling has been linked to prostate cancer, breast cancer and colorectal cancer. BMPs are part of the transforming growth factor β (TGFβ) superfamily of morphogenic proteins. Members of this family bind to a complex of transmembrane serine threonine kinase receptors type I and II, triggering the phosphorylation and activating of the type I receptor by the type II receptor kinase. Activation of this pathway by BMPs leads to the inhibition of intestinal stem cell activation and promotion of intestinal differentiation. A study by Kosinki *et al*. (74) has characterised the gene expression profiles of the human colon by comparing the gene expression pattern between the top and basal crypt compartments (Fig 1.19). They found differential expression of multiple BMP components along the colon crypt axis. *BMP1, BMP2, BMP5, BMP7, SMAD7* and *BMPRII* were highly expressed in colon tops, whereas the BMP antagonists *CHRDL1 (*chordin-like 1), *GREM1* (gremlin 1) and *GREM2* (gremlin 2) were expressed in basal colon crypts. This would suggest that BMP signalling is activated in the upper crypt and its inhibitors *CHRDL1, GREM1* and *GREM2* are located at the bottom to antagonise BMP signalling in the intestinal epithelial stem cell niche. They also demonstrate that CHRDL1, GREM1 and GREM2 likely originate from myofibroblasts and smooth muscle cells, which are both located at the crypt base close to the stem cell niche. Therefore by inhibiting BMP signalling at the crypt base, the antagonists maintain Wnt signalling and inhibit differentiation.

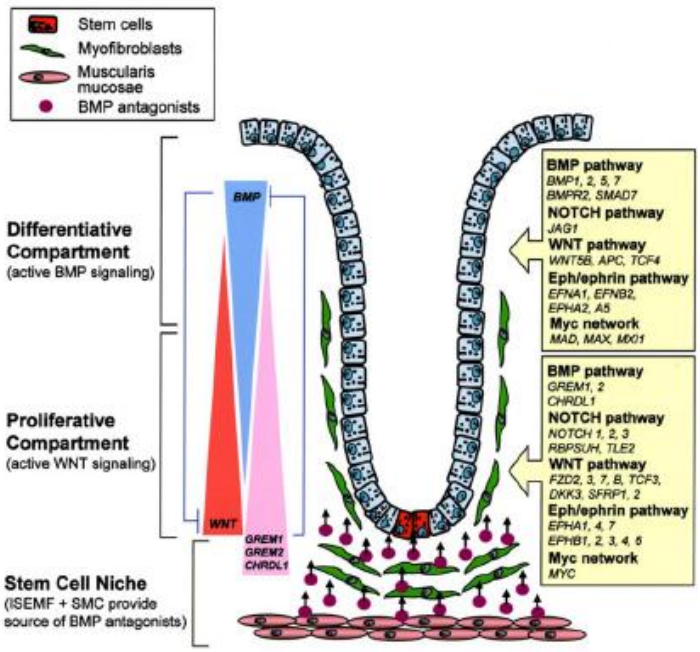


Figure 1.19 Gene expression profile along the crypt axis showing Wnt signalling components at the base of the crypt and BMP signalling components at the top of the crypt. *Figure from* (74)**.**

He *et al*. (8) also investigated the role of BMP signalling in regulating intestinal development and determined the expression patterns of BMP4, its antagonist Noggin, BMPRIA and phosphorylated SMAD 1,5,8 (pSMAD 1,5,8). They found that BMP4 was expressed in the intravillus and intercrypt mesenchymal cells, including those adjacent to intestinal stem cells. BMPRIA had a gradient distribution in epithelial cells along the crypt-villus axis and was highly expressed in intestinal stem cells but not in the cells in the proliferation zone. Noggin was expressed in the submucosal region adjacent to the crypt base and in only some cells in the intestinal stem cell position or the surrounding cells. pSMAD 1,5,8, the sign of active BMP signalling was found in both villi and intestinal stem cells. However, the transient expression of Noggin is required to override the BMP signal and the release β-catenin inhibition by PTEN to activate stem cells. This balancing role between BMP and Wnt signalling inhibits aberrant proliferation of stem cells, thereby preventing crypt fission and increase in crypt number. Lombardo *et al*. (165) also found

that in colorectal cancer colonic crypts, BMP4 was expressed along the whole crypt axis apart from a few cells at the very base of the colonic crypt where stem cells are located. This was confirmed by the lack of BMP4 in $CD133^+$ cell populations, whereas it was expressed in the $CD133^-$ cell populations suggesting functional divergence between the cancer stem cells ($CD133^+$) and the differentiated $CD133^-$ cells. They also found the receptors BMPRIA, BMPRIB and BMPRII in these stem cell fractions and that exogenous activation of BMP signalling with BMP4 led to a rapid and massive differentiation of the cancer stem cells. BMP treatment also increased PTEN levels and consequently inhibited the PI3K/AKT pathway in the cancer stem cells which inhibits the release of β-catenin that would otherwise activate stem cell self-renewal.

Farrall *et al*. (166) generated spheroids from APC^{min} mice which upon activation of β-catenin by loss of APC or transgenic induction of mutant β-catenin initiated the conversion of untrasformed intestinal cells to tumour cells. They found that the spheroid cells produced Wnt and Notch ligands as well as BMP4 which was also found in adenomas. High BMP4 expression and pSMAD1,5,8 activity overlapped with high β-catenin levels in the adenoma but there was relatively low expression of the stem cell marker CD133. Spheroids were cultured in noggin to inhibit differentiation, and when this is removed the levels of pSMAD1,5,8 were increased, suggesting autocrine BMP signalling. Addition of recombinant BMP4 led to the spheroids displaying a "dimpling" on their surface and attenuated growth rates. Cells from rBMP4 pre-treated spheroids had also lost their ability to self-renew in clonoginecity assays demonstrating that BMP signals modulate the CSC-like/progenitor characteristics of spheroid cells and beyond a threshold, induce irreversible differentiation.

BMP signals are mediated by type I and type II serine/threonine kinase receptors, see Fig 1.20. There are two subclasses of type I receptors, BMPRIA and BMPRIB. Upon ligand binding, the type II receptor forms a heterodimer with the type I receptor that results in the phosphorylation of downstream SMAD proteins. pSMAD1,5,8 associates with SMAD4

and the heterodimeric complex translocates to the nucleus to activate or inhibit transcription (167). BMPs can also bind to activin type II (ACVR2) and type IIB (ACVR2B) receptors. BMP7 preferentially binds to ACVRII and ACVRI but also has affinity for BMPRII, BMPRIA, and BMPRIB, whereas BMP2 and BMP4 appear to bind to BMPRIA and BMPRII preferentially (168). In the nucleus, the SMAD1,5,8/SMAD4 complex can bind to DNA sequences directly or interact with transcription factors such as Runx-2, Menin, Yin Yang 1 (YY1) or *Hoxc8* (169). SMAD transcriptional activity can be also regulated by the association with transcriptional co-activators/repressors such as p300/CREB binding protein (CBP) and the histone acetyltransferase GCN5 or the homolog of the transforming protein of the avian Sloan-Kettering retrovirus (c-Ski) and the Ski-related novel protein (SnoN) (169), (167).

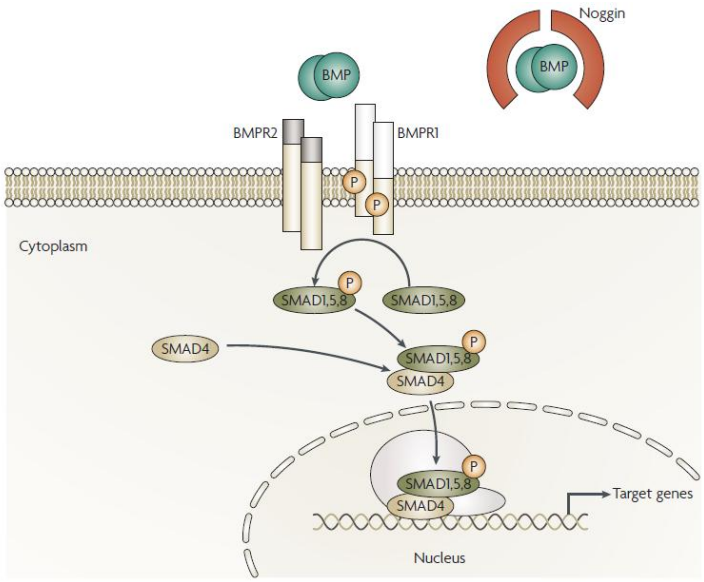


Figure 1.20 The BMP signalling pathway. *BMP ligands bind to the BMP receptors BMPRI and BMPRII leading to BMPRII phosphorylating and activating BMPRI. Phosphorylated BMPRI subsequently phosphorylates SMAD1,5,8 which associates with SMAD4, enters the nucleus and activates gene expression. The BMP signal can be blocked by extracellular antagonists such as noggin, which bind BMP ligands and prevent their association with the BMP receptors. Figure from* (170).

BMP activity is controlled by intracellular and extracellular factors that modulate BMP action. BMP effects can be regulated by inhibition of BMP-BMP receptor interaction by extracellular BMP binding proteins, presence of dominant negative non-signalling membrane pseudoreceptors, blocking of BMP signalling by inhibitory SMADs, blocking of BMP signalling by intracellular SMAD binding proteins and ubiquitination and proteosomal degradation of BMP signalling effectors (167). The protein sequence of BMP antagonists is characterised by conserved cysteine-rich domains that form cysteine knot structures. Knots are functional motifs that determine the folding of the peptide and exposure of specific hydrophobic residues, facilitating diverse protein-protein interactions (167). There are three main BMP antagonists, Noggin, Gremlin and Chordin that maintain Wnt signalling and inhibit differentiation at the crypt base.

Noggin, encoded by the *NOG* gene, is secreted as a glycosylated protein, covalently linked homodimer of 64 kilodaltons (kDa). The primary structure of noggin consists of an acidic aminoterminal region and a CR carboxyterminal region containing a cystine knot. A central, highly basic heparin-binding segment retains noggin at the cell surface. Noggin binds with various degrees of affinity to BMP2, 4, 5, 6, 7, GDF5, 6 and vegetally localised protein 1 (Vg 1), but not other members of the TGFβ family of peptides (167). By diffusing through extracellular matrices more efficiently than members of the TGFβ superfamily, noggin has a principal role in creating morphogenic gradients by inhibiting the BMP signalling pathway (171).

Gremlin, also known as Drm, is part of the Dan (Differential screening-selected gene aberrative in Neuroblastoma) family and is a cysteine knot-secreted protein. The *gremlin* gene was first cloned from a Xenopus ovarian library for its axial patterning activities and encodes for a glycosylated homodimeric peptide of 20.7-kDa. Gremlin binds BMP2, 4, and 7 with high affinity, but does not interact with other members of the TGFβ superfamily (172).

Chordin was initially identified in the Spemann Organizer for its ability to antagonise BMP signalling and is the *Xenopus* homologue for Short Gastrulation (Sog) in *Drosophila*. It is secreted as a glycosylated homodimer of 120kDa and is characterised by four CR domains which are the sites of interaction with BMPs. Chordin binds specifically to BMP 2, 4, and 7 and does not bind other members of the TGFβ superfamily. Chordin is regulated by interaction with other secreted proteins of the extracellular matrix. The chordin-BMP complex is a substrate for the zinc metalloprotease BMP1/tolloid which cleaves chordin, inactivating its biological activity thus releasing BMPs into the extracellular space (167) (173).

BMP signalling is also regulated by inhibitory SMAD 6 and 7 (i-SMADs) which bind to type I BMP receptors, thereby interfering with SMAD 1,5,8 phosphorylation and heterodimerisation with SMAD4. SMAD6 can inhibit BMP effects by modifying the interaction of SMAD1,5,8 with co-repressors. SMAD1 induces transcription by dislodging transcriptional repressors such as *Hoxc8*, and SMAD 6 prevents this dislodging from DNA binding sites by binding itself to *Hoxc8* so that the repression persists (174). The pseudorecetor BMP and activin bound protein (BAMBI) is a transmembrane glycoprotein with an extracellular domain similar to that of type I TGFβ and BMP receptors. BAMBI associates to type IA and IB BMP receptors and inhibits the effects of the activated receptors without direct interaction with either TGFβ or BMP (167).

BMP signalling can also be regulated by intracellular binding proteins through the low affinity binding to the GCCG, CAGA or GC rich DNA sequence motifs in the promoter regions of BMP responsive genes and through interaction with transcription factors or transcriptional co-activators/repressors such as p300/CBP, c-Ski, SnoN and Tob. Ski, a nuclear oncoprotein homologous to the transforming protein v-Ski of the avian Sloan-kettering retrovirus, interacts with the TGFβ specific SMAD2,3 and with SMAD4, thereby interfering with the formation of functional SMAD complexes (175). The transducer of Erb B-2 (*Tob*) gene is a member of the PC3/BTG/Tob family of genes which are involved

in cell replication and differentiation. Tob decreases BMP signalling by binding SMAD1,5,8 and by interacting with BMP type I receptors (167).

Finally, BMP signalling is also regulated by the ubiquitin-mediated proteosomal system which is important in cell cycle progression, gene transcription and signal transduction. The formation of ubiquitin-protein complexes requires three enzymes that are involved in a cascade of ubiquitin transfer reactions: ubiquitin-activating enzyme (E1), ubiquiting-conjugating enzyme (E2) and ubiquiting ligases (E3). The specificity of protein ubiquitination is determined by E3 which defines the substrate specificity and subsequent protein degradation by the 26S proteosome. SMAD-ubiquitination regulatory factor (Smurf) 1 and 2 are SMAD specific E3 ubiquitin ligases which interact with SMAD 1, 5, 6, and 7. Smurf1 binds to ubiquitin through a conserved cysteine located at the carboxy terminus of the molecule and binds to SMADs through a WW domain. Smurf1 is located in the nucleus and is exported to the cell membrane and cytoplasm where it induces the proteosomal degradation of type I TGFβ, BMP receptors and SMAD 1 and 5. Smurf1 also enhances the interaction of i-SMADs with type I receptors to amplify the repression of BMP signalling (167) (176).

1.4.2 BMP signalling and cancer

Patients with juvenile polyposis, a rare autosomal dominant hamartomatous polyposis syndrome have an increased risk for the development of colorectal cancer. The polyps are mostly found in the colorectum and sometimes in the proximal gastrointestinal tract. It was found in patients with this syndrome that there were mutations in BMPRI and SMAD4, accounting for approximately half of all juvenile polyposis cases, which suggested a role for the BMP pathway in the initiation of colorectal neoplasia. Kodach *et al*. (177) have shown that there was reduced expression of BMPRs and SMAD 4 in colorectal cancers but not adenomas and that the expression of pSMAD1,5,8 was

inactivated in 80% of colorectal cancers but was active in the adenomas. Selective loss of pSMAD1,5,8 in areas of high-grade dysplasia/carcinoma within adenomas indicates that loss of BMP signalling is associated with the progression of adenomas to carcinomas.

Inhibition of BMP signalling in mice via transgenic expression of noggin in the intestinal epithelium leads to formation of ectopic crypts and a phenotype similar to human juvenile polyposis in both small intestine and the colon (7). Conditional inactivation of BMPRIA in mice also disturbs the homeostasis of intestinal epithelial regeneration with an expansion of the stem and progenitor cell populations, eventually leading to intestinal polyposis similar to human juvenile polyposis (8). Beppu *et al*. (178) have shown that inactivation of BMPRII in the stromal cells of the colonic mucosa leads to overgrowth of the epithelium and the development of hamartomatous polyps. Although epithelial hyperplasia was observed along the entire colorectum, polyp formation was limited to a small portion of the epithelial surface, which suggests that the stromal BMPRII mutation alone may not be sufficient to initiate polyposis. It is possible that secretion of growth factors such as hepatocyte growth factor (HGF) and transforming growth factor β1 (TGFβ1) may act on the epithelial cells to lead to epithelial hyperplasia or BMPRII deletion may act indirectly by increasing myofibroblast proliferation, which in turn promotes epithelial cell proliferation.

The role of the BMP pathway in sporadic colorectal cancers has been difficult to identify due to the usual screening methods such as loss of heterozygosity, point mutations and promoter methylation not identifying any specific members of the BMP pathway that may be involved. Although SMAD4 was frequently deleted in colorectal cancers, it has been attributed to the loss of TGFβ signalling rather than BMP signalling (170). Recently, a number of studies have tried to investigate a possible role for the BMP pathway in sporadic colorectal cancer. Hardwick *et al*. (179) found that BMP2 inhibits colonic epithelial cell growth *in vitro*, promoting apoptosis and differentiation and inhibiting proliferation and that BMP2 expression is lost in the microadenomas of familial adenomatous polyposis

patients suggesting that BMP2 acts as a tumour suppressor. Loh *et al*. (180) have found BMP3 to be growth suppressive and its expression is frequently lost through promoter methylation, again suggesting a tumour suppressor role for BMPs in colorectal cancers. However, BMP4 expression has been shown to be up-regulated in the transition from primary colorectal adenomas to adenocarcinomas (181). Deng *et al*. (181) demonstrated that overexpression of BMP4 can protect colon cancer cells from apoptotic death under stress environment and drive these cancer cells to a more migratory and invasive phenotype through induction of uPA activity. This suggests that BMP4 promotes invasive behaviour of colon cancer cells. BMP7 has also been found to increase with progression through the adeno-carcinoma sequence and to correlate with worse prognosis (182). By examining pSMAD1,5,8 expression levels, Kodach *et al*. (177) found that loss of BMP signalling occurs during the transition from late adenoma to early carcinoma suggesting that BMP signalling is involved in tumour progression rather than as an initiator of carcinogenesis.

1.5 TGFβ/Activin Signalling Pathway

1.5.1 Role in tissue renewal

TGFβ (Transforming growth factor β) signalling is involved in a variety of biological functions, including cell growth, cell differentiation and apoptosis. TGFβ promotes growth and development during early embryogenesis and in some adult mesenchymal cells, whilst in mature tissues, cells respond with cytostasis and apoptosis (109). There are 5 isoforms of TGFβ (TGFβ1-5) which belong to a large superfamily that includes activins, inhibins, BMPs and myostatin. Each of the TGFβ ligands is encoded by different genes which act through the same receptor signalling cascade. The TGFβ ligands are stored in the extracellular matrix, attached to latent TGFβ binding proteins (LTPBs). This prevents the binding of the molecule to its receptor (183). TGFβ ligands bind to type II receptor TGFβRII which in turn attracts and activates type I receptor TGFβRI by phosphorylation. SMAD2 and SMAD3 are then phosphorylated at the carboxyl-terminal serines by the activated TGFβRI receptor and form heteromeric complexes with SMAD4. The SMAD2/3/4 complex translocates to the nucleus and binds to specific regulatory sites on target genes (Fig 1.21). SMAD4 can only translocate to the nucleus when it is in a complex with R-SMADs, whereas SMAD2 and SMAD3 can translocate in a SMAD4 independent manner. The activity of the SMADs is modulated by inhibitory SMAD6 and SMAD7 as well as adaptors such as SARA (SMAD-anchor for receptor activation) for SMAD2 and ELF for SMAD3 and SMAD4. Once in the nucleus, the SMAD2/3/4 complex induces target genes involved in tumour promotion and tumour suppression as well as a cyclin-dependent kinase (CDK) inhibitor p21 which leads to cell cycle arrest. (183). P21 is the product of *waf/cip1* gene which is an inhibitor of CDK as well as inhibitor of propagation of cell cycle at G1 and G2 activated upon DNA damage. P21 interacts with complexes of CDK2 and cyclin E and inhibiting CDK2 activity, thus preventing progression of the cell cycle (184).

TGFβ also activates non SMAD pathways that include three distinct MAP kinase pathways: Erk, c-Jun N-terminal kinase (JNK) and p38 MAP kinase pathways. JNK and p38 MAP kinase phosphorylate c-Jun and ATF-2 respectively. C-Jun is a component of the AP-1 transcription factor, whereas ATF-2 acts as a homodimer and heterodimer with c-Jun. SMAD3 interacts with phosphorylated c-Jun and ATF-2. SMAD3 and SMAD4 then act together with c-Jun and ATF-2 in transcriptional activation of target genes (185) (186) (187).

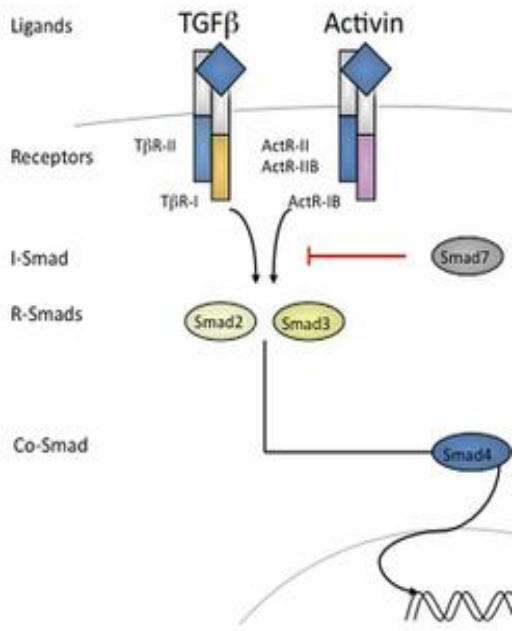


Figure 1.21 TGFβ and activin signalling pathways. *TGFβ ligand binds to type II receptor TGFβRII which associates with type I receptor TGFβRI and causes its phosphorylation and activation. This in turn recruits SMAD2 and SMAD3 which complex with SMAD4 and translocate to the nucleus. In a similar fashion, activin ligands bind to type II receptor ACTRII which associates with type I receptor ACTRI. This binding causes its phosphorylation and recruitment of SMAD2 and SMAD3 which in turn complex with SMAD4 and translocate to the nucleus. Figure from* (188)

As well as the TGFβ ligands, the 3 activin ligands activin A, activin B and activin AB are also part of the TGFβ superfamily. Like TGFβ ligands, activins bind to a type II receptor

ACTRIIA or ACTRIIB which subsequently associates with the type I receptor ACTRIB (Fig 1.21). This association causes the phosphorylation of the type I receptor which in turn recruits the regulatory SMAD2 and SMAD3. SMAD4 then complexes with SMAD2/3 and translocates to the nucleus.

TGFβ signalling is regulated by inhibitory SMADs (i-SMADs) which interact with type I receptors that have been activated by the type II receptors causing the dissociation of the R-SMADs 2 and 3. I-SMAD expression is upregulated by TGFβ and activins and a potent inhibitor of both TGFβ and activin signalling is SMAD7. The expression of SMAD7 is induced by direct effects of the SMAD3 and SMAD4 on the *SMAD7* promoter (189).

Several transcriptional co-repressors interact with SMADs to modulate TGFβ signalling. TGIF, a homeodomain of the TALE class was the first transcriptional co-repressor shown to interact with SMADs (190). C-Ski and its related protein SnoN (Ski-related novel gene) are also SMAD-binding transcriptional co-repressors with c-Ski binding to SMAD2, SMAD3 and SMAD4 (175). TGIF and c-Ski compete with P300/CBP for interaction with TGFβ-specific R-SMADs and they repress the transcription of target genes induced by TGFβ. Both TGIF and c-Ski recruit histone deacetylates (HDACs) to SMAD complexes which lead to the transcriptional repression of target genes (185). SnoN is a more effective repressor of transcription induced by SMAD2 than of that induced by SMAD3. In the absence of ligand stimulation, SnoN represses the spontaneous activation of TGFβ-responsive genes. Upon TGFβ stimulation and nuclear accumulation of SMAD3, SnoN is rapidly degraded by cellular proteasomes. After a while, TGFβ signalling induces SnoN expression, which terminates TGFβ signalling through negative feedback regulation (185) (191).

The linear migration, differentiation and compartmentalisation along the crypt-villus axis is controlled by several gradients including Wnt and TGFβ. TGFβRII receptors have been shown to be localised in both the differentiated cells of crypt villi as well as the

undifferentiated crypt cells at the base of the crypts (192) (193) and SMAD4 and ELF expression is observed at both the top and base of the crypts (194). The presence of TGFβ signalling and the absence of Wnt signalling in the villus compartment results in rapid cell cycle arrest and differentiation. Therefore, SMAD4 and TCF4 constitute a dominant switch between the proliferative progenitor and differentiated epithelial cell (109). In colorectal cancers this switch is permanently reversed with TGFβ signalling being inactivated and TCF4 is constitutively activated by mutations in the Wnt cascade. Letamendia *et al*. (195) show that there is interaction and crosstalk between the TGFβ and Wnt signalling pathways. They show that SMADs physically interact with LEF1/TCF transcription factors and that TGFβ-dependant activation of LEF1/TCF target genes can occur independently of β-catenin. Furuhashi *et al*. (196) have also shown that axin has a role in SMAD3 mediated signalling by associating with SMAD3 in the cytoplasm and facilitating phosphorylation by TGFβRI/II, then dissociating when p-SMAD3 associates with SMAD4. Furukawa *et al*. (197) have found that $SMAD3^{-/-}$ mice had an increased number of proliferating cells with activation of the Wnt pathway as well as loss of EphB receptor expression. This suggests that SMAD3 in the normal colon negatively regulates proliferation and plays a role in determining their location along the crypt axis. Satterwhite *et al*. (198) found that TGFβ rapidly reduced APC protein levels and increased β-catenin mRNA and protein levels as well as its nuclear accumulation. Retrovirus mediated overexpression of β-catenin enhanced the ability of TGFβ to induce cell cycle arrest. This demonstrates that TGFβ mimics the effect of Wnt signalling on β-catenin and its accumulation along with the reduction of APC have cooperative effects on mechanisms that mediate TGFβ-induced cell cycle arrest. Edlund *et al.* (199) also show that SMAD7 interacts with β-catenin and LEF1/TCF in a TGFβ dependent manner. When endogenous SMAD7 was suppressed with siRNA, TGFβ induced increase in activated p38, AKT phosphorylated on Ser473, GSK3β phosphorylation on Ser9 was prevented as well as the TGFβ-induced association between β-catenin and LEF1/TCF. Edlund *et al*. (199) propose a mechanism by which TGFβ signalling, dependent on SMAD7, activates p38 MAP

kinase, which in turn activates AKT to inhibit GSK3β, allowing accumulation of β-catenin. B-catenin then builds up in the cytoplasm and translocates to the nucleus where it partners with LEF1 and SMAD7 to promote apoptosis (109) (Fig 1.22).

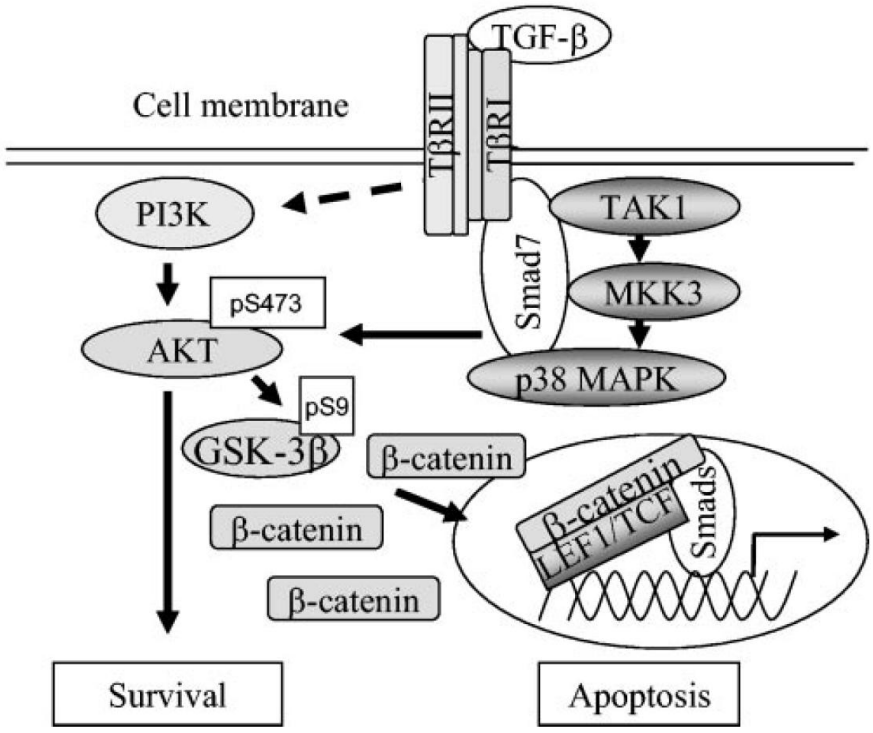


Figure 1.22 TGFβ/SMAD7 signalling pathway. *TGFβ-SMAD7 signalling pathway results in the activation of p38 MAP kinase which in turn activates AKT to inhibit GSK3β, thus allowing accumulation of β-catenin. β-catenin then builds up in the cytoplasm and translocates to the nucleus where it partners with LEF1 and SMADs to promote apoptosis. Figure from* (199).

The TGFβ signalling pathway has also been shown to play an important part in epithelial to mesenchymal transition (EMT). In this process the cells lose their epithelial characteristics including polarity and cell-cell contacts and acquire a migratory behaviour, allowing them to move away from their epithelial cell community and integrate into surrounding tissue (200). TGFβ was first shown to induce EMT in immortalized mammary epithelial NMuMG cells. This differentiation from epithelial to fibroblastic

phenotype was observed within 16 hours after addition of TGFβ1 and was accompanied by a decreased expression of the epithelial markers E-cadherin, ZO-1, and desmoplakin I and II, an increased expression of mesenchymal fibronectin markers and by a reorganization of actin stress fibers (201). The EMT effect can be fully reversible as removal of TGFβ1 restored the epithelial phenotype within two days. The reversibility of the TGFβ-dependent EMT was also demonstrated in dedifferentiated mouse colon carcinoma cells by the over-expression of a dominant-negative TGFβRII which induced mesenchymal to epithelial transition (MET) and inhibited *in vitro* invasiveness formation along with the abolishment of metastasis formation in these cells (202).

1.5.2 TGFβ signalling and cancer

The TGFβ signalling pathway is a double edged sword, with a role in both tumour suppression as well as tumour progression. TGFβ1 switches from an inhibitor of tumour cell growth to a stimulator of growth and invasion during human colon carcinoma progression (183). Schroy *et al*. (203) show that metastatic colon carcinoma cells respond to TGFβ by proliferation, whereas moderate to well-differentiated primary site colon carcinomas were growth inhibited by TGFβ.

Inactivating TGFβRII mutations account for 30% of all colorectal cancers and occur in most human colon and gastric carcinomas with microsatellite instability (183). The *TGFβRII* gene is a tumour suppressor as demonstrated by Wang *et al*. (204) who show that restoration of TGFβRII expression resulted in suppression of tumourigenicity. MacKay *et al*. (205) show that transfection of colon cancer cells with TGFβRII causes growth inhibition and reduction of malignant properties, demonstrating again that the *TGFβII* gene is a tumour suppressor. Trobridge *et al*. (206) have demonstrated that a combination of inactivation of the TGFβ signalling pathway and expression of oncogenic Kras leads to the formation of invasive intestinal neoplasms through a β-catenin independent pathway and

that these adenocarcinomas have the capacity to metastasize. Munos *et al*. (207) also found that the loss of TGFβRII in intestinal epithelial cells promotes the invasion and malignant transformation of tumours initiated by APC mutation suggesting that Wnt signalling deregulation and TGFβ signalling inactivation cooperate to drive the initiation and progression, respectively, of intestinal cancers *in vivo*.

Pasche *et al.* (208) have described aTGFβRI polymorphic allele, TGFβRI(6A) that has a deletion of three alanines from a nine-alanine stretch and observed a higher than expected number of TGFβRI(6A) homozygotes among tumour and non-tumour DNA from patients with a diagnosis of cancer. They conclude that TGFβRI(6A) acts as a tumour susceptibility allele that may contribute to the development of cancer, especially colon cancer, by means of reduced TGFβ-mediated growth inhibition.

SMAD4 and SMAD2 mutations are also involved in development of cancer by disrupting the TGFβ signalling pathway. SMAD4 undergoes biallelic loss in a third of metastatic colorectal cancers and SMAD2 is also a target of inactivating mutations in a small proportion of colorectal cancers (209) (210). SMAD4 mutations have also been associated with juvenile polyposis syndrome, an autosomal dominant disorder characterised by hamartomatous intestinal polyps and an increased risk of gastrointestinal cancers (10). Zhang *et al*. (211) show that in SMAD4 null cell lines, TGFβ induced invasion, migration, tumourigenicity and potentiality for metastasis, while incubation with a potent TGFβ-receptor kinase inhibitor reversed these effects, suggesting that loss of SMAD4 may underlie the functional shift of TGFβ from a tumour suppressor to a tumour promoter. Yang *et al*. (212) show that although mice with homozygous loss of SMAD2 and SMAD4 die in utero, their heterozygous counterparts are viable and Taketo *et al*. (213) also show that SMAD4 heterozygously null mice develop gastric polyps that can develop into tumours at a late age. When mice mutated with one APC allele are crossed with heterozygous null SMAD4 mice, the mice develop larger polyps that progress into malignant adenocarcinomas with loss of the remaining copies of both APC and SMAD4

(214). Freeman *et al*. (9) have found that decreased expression of SMAD4 in human colon cancer is associated with increased expression of β-catenin mRNA. There was also increased expression of c-Myc and Axin2 mRNA in lesions where SMAD4 expression is lost. In HEK293T cells, there was a significant decrease in β-catenin mRNA levels after treatment with BMP2 and an increase in β-catenin mRNA levels after Noggin treatment, thus suggesting that SMAD4 restoration/expression enables canonical BMP signalling to decrease β-catenin expression and inhibit Wnt signalling.

1.6 Notch Signalling Pathway

1.6.1 Role in tissue renewal

The Notch signalling pathway, in combination with Wnt signalling is important in regulating proliferation as well as lineage specification. In the intestine, Notch activity determines lineage decisions between enterocytes and secretory cell differentiation. Inhibition of the Notch pathway results in an increase in goblet cells whereas its activation results in goblet cell depletion (11).

The four different receptors are Notch1, Notch2, Notch3 and Notch 4 with the ligands being Jagged1, Jagged3, (Delta-like) Dll1, Dll3 and Dll4. Schroder *et al*. (215) determined expression of Notch pathway components in mouse intestine and found transcripts of Notch receptors 1-3 in both epithelium and mesenchyme whilst Notch 4 was only in the mesenchyme. Low amounts of *Dll1* and *Dll4* were found in some cells in the epithelium and *Dll1* was also expressed in the mesenchyme underlying the gut epithelium. *Dll3* and *Dll4* were restricted to a few cells in the mesenchyme. *Jag-1* and *Jag-2* were also expressed at low levels in the mesenchyme. Kosinki *et al.* (74) also carried gene expression patterns of human colon tops and basal crypts and found that the receptors Notch 1, Notch 2, Notch 3, RBPSUH and TLE2 were highly expressed at the basal crypt and the Notch ligand Jagged 1 was expressed at the top of the crypt.

The Notch pathway is activated when Notch ligands bind to Notch receptors on an adjacent cell which activate two proteolytic events catalysed by a disintegrin and metalloprotease (ADAM) and γ-secretase proteases which cleave the transmembrane Notch receptor liberating the constitutively active Notch intracellular domain (NICD) (Fig 1.23). NICD then translocates to the nucleus, forms a complex with one of three transcriptional regulators CSL, MAML-1 or p300/CBP and induces the expression of downstream transcriptional factors such as Hes-1 (hairy-enhancer-of-split-1) and MAML-1

(Mastermind-like-1) (26). Hes-1 and MAML-1 in turn activate target gene expression that regulate proliferation and differentiation.

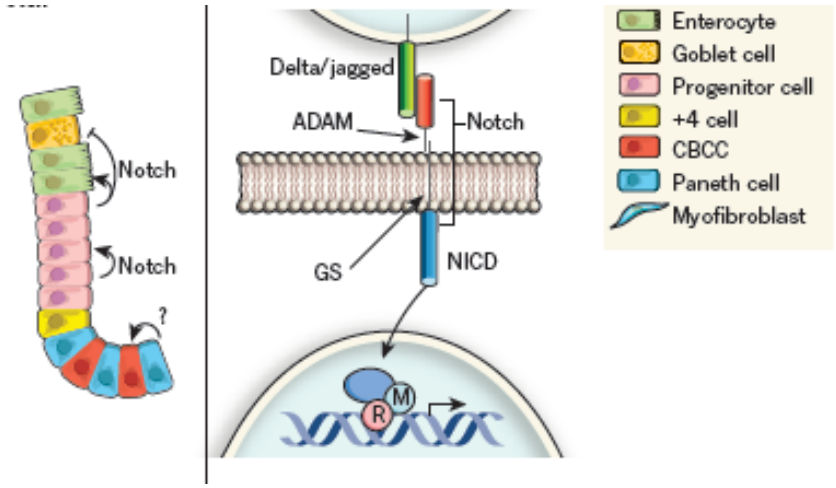


Figure 1.23 Notch signalling pathway. *Jagged and Delta ligands bind to Notch receptors on the surface of a neighbouring cell which then activates the γ-secretase protein complex and releases NICD. NICD translocates to the nucleus and drives the transcription of Notch gene targets aided by mediators such as recombining binding protein suppressor of hairless (R) and mastermind-like protein 1 (M). Figure from* (11).

To determine whether Notch signalling has a direct effect on intestinal development and adult intestinal cell turnover, Stanger *et al*. (87) used a gain-of-function approach to activate Notch. They found that ectopic Notch signalling in adult intestinal progenitor cells leads to a bias against secretory fates, whilst ectopic activation of Notch in the embryonic foregut results in reversible defects in villus morphogenesis and loss of the proliferative progenitor compartment. Therefore Notch regulates adult intestinal development by controlling the balance between secretory and absorptive cell types. By affecting stem or progenitor cells Notch activation may perturb morphogenesis in the embryo. Fre *et al*. (12) generated transgenic mice that allowed them to assess the expression and activity of Notch receptors in intestinal stem cells. They found that both Notch 1 and Notch 2 receptors are specifically expressed in crypt stem cells and that Notch signalling is also active in these stem cells and well as in absorptive progenitors. However

cells destined to adopt a secretory fate and in terminally differentiated cells, Notch activity was undetectable. Kwon *et al*. (216) suggest that Notch and Wnt signalling often intersect in stem and progenitor cells and regulate each other transcriptionally. They show that membrane-bound Notch physically associates with unphosphorylated (active) β-catenin in stem and colon cancer cells and negatively regulates post-translational accumulation of active β-catenin protein. Notch-dependent regulation of β-catenin protein did not require ligand-dependent membrane cleavage of Notch or GSK3β-dependent activity of the β-catenin destruction complex. However, it required the endocytic adaptor protein Numb and lysosomal activity. This suggests a function of Notch in negatively titrating active β-catenin protein levels in stem and progenitor cells.

1.6.2 Notch signalling and cancer

Aberrantly activated Notch signalling has been observed during the carcinogenesis of human cancers such as colon, pancreatic, breast, prostate, liver and lung and over-expression of Notch signalling is associated with poor prognosis in breast tumours and prostate cancers (14). Precancerous conditions such as Crohn's and Ulcerative Colitis have been found to have up-regulated transcription factors that function downstream of Notch signalling, such as KLF4 and Hes-1, which may be responsible for the altered goblet cell differentiation and mucin formation in these patients (13) (14). Rodilla *et al.* (217) identified a group of genes downstream of Wnt/β -catenin that are directly regulated by Notch which are repressed by γ-secretase inhibitors and up-regulated by active Notch 1 in the absence of beta-catenin signalling. They demonstrate that Notch is downstream of Wnt in colorectal cancer cells through β-catenin-mediated transcriptional activation of the Notch ligand Jagged 1 and expression of activated Notch 1 partially reverts the effects of blocking Wnt/β-catenin pathway in tumours implanted s.c. in nude mice. Qiou *et al.* (14) found that the Notch ligand *Jagged 1* is expressed at a significantly higher level in CRC tissues than in their matched normal colonic mucosa. They also observed that higher level

of *Jagged1*, *Jagged2*, *DLL1*, *DLL3*, *DLL4*, *Notch* receptors 1–4 and some downstream targets of Notch signalling (*Hes-1*, *Deltex* and *NICD*) are present in >75% of CRC tissues compared with normal colonic tissues. Galeb *et al*. (218) found that the γ-secretase inhibitor dibenzazepine reduced the rate of proliferation in colon cancer cells and levels of KLF were increased. Conversely, over-expression of Notch in these colon cancer cells reduced KLF4 levels, suppressed KLF4 promoter activity, and increased proliferation rate. Treatment of Apc(Min/+) mice with dibenzazepine also resulted in a 50% reduction in the number of intestinal adenomas compared with the vehicle-treated group. This suggests that Notch signalling suppresses KLF4 expression in intestinal tumours and colorectal cancer cells and inhibition of Notch signalling increases KLF4 expression and goblet cell differentiation and reduces proliferation and tumour formation.

1.7 Colonic crypt culture models

During the course of research for this thesis, the first long-term culture system that maintained basic intestinal crypt-villus physiology was established by Sato *et al.* (67). Growth requirements of the intestinal epithelium included R-Spondin 1, EGF (Epidermal Growth Factor) and Noggin which are associated with crypt proliferation. Mouse small intestinal crypts were isolated and placed into a matrigel-based culture containing the growth factors, leading to sealing of the crypts. The lumen then filled with apoptotic cells and the crypt region underwent continuous budding events which grew into structures termed 'organoids'. These organoids cultured for more than 8 months with no loss of characteristics and expression analysis revealed they were very similar to freshly isolated crypts. Culturing crypts from *Lgr5-EGF-ires-CreERT2* mice revealed that the bases of the crypts contained $Lgr5^+$ stem cells (Fig 1.24). These Lgr5-GFP^+ cells were sorted, and with the addition of the Rho kinase inhibitor Y-27632 to inhibit anoikis, the $Lgr5^+$ stem cells grew into organoids that were indistinguishable from whole crypt derived organoids.

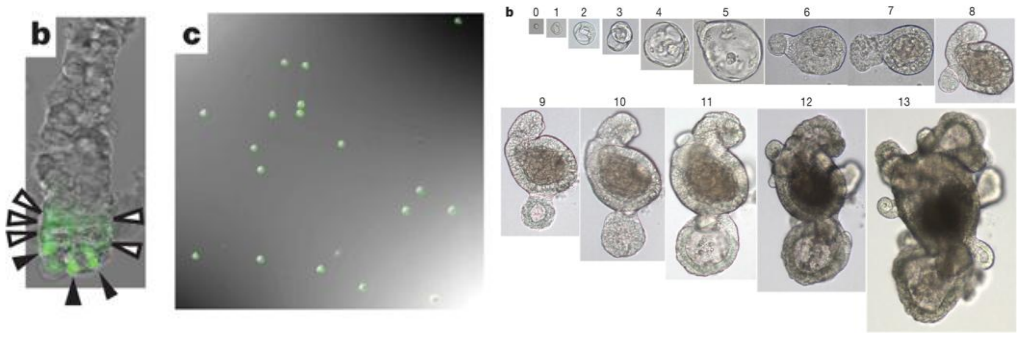


Figure 1.24 Small intestinal organoid culture. *Sorted Lgr5-GFP^+ stem cells (green) were cultured in media containing R-Spondin 1, EGF, Noggin and Y-27632. These single cells grew into organoids that could be passaged and replated to form new organoids. Figure from* (67).

Using the same culture conditions, Gracz *et al.* (71) found that isolated cells from a Sox^{EGFP} mouse sorted for Sox^{EGFPlo} cells grew into organoids over a period of one week

without a mesenchymal niche. These organoids contained all the differentiated small intestine cell types, expanded in culture and could be passaged numerous times.

These organoid cultures were formed *in vitro* without the need of mesenchymal niche, but substitution of growth factors normally secreted by the niche was required. Ootani *et al.* (219) developed a long term small and large intestinal epithelial culture system that still contained the mesenchymal niche. Using neonatal mouse tissue that was minced into small pieces, Ootani *et al*. embedded the tissue in a 3D collagen gel and cultured with mouse R-Spondin 1. The tissue cultures grew into spheres that lasted for more than 350 days *in vitro* and consisted of a polarised epithelial monolayer that was in close proximity with myofibroblasts and collagen matrix (Fig 1.25). Tissue fragments that did not have close proximity with myofibroblasts did not form spheres and eventually died, demonstrating their essential need in maintaining cells in the stem cell niche.

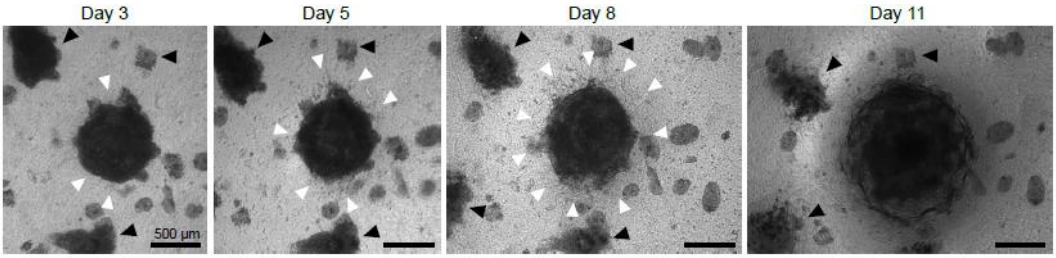


Figure 1.25 Intestinal epithelial culture within a Wnt-dependent stem cell niche. *Growth of myofibroblasts is essential for growth of spheres (white arrows). Tissue fragments without myofibroblasts in close proximity do no form spheres and eventually die (black arrows). Figure from* (219).

Most recently, Sato *et al*. (20) expanded on their small intestinal organoid culture and adapted the growth conditions for both mouse and human colonic organoid culture. They found that with mouse colon, the EGF, Noggin and R-Spondin 1 growth factors were not enough to maintain organoids longer term. Unlike small intestinal crypts, colonic crypts do not contain Paneth cells that have been shown to produce Wnt ligands which are essential for maintenance of stem cells (34). In the colonic organoid culture, it was found

that addition of recombinant Wnt3a was required to overcome this lack of Wnt ligand insufficiency. Removal at a later stage of Wnt3a caused differentiation of all epithelial lineages. This new culture condition was applied to human isolated colonic crypts and although initially survived, most disintergrated within 7 days. Sato *et al*. (20) found that addition of gastrin, nicotinamide, A83 (Alk4/5/7 inhibitor) and SB202190 (p38 inhibitor) significantly improved plating efficiency and longevity of organoids (Fig 1.26). Addition of these factors maintained the organoid cells in an undifferentiated state which only with their removal could cells differentiate to goblet, enteroendocrine and enterocytes fates. Sato *et al*. (20) also cultured human colorectal cancer samples and found that since the Wnt pathway is constitutively active in colorectal cancers, no Wnt, R-Spondin 1 or Noggin was required in colon cancer organoids.

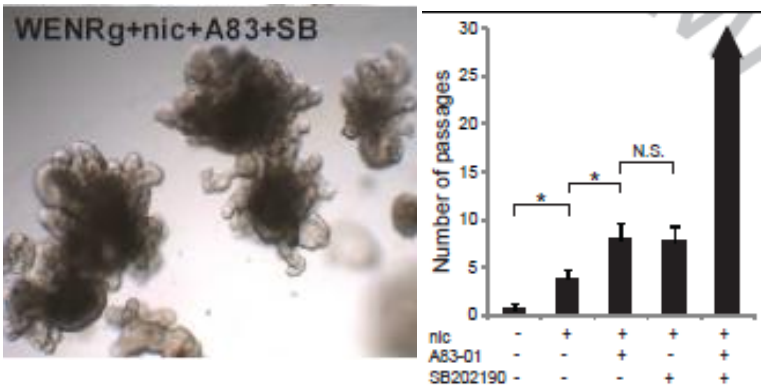


Fig 1.26 Human colonic organoid culture. *Addition of Wnt3a, gastrin, nicotinamide, A83 and SB202190 improved organoid plating efficiency and longevity. Figure from* (20)*.*

Jung *et al*. (220) also modified the original small intestinal organoid culture and adapted it for human colonic organoid culture. Like Sato *et al*., Jung *et al*. confirmed the requirement of Wnt3a, R-Spondin 1, EGF, Noggin, gastrin, nicotinamide, SB202190 and also the need for Prostaglandin E_2 (PGE_2). Under their conditions though, the isolated crypts did not form organoids but spheroids. Jung *et al*. (220) also sorted isolated $EPHB2^{high}$ vs $EPHB2^{medium}$ and $EPHB2^{low}$ cells to differentiate between stem cells and differentiated cells cells in their spheroid culture conditions. The highest EPHB2 surface levels correspond to

colonic epithelial cells with the longest telomeres and elevated expression of intestinal stem cell marker genes thus could be used to as a substitute for $Lgr5^{+}$ stem cells. $EPHB2^{high}$ cells grew into spheroids after 9-14 days and could be passaged and cultured for more than 4 months (Fig 1.27). Again, this culture condition allows the maintenance of an undifferentiated and multipotent intestinal stem cell like phenotype *in vitro*.

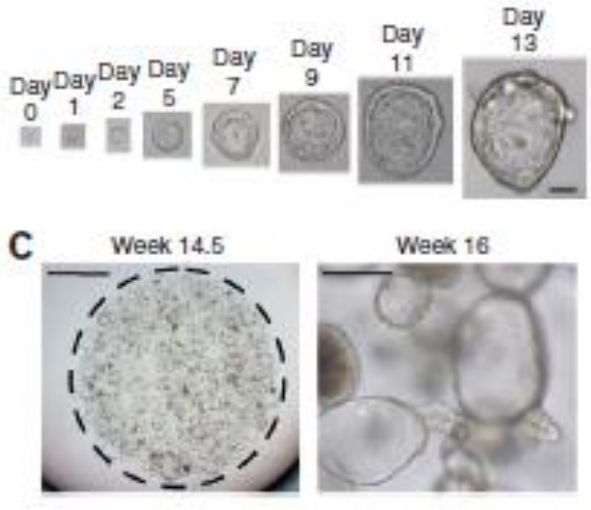


Figure 1.27 Human colonic spheroid culture. *Isolated $EPHB2^{high}$ stem cells grew into spheroids which could be passaged and cultured for more than 4 months. Figure from* (220).

These tissue culture conditions that favour activation of the Wnt pathway and inhibition of TGFβ/BMP pathways have led to the expansion of mouse and human intestinal organoids *ex vivo* that are composed predominantly of immature stem cells that can be induced to differentiate by withdrawl of Wnt stimulation or therapeutic transplantation (68). However, the processes and signalling pathways involved in stem-cell driven tissue renewal in the human colonic epithelium are yet to be determined and are required to understand the risk and pathogenesis of colorectal cancer and inflammatory diseases.

1.8 Intestinal Metaplasia: Development of Barrett's oesophagus culture model

1.8.1 Signalling pathways in Barrett's oesophagus

Barrett's oesophagus is a precursor of oesophageal adenocarcinoma that is identified by the presence of columnar epithelium in the lower oesophagus which has replaced the normal squamous cell epithelium as a result of metaplasia (Fig 1.28). The histological hallmark of Barrett's oesophagus is the presence of intestinal goblet cells in the oesophagus (20). It has been suggested that Barrett's oesophagus arises as an adaptation to the harsh intra-oesophageal environment of chronic gastroesophageal reflux disease and acquires functions that participate in mucosal defence such as secretion of bicarbonate and mucous, expression of claudin 18 tight junctions, overexpression of defence and repair genes and resistance to prolonged and repeated acid exposure (221).

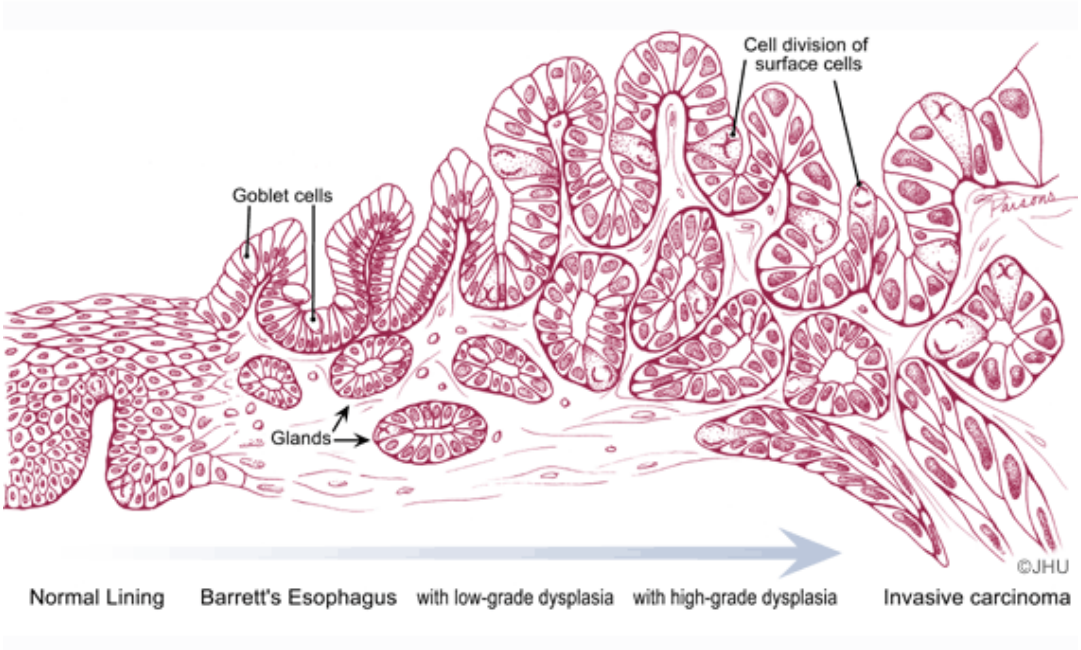


Figure 1.28 Barrett's oesophagus metaplasia sequence. *Normal squamous epithelium is replaced by columnar epithelium as a response to acid and bile acid injury. This metaplasia predisposes the oesophageal tissue to adenocarcinoma though a sequence of low-to high grade dysplasia. Figure from* (222).

The events in the pathogenesis of Barrett's metaplasia are not well understood but several signalling pathways such as Wnt, BMP and Hedgehog may be involved in the development of Barrett's oesophagus. Abnormal activation of β-catenin is found to be common during neoplastic progression of Barrett's oesophagus and is associated strongly with development of neoplasia (15). However, mutations in β-catenin, axin and APC are rarely detected in Barrett's oesophagus (223) (224). Clement *et al*. (224) therefore investigated wether activation of Wnt and Frizzled proteins and/or silencing of APC and down-regulation of Wnt antagonists through promoter methylation is responsible for the activation of the Wnt pathway in the development of oesophageal adenocarcinoma in Barrett's oesophagus. They found that *APC* and *SFRP1* silencing by promoter hypermethylation occurred frequently and the *Wnt2* gene was up-regulated when low-grade dysplastic Barrett's oesophagus progressed to adenocarcinoma. Moyes *et al.* (16) also wanted to determine whether increased Wnt signalling in the oesophagus contributes to the development of Barrett's oesophagus and dysplasia. They found that overall Ki67, a marker of proliferation, was increased with progression from metaplasia to high grade dysplasia and cancer. β-catenin in normal squamous epithelium was predominantly membranous withno definite nuclear accumulation. In Barrett's metaplasia, membranous localisation of β-catenin was also observed and an increased cytoplasmic β-catenin was seen in low grade dysplasia with nuclear accumulation in both low grade and high grade dysplasia. This suggests that Wnt signalling is markedly activated in high grade dysplasia compared to earlier stages of disease, metaplasia and low grade dysplasia. Moyes *et al.* (16) also analysed the expression of the three Wnt target genes cyclin D1, Sox9 and c-Myc. There was an increase in all three target gene expressions from normal squamous epithelium to Barrett's metaplasia and high grade dysplasia, suggesting that activated Wnt signalling could be a contributing factor to neoplastic progression of Barrett's oesophagus. The expression of c-Myc in patients with Barrett's oesophagus and adenocarcinoma was also analysed by Schmidt *et al*. (225) who found a linear correlation of c-Myc over-

expression along the metaplasia-dysplasia-adenocarcinoma sequence and a significant increase in expression compared to the control group.

Recent studies suggest a possible role for BMP signalling and especially increasing BMP4 expression in the squamous to columnar metaplasia of Barrett's oesophagus. Zhou *et al.* (226) show that acid and bile salt increase the expression of BMP4. In addition, recombinant human BMP4 induced villin expression in human oesophagus epithelial cells, as did chronic acid exposure, which can be effectively inhibited by noggin. BMP4 also induced activation of SMAD1 and promoted protein expression of ID2 and CDX2. BMP4 mRNA and CDX2 mRNA levels were shown by Castillo *et al.* (227) to be significantly greater in non-specialized columnar type of metaplasia (NSCM) than in squamous epithelium suggesting that BMP4 activation in NSCM and early expression of CDX2 are involved in the columnar epithelial differentiation of Barrett's oesophagus. Milano *et al.* (17) found that in both human and rat tissue the BMP pathway was activated in oesophagitis and Barrett's oesophagus. Upon incubation of squamous cell cultures with BMP4, the cytokeratin expression pattern showed a shift that was consistent with columnar epithelium. There was up-regulation of p-SMAD1,5,8 that was effectively blocked by noggin. Comparison of the gene expression profiles of squamous cells, BMP4-treated squamous cells, and Barrett's oesophagus cells showed a significant shift in the profile of the BMP4-treated squamous cells toward that of the cultured Barrett's oesophagus cells thus suggesting that the BMP pathway could play a role in the transformation of normal oesophageal squamous cells into columnar cells. Wang *et al.* (18) have also recently shown that epithelial Hedgehog ligand expression may contribute to the initiation of Barrett's oesophagus through induction of stromal BMP4 which triggers reprogramming of oesophageal epithelium in favour of a columnar phenotype. They found that *SHH* (Sonic Hedgehog) and *IHH* (Indian Hedgehog) expression was up-regulated markedly in response to acid. *Sox9*, which is downstream of the Hedgehog pathway during columnar epithelial phase of oesophageal development, was increased in Barrett's oesophagus tissue compared

to squamous tissue. Knowing that stromal Hedgehog target genes signal back to epithelium during gut development, Wang *et al*. (18) show that BMP4 can induce expression of Sox9 in oesophageal epithelial cells. DMBT1 (Deleted in Malignant Brain Tumours 1), the human Hensin homologue expressed in intestinal crypt cells was increased in Barrett's oesophagus and was found to be regulated by Sox9 and induces a columnar-like phenotype (Fig 1.29).

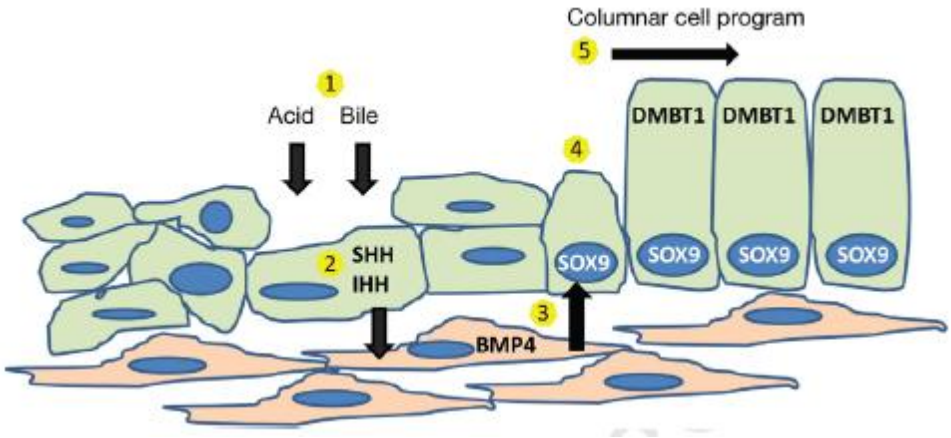


Figure 1.29 Molecular model of metaplasia proposed by Wang et al. *Acid and bile acids injure oesophageal squamous epithelium which secrete SHH and IHH causing mesenchymal secretion of BMP4. BMP4 signals to epithelium activating Sox9 which in turn activates a columnar cell transcriptional program including DMBT1. Figure from* (18).

TGFβ signalling also plays a part in Barrett's oesophagus related adenocarcinoma. TGFβ1 overexpression is found to be associated with advanced stage of oesophageal adenocarcinoma and has a negative impact on survival (228). SMAD4 mRNA had been found to be progressively reduced in the metaplasia-dysplasia-adenocarcinoma sequence along with SMAD4 promoter methylation in majority of Barrett's adenocarcinomas (229). Mendelson *et al*. (19) also observed loss of SMAD4 and TGFβRII in Barrett's oesophagus and adenocarcinoma tissues. They also demonstrated that Hes-1, a Notch signalling target and mediator, is up-regulated in Barrett's oesophagus related adenocarcinoma. Sato *et al.*

(20) have shown that Notch inhibition with a γ-secretase inhibitor converts the proliferative Barrett's epithelial cells into terminally differentiated goblet cells.

Since Barrett's oesophagus develops as a result ofchronic gastroesophageal reflux, irritation of the squamous epithelium leads to the accumulation of inflammatory cytokines in the epithelium. Some of the cytokines implicated in inflammatory and malignant processes are Tumour Necrosis Factor-α (TNF-α) and Interleukin 1-β (IL1-β). TNF-α has been found in malignant and/or stromal cells in colorectal, ovarian, breast, prostate and bladder cancers and its production seems to correlate with tumour progression or worsening prognosis in prostate and haematogenous malignancies (230). Tselepis *et al.* (230) have shown elevated levels of TNF-α in Barrett's metaplastic epithelium compared to normal squamous epithelium and that TNF-α expression persisted and intensified during the progression of Barrett's oesophagus to adenocarcinoma. This increased expression of TNF-α along the metaplasia-dysplasia-adenocarcinoma sequence suggests a relatively early role for it in the formation of the adenocarcinoma. They also demonstrated that the TNFR1 cytokine receptor was also increased in abundance during the progression of the disease, suggesting that the TNF-α signal may be amplified. TNF-α signalling occurs though several intracellular pathways including Nuclear Factor κB (NFκB) activation (Fig 1.30) and initiation of the Mitogen activated protein kinase (MAPK) cascade that includes Extracellular signal regulated kinase (ERK) and p38 activation.

NFκB is a protein complex that acts as a transcription factor and plays a key role in regulating the immune response to infection. However, dysregulation of NFκB has been linked to cancer, inflammatory and autoimmune diseases. The TNF-α induced NFκB activity involves five mammalian NFκB/Rel proteins: c-Rel, NF-κB1 (p50/p105), NFκB2 (p52/p100), RelA(p50/p65), RelB. In the absence of TNF-α stimulation, NFκB is associated with the inhibitor IκB in the cytoplasm. TNF-induced activation of NFκB largely relies on phosphorylation dependant ubiquitinnylation and degradation of inhibitor of κB (IκB) proteins (231). This occurs primarily via activation of a kinase called the IκB

kinase (IKK). IKK is composed of a heterodimer of the catalytic IKK alpha and IKK beta subunits and a master regulatory protein called NEMO (NFκB essential modulator) or IKK gamma. When activated, the IκB kinase phosphorylates two serine residues located in an IκB regulatory domain, thus ubiquitinylating the protein leading to its degradation by the proteosome (231). With the degradation of the IκB inhibitor, the free NFκB translocates to the nucleus and induces expression of certain genes. The activation of these genes leads to an inflammatory or immune response, cell survival response or cellular proliferation. NFκB also turns on the expression of its own repressor IκB. This newly re-synthesised IκB then re-inhibits the NFκB, causing an auto-feedback loop.

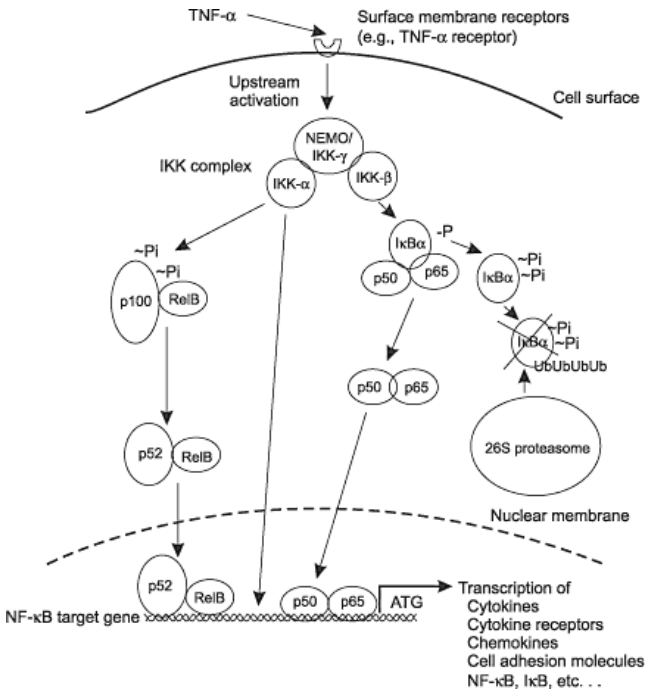


Figure 1.30 NFκB activation by TNF-α*. NFκB subunits RelA(p50/p65) are sequestered in the cytoplasm to the inhibitor molecule IκB. Upon stimulation with TNF-α, the IκB kinase complex of IKK α+β+γ (NEMO) is activated; IκB is phosphoylated, which causes its ubiquitinylation and degradation by the proteosome. This releases NFκB which then translocates to the nucleus to initiate transcription of target genes. Figure from* (232).

Leedham *et al*. (233) propose that the cellular origin of Barrett's metaplasia is that stem cells within the submucosal gland ducts regenerate the damaged oesophageal mucosa. Leedham *et al*. (233) investigated whether the oesophageal gland duct was the source of both Barrett's metaplasia and neo-squamous islands by determining tumour suppressor gene loss of heterozygosity patterns and p16 and p53 point mutations on a crypt-to-crypt basis. They identified a p16 point mutation arising in the squamous epithelium of the oesophageal gland duct which was also present in the adjoining metaplastic crypt. The presence of an identical mutation in the two different epithelium types suggests that the origin of the Barrett's metaplastic tissue is a progenitor located in the oesophageal gland duct. Nicholson *et al*. (234) used mtDNA mutations as markers of clonal expansion to investigate the stem cell architecture of the normal oesophagus and the glands (crypts) in Barrett's metaplasia. They found CCO-deficient areas in glandular tissue as well as the squamous epithelium suggesting that the glandular tissue and squamous epithelium were derived from the same precursor cell. They also show that individual Barrett's crypts contained clonal populations of CCO-deficient cells which contained all the differentiated cell lineages indicating that the crypt contained a multipotent stem cell. They also identified partially CCO-deficient crypts indicating the presence of multiple stem cells in each crypt. Nicholson *et al.* (234) propose that these partially mutated crypts are in the process of clonal conversion where the CCO-deficient clone is in the process of non-mutated cells, as found in colonic crypts (235). They also show that Barrett's crypts can form large clonal patches of several crypts, suggesting that Barrett's metaplastic crypts can divide by fission, as seen in the colonic epithelium (236).

No robust markers of stem cells in Barrett's oesophagus have been identified. However, since Barrett's oesophagus is a type of intestinal metaplasia, the intestinal stem cell marker Lgr5 could be used to identify stem cells in Barrett's oesophagus. Becker *et al*. (237) examined the pattern of immunostaining of Lgr5 in Barrett's oesophagus and found $Lgr5^{+}$ cells at the base of crypts although not all cells at the very base were positive. They later

found that there was variable staining of Lgr5, ranging from predominantly low intensity in non dysplastic Barrett's oesophagus to high intensity in dysplasia. No Lgr5 expression was seen in normal squamous tissue and high Lgr5 expression in adenocarcinoma was associated with worse survival. They suggest that the high $Lgr5^{+}$ stem cells seen with dysplasia may represent stem cells prone to becoming cancer stem cells (238). Increased expression of Lgr5 in Barrett's oesophagus compared to squamous tissue was also seen by von Rahden *et al*. (239) and high expression of Lgr5 in oesophageal adenocarcinoma also had an association with poorer survival. Vega *et al.* (240) found that there was a fivefold increase in Lgr5 mRNA human Barrett's oesophagus compared to paired normal tissue. Immunohistochemical analysis also revealed an increase in expression of Lgr5 in Barrett's oesophagus tissue compared to normal. They also found that two other stem cell markers DCAMKL-1 and Msi-1 were also up-regulated in Barrett's oesophagus compared to normal squamous tissue.

1.8.2 Culture models of Barrett's oesophagus

Surgical manipulation of the upper GI tract in animal models is frequently used in the development of therapeutics. Buttar *et al*. (241) have shown using a rat model of gastro-oesophageal reflux, where a surgical oesophago-jejunostomy predisposes animals to Barrett's and oesophageal adenocarcinoma, that treatment with COX-2 inhibitors reduced the risk of oesophageal adenocarcinoma, suggesting a link between COX-2 activity and progression from Barrett's to cancer (242). *In vitro* models have been used to understand the molecular basis of carcinogenesis from which data such as viability, apoptosis, tracking signalling molecules and identifying transcripts and proteins can be identified (242). However, this model's drawback is the lack of conditions and interactions that the cells would be subjected to in their natural environment. Also, due to the lack of an identifiable gatekeeper mutation such as APC and KRAS and oesophageal-specific promoters, no suitable transgenic mouse models have been engineered so far. Another model used in

research is the *ex vivo* culture approach that allows oesophageal cells to be studied in their original environment. These *ex vivo* cultured cells can be exposed to agents such as acids and bile acids. So far the main obstacle to this approach is the fact that the ex *vivo* cultures can only be kept alive for up to a few hours, thus allowing only certain questions to be addressed. However, since Barrett's oesophagus is the replacement of the normal stratified squamous epithelium of the oesophagus with a metaplastic glandular epithelium, this glandular epithelium forms invaginations that resemble those of crypts found in the small intestine and colon. Recently, Sato *et al.* (20) have used their human colon organoid culture model to try and establish a Barrett's oesophagus culture model. Due to the similarity of these crypt structures, isolated Barrett's crypts started to form cystic organoid structures that were very similar to the colon organoids. However, addition of FGF10 to the culture medium was required to enable the Barrett's oesophagus organoids to form budding structures and prolong the culture duration for more than 3 months (Fig 1.31).

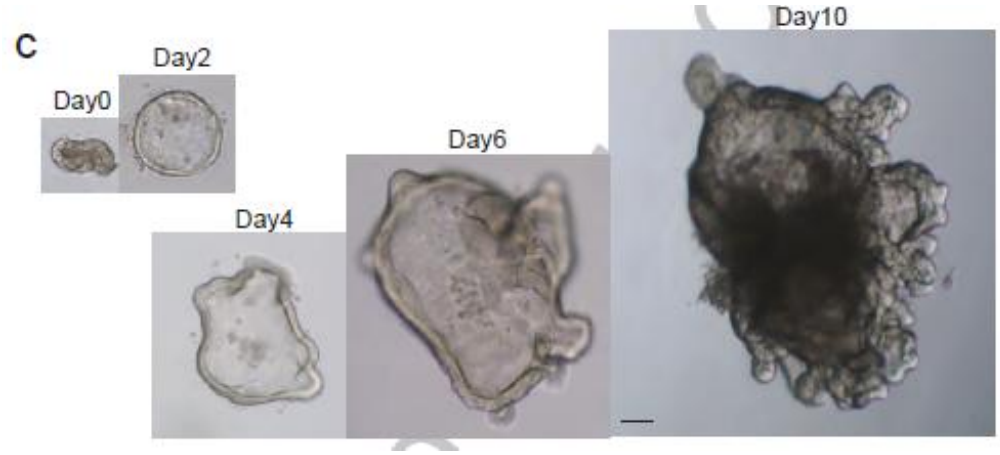


Figure 1.31 Barrett's oesophagus organoid culture. *Isolated Barrett's crypts form budding structures that expand, passage and culture for more than 3 months. Figure from* (20).

As with the colonic organoids, these Barrett's organoid structures are composed of stem/progenitor cells that can be induced to differentiate with treatment of Notch inhibitor DBZ. The processes and signalling pathways involved in the development and stem-cell

driven tissue renewal of Barrett's oesophagus are yet to be determined and are required to understand the risk and pathogenesis of Barrett's metaplasia and oesophageal adenocarcinoma. Therefore a culture model of near-native human Barrett's epithelium is required that demonstrates stem-cell driven tissue renewal *ex vivo*.

1.9 Hypothesis

Renewal of the human colonic epithelium and Barrett's oesophagus epithelium is maintained by crosstalk between Wnt and BMP/TGFβ signalling pathways.

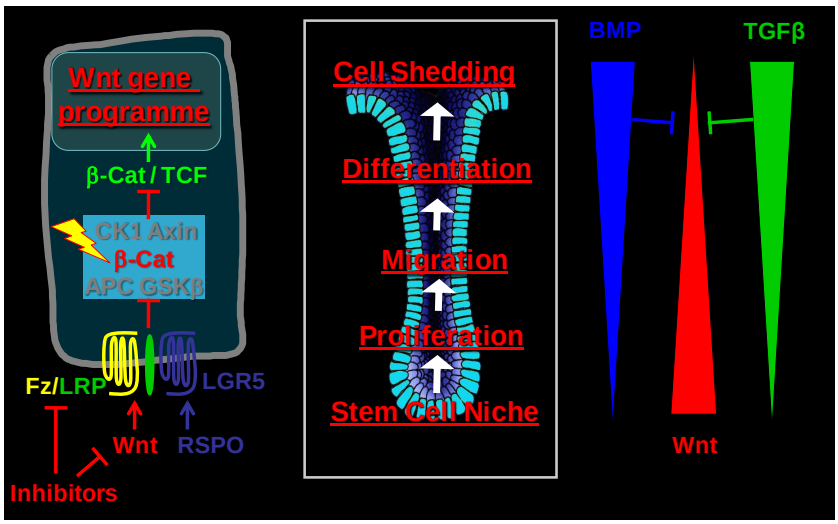


Figure 1.31 Hypothesis. *Cross-talk between Wnt and BMP/TGFβ signalling pathways maintains crypt renewal.*

1.10 Aims

- Determine Wnt, BMP and TGFβ signalling status in native human colonic epithelium.
- Investigate consequences of Wnt activation and inhibition on colonic crypt morphology, proliferation, Wnt target gene expression and stem cell number.
- Investigate consequences of Notch signalling inhibition on stem cell number.
- Investigate consequences of BMP signalling activation and inhibition on colonic crypt morphology, proliferation, pSMAD1,5,8, expression, Wnt target gene expression and stem cell number.
- Investigate consequences of TGFβ signalling activation and inhibition on colonic crypts morphology, proliferation, pSMAD2,3 expression, Wnt target gene expression and stem cell number.
- Develop 3D *ex vivo* culture model for Barrett's oesophagus crypts.

Chapter 2 Materials and Methods

2.1 Cell culture

2.1.1 Crypt isolation from colonic and Barrett's epithelium

For isolation of colonic crypts, biopsy size tissue samples were obtained from the sigmoid colon of patients undergoing a colonoscopy (3 biopsies per patient) or from macroscopically normal regions of the sigmoid colon of patients undergoing surgery for cancer (75 patients, 20-85yrs old). Barrett's epithelium biopsy samples were also obtained either from patients undergoing a gastroscopy or an oesophagectomy for oesophageal cancer (15 patients, 50-80 years old). This is with the approval of the Norwich District Ethics Committee, Norfolk and Norwich University Hospital and informed consent in each case. Human colonic and Barrett's crypts were isolated in a similar method to that previously described (243). Samples were washed in HEPES (N-2-Hydroxyethyl piperazine –N-2-ethanesulphonic acid)-buffered saline (HBS) pH=7.4 (mM): NaCl 140, KCl 5, HEPES 10, D-Glucose 5.5, Na_2HPO_4 1, $MgCl_2$ 0.5, $CaCl_2$ 1, placed in HBS devoid of both Ca^{2+} and Mg^{2+} and supplemented with EDTA (Diaminoethanetetra-acetic acid disodium salt) 1mM and DTT (Dithiothreitol) 1mM for 1 hr at room temperature. (All reagents were from Fisher Scientific). Crypts were liberated by vigorous shaking, embedded in matrigel (growth factor reduced, phenol free: BD Bioscience) and seeded onto non-fluorescent glass coverslips (Sigma) in a 12-well plate (Sigma). The matrigel was polymerized for 10 mins at 37°C in a humidified 95% air, 5% CO_2 incubator. 200µl of culture medium (Advanced DMEM/F12: Invitrogen) supplemented with penicillin/streptomycin (100 U/ml) (Invitrogen), L-Glutamine (2mM) (Invitrogen), N2 (Invitrogen), B27 (Invitrogen) and 1mM *N*-acetylcysteine (Sigma) was placed on the crypts containing growth factor combinations required for the experimental groups.

2.1.2 Crypt long term culture

Crypts for experimentation were kept in culture for 3 days then fixed for immunocytochemistry, or for 1-2 weeks for longer term experiments. The crypt culture media was replaced every 2 days and contained the following growth factors and agonists/antagonists that were used in different combinations depending on the experimental group. For short term experiments IGF, Noggin and R-Spondin 1 were used as the control media with Wnt3a, Dkk1 and IWP2 used to stimulate/inhibit the Wnt signalling pathway and BMP4, TGFβ and Activin used to stimulate the BMP/TGFβ signalling pathways. Longer term experiments contained IGF, Noggin, R-Spondin1 and Wnt3a in the media.

Growth factor	***Concentration used***	***Supplier***
Recombinant human Activin	50ng/ml	Peprotech
Recombinant human BMP4	100ng/ml	Peprotech
Recombinant human Dkk1	800ng/ml	R&D Systems
Recombinant human EGF	50ng/ml	R&D Systems
Recombinant human IGF1	50ng/ml	Sigma
Recombinant human Noggin	100ng/ml	Peprotech
Recombinant human R-Spondin 1	500ng/ml	Sino Biological
Recombinant human TGFβ1	20ng/ml	R&D Systems
Recombinant human Wnt3a	100ng/ml	R&D Systems

Agonist/Antagonist	*Concentration used*	*Supplier*
A83-01	0.5μM	Tocris Bioscience
DBZ	10mM	Calbiochem
IWP2	5μM	Stemgent
anti-TGFβ1,2,3 antibody	10μg/ml	R&D Systems
COX2 antibody	10μg/ml	Cayman Chemicals

Table 2.1 Agonists and antagonists used during experiments.

2.2 Crypt renewal experiments

2.2.1 Crypt morphology

The morphology of colonic crypts maintained in culture under different experimental conditions was visualised with a Nikon Ti-E SuperResearch Inverted Motorised Time-Lapse System microscope fitted with a humidified chamber at x10 objective. DIC images of crypts were taken every 24 hrs for a period of 1-2 weeks or until the crypts died. Length measurements for each day were taken using the Nikon NIS Viewer software and normalised to day 1 measurements. Statistical differences between groups were determined using one-way ANOVA and Tukey's post-hoc analysis.

2.2.2 Cell proliferation

2.2.2.1 BrdU incorporation

Cultured human colonic crypts were incubated with 10μM BrdU (Bromodeoxyuridine) at day 2 in media with growth factors required for each experimental group at 5% CO_2, 37°C

for 24 hrs. Crypts were then fixed and processed for immunocytochemistry (as described in section 2.4).

2.2.2.2 Real-time digital time-lapse microscopy

Crypt mitotic events (Fig 2.1) were visualised with a Nikon -E SuperResearch Inverted Motorised Time-Lapse System microscope fitted with a humidified chamber at x20 objective. DIC images of selected crypts were taken at an interval of 5mins over a period of 24 hrs and the number of mitoses was manually counted over the 24 hr period using the Nikon NIS Viewer software. The number of mitoses per hour was then subsequently calculated and one-way ANOVA with Tukey's post-hoc analysis was performed to determine any differences between groups.

Figure 2.1 Time-lapse image of mitosis. *Number of mitoses over a period of 24 hrs were counted and analysed.*

2.2.3 Cell migration

Cell migration within a crypt was determined using time-lapse microscopy on the Nikon -E SuperResearch Inverted Motorised Time-Lapse System microscope. Over a period of 24hrs, at an interval of every 5mins, images were taken of crypts within different experimental groups. The rate of migration per hour was then determined by following a selected cell in the upper region of the crypt and tracking its migration over the 24hr period. 3 cells per crypt were tracked and the distance migrated was measured using the Nikon NIS Viewer software.

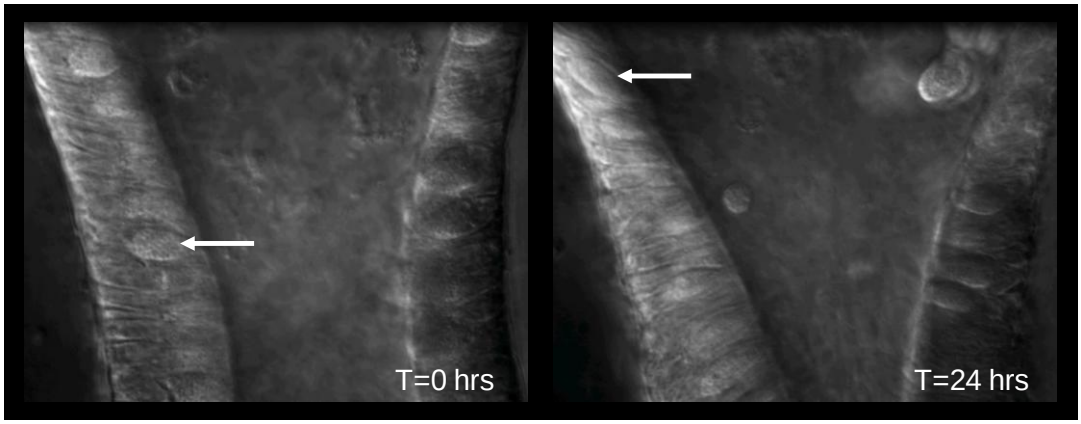


Figure 2.2 Time-lapse image of cell migration. *A distinctive cell was tracked over a period of 24hrs and the distance migrated was measured.*

2.2.4 Cell viability

Cell viability was determined by loading crypts with Calcein-AM (5μM) and PI (Propidium Iodide) (1μg/ml) and visualising using a Zeiss 510 Meta confocal fluorescence microscope at x20 objective.

2.3 Crypt transduction

2.3.1 Lentivirus TOP-GFP transduction

Freshly isolated human colonic crypts were transduced with TOP-GFP lentivirus (an inducible TCF/LEF-responsive firefly luciferase reporter) (Qiagen) placed into matrigel, and seeded onto 12-well plates and left to polymerise for 10mins at 37°C in a humidified 95% air, 5% CO_2 incubator. 200µl of culture medium (Advanced DMEM/F12: Invitrogen) supplemented with penicillin/streptomycin (100 U/ml), L-Glutamine (2mM), N2, B27 and 1mM *N*-acetylcysteine was placed on the crypts containing recombinant human IGF, recombinant human Noggin, recombinant human R-Spondin 1 and recombinant human Wnt3a (concentrations in section 2.1.2). Crypts were then left in culture for 3 days, fixed and processed for immunocytochemistry (as described in section 2.4). Lenti-CMV-GFP was used as the positive control transduction that was carried out in parallel (Fig 2.3). The multiplicity of infection (MOI) for both the Lenti-TOP-GFP and it positive control Lenti-CMV-GFP was calculated:

[$0.8x10^7$ TU/ml / 200µl] / [200 crypts x 1000 cells]

MOI= 0.2 TU / crypt cell

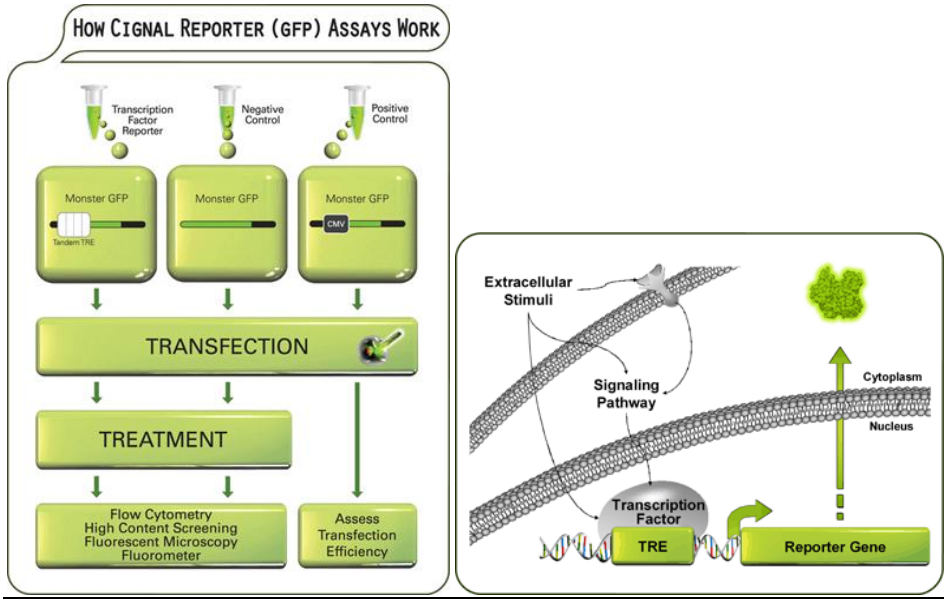


Figure 2.3 *The Cignal Reporter Assays (GFP) kit includes pre-formulated, transfection-ready reporter, negative control, and positive control. The transcription factor reporter and negative control are transfected and subjected to experimental treatments, in parallel. GFP expression is quantified using a fluorescent microscope. Change in the activity of the Wnt signaling pathway is determined by comparing the GFP fluorescence in treated versus untreated transfectants. The positive control serves as a control for transfection efficiency, by monitoring GFP expression from the constitutively expressing CMV-GFP reporter. Figure from* (244).

2.3.2 Adenovirus dnTCF4 transduction

Transduction of Ad-TCF4 (dn) (Ready-use human Adenovirus Type5 (dE1/E3)) (Vector Biolabs) was carried out in the same manner as that described for TOP-GFP lentivirus. Adeno-CMV-GFP (Ready-use human Adenovirus Type5 (dE1/E3)) was used as a positive control. The multiplicity of infection (MOI) for both the Ad-TCF4 (dn) and it positive control AD-CMV-GFP was calculated:

[$1x10^{10}$ TU/ml / 200µl] / [200 crypts x 1000 cells]

MOI= 250 TU / crypt cell

2.4 Immunocytochemistry

2.4.1 Native human colonic crypts and Barrett's epithelium

Biopsy size human tissue samples were immediately fixed in 4% paraformaldehyde (PFA) (Sigma) following removal from the patient either undergoing a colonoscopy/gastroscopy or surgery and microdissected into single crypts. Following embedding in matrigel, microdissected-native crypts were fixed with 4% PFA for 1 hr and permeabilized with either 1% SDS or Triton-X (0.5% w/v PBS) for 30 mins. Non-specific binding sites were blocked with blocking serum composed of 10% goat (Abcam) or donkey serum (Sigma) and 1% bovine serum albumin (Sigma) for 2hrs. The protein of interest was labelled by incubating the crypts overnight with one or more of the antibodies listed in Table 2. Secondary antibodies, listed in Table 3 (all from Molecular Probes), were added for 2 hrs, followed by a PBS wash before mounting on glass slides (BDH) in vectashield mounting media (Vectorlabs) with DAPI or PI (Propidium Iodide) stain.

2.4.2 Near-native cultured human colonic crypts and Barrett's epithelium

Following live experimentation, cultured human colonic crypts or Barrett's crypts were fixed in 4% paraformaldehyde for 1hr and permeabilized with either SDS (1%) or Triton-X (0.5% w/v). For BrdU epitope retrieval, crypts were denatured in 1M HCl for 10mins. Non-specific binding sites were blocked with blocking serum composed of 10% goat or donkey serum and 1% bovine serum albumin for 2hrs. Crypts with primary antibody were incubated overnight and secondary antibodies added for 2hrs, before mounting on glass slides in vectashield mounting media with DAPI or PI stain. Primary and secondary antibodies (Molecular Probes) used to detect protein of interest are listed in Table 2.2 and 2.3 respectively. Non-specific labelling of secondary antibodies was determined by omitting the primary antibody.

2.4.3 Antibodies

Primary antibody target	***Species origin***	***Concentration***	***Supplier***
Axin II	Rabbit polyclonal	1:200	Abcam
Active β-Catenin	Mouse monoclonal	1:200	Millipore
Active Caspase-3	Rabbit polyclonal	1:200	Cell Signalling
BrdU	Rat polyclonal	1:200	Abcam
C-Myc	Mouse monoclonal	1:200	Abcam
Chromogranin A	Rabbit polyclonal	1:400	Abcam
E-Cadherin	Goat polyclonal	1:200	R&D Systems
GFP	Goat polyclonal	1:200	Abcam
Ki67	Mouse monoclonal	1:200	Dako
LGR5	Mouse monoclonal	1:200	Origine
Mucin2	Rabbit polyclonal	1:200	Santa Cruz
NFκB p65	Rabbit monoclonal	1:200	Cell Signalling
OLFM4	Mouse monoclonal	1:200	Yasui Lab, Japan
OLFM4	Rabbit polyclonal	1:200	Abcam
pSMAD 1,5,8	Rabbit polyclonal	1:100	Cell Signalling
pSMAD 2,3	Rabbit polyclonal	1:100	Cell Signalling
TGFβ 1,2,3	Mouse monoclonal	1:200	R&D Systems

Table 2.2 Antibodies used during experiments.

<u>Secondary antibodies</u>

AlexaFluor 568nm goat anti rabbit IgG (H+L) 2mg/ml

AlexaFluor 488nm goat anti mouse IgG (H+L) 2mg/ml

AlexaFluor 488nm goat anti rat IgG (H+L) 2mg/ml

AlexaFluor 568nm donkey anti mouse IgG (H+L) 2mg/ml

AlexaFluor 647nm donkey anti rabbit IgG (H+L) 2mg/ml

Table 2.3 Secondary antibodies used during experiments.

2.4.4 Visualisation and semi-quantitative analysis

Colonic or Barrett's crypts mounted on slides were visualised with a Zeiss 510 Meta confocal fluorescence microscope and optical slices of 1μm were taken through the crypt at x63 objective. Analysis was performed using ImageJ software and fluorescence intensity levels of protein were taken by placing ROIs on the cell nuclei or cell basal membrane (Fig 2.4). For each crypt, 10 representative cells from each region of the crypt (Fig 2.4) were analysed and the mean readings for each region for each crypt was calculated and normalised against the mean intensity level of the base of the control group. For proliferation experiments, the number of BrdU positive cells were manually counted for each region of the crypt: base, mid and top (Fig 2.4) and their percentage of the total number of cells was calculated. Statistical differences between groups were determined using one-way ANOVA and Tukey's post-hoc analysis. N= number of crypts analysed with at least 3 crypts in each group from each patient.

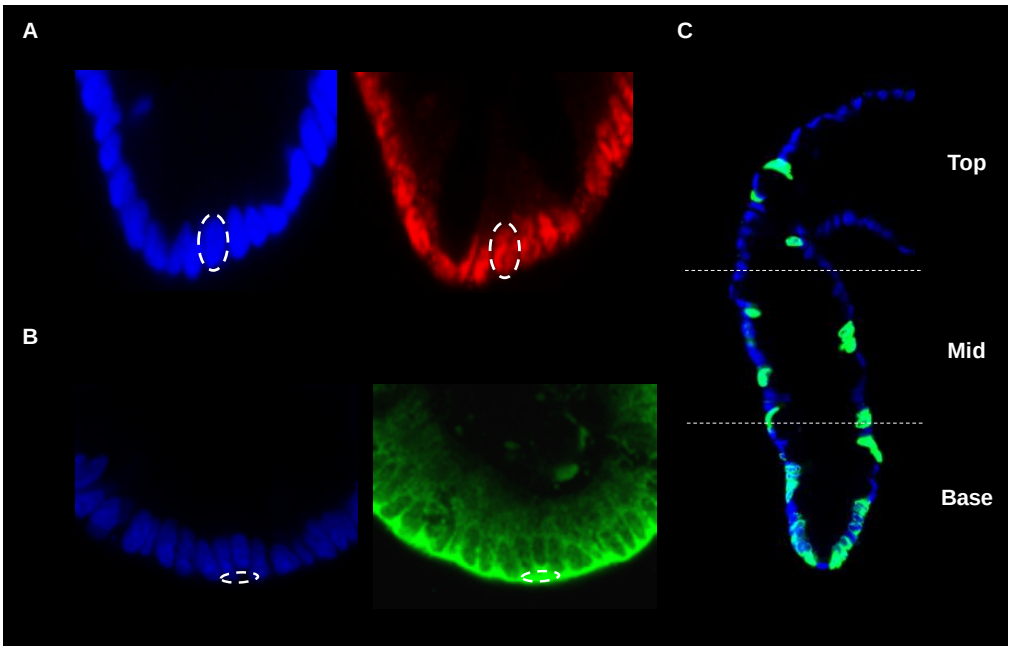


Figure 2.4 Crypt analysis. *A) Region of interest (ROI) placed around the nucleus to measure fluorescence intensity levels. B) ROI placed around basal cell membrane to measure fluorescence intensity levels. C) BrdU positive cells (green) in the 3 regions of the crypt used for analysis.*

Chapter 3 Results

3.1 Wnt signalling regulated tissue renewal

3.1.1 Introduction

The colonic epithelium undergoes constant homeostatic renewal with 10 billion cells being shed and replaced by intestinal stem cell progeny every day. The self-renewing unit of the tissue is exquisitely organised into invaginations known as crypts. This perpetual tissue renewal is thought to minimise the accumulation of (epi)genetic changes that would otherwise occur in a more stable population of intestinal epithelial cells. Tissue renewal of the crypt involves the asymmetric or symmetric division of stem cells at the base of the crypt, with the transit amplifying progeny migrating up the crypt-axis and differentiating into one of four cell lineages: absorptive enterocyte cells, mucous secreting goblet cells, hormone secreting enteroendocrine cells, prostanoid secreting tuft cells, and in the small intestine Paneth cells which are involved in innate immune response and stem cell niche maintenance. On reaching the top of the crypt, these cells are shed into the gut lumen and undergo apoptosis.

One of the major regulators involved in the establishment of crypt renewal and homeostasis is the canonical Wnt signalling pathway. The canonical Wnt pathway is involved in processes such as the maintenance of stem/progenitor cells via cell cycle control and inhibition of differentiation, regulation of migration and localization of epithelial cells along the crypt-axis and directing early secretory lineage development. The importance of the Wnt signalling pathway in maintaining crypt homeostasis was shown by Kuhnert *et al*. (2) who demonstrated that adenoviral expression of Dkk1, a Wnt antagonist resulted in inhibition of proliferation and loss of crypts in the colon and small intestine. Loss of TCF4 early in development and in adult colon also results in increased cell proliferation and leads to colon tumourigenesis (164).

3.1.2 Wnt signalling status in native colonic crypts

To investigate Wnt signalling status in native human colonic crypts, biopsy size tissue samples were obtained from the sigmoid colon of patients undergoing a colonoscopy or from the macroscopically normal regions of the sigmoid colon of patients undergoing surgery for cancer. Fixed biopsies were then microdissected into individual crypts and immunolabelling for β-catenin was performed on the tissue to determine the *in vivo* status of Wnt signalling pathway (Fig 3.1). Nuclear immunofluorescence intensity was measured using Image J software and the measurements were normalised to the immunofluorscence intensity at the base of the crypt. Nuclear β-catenin predominated at the crypt base and exhibited an immunofluorescence intensity gradient that diminished along the crypt-axis.

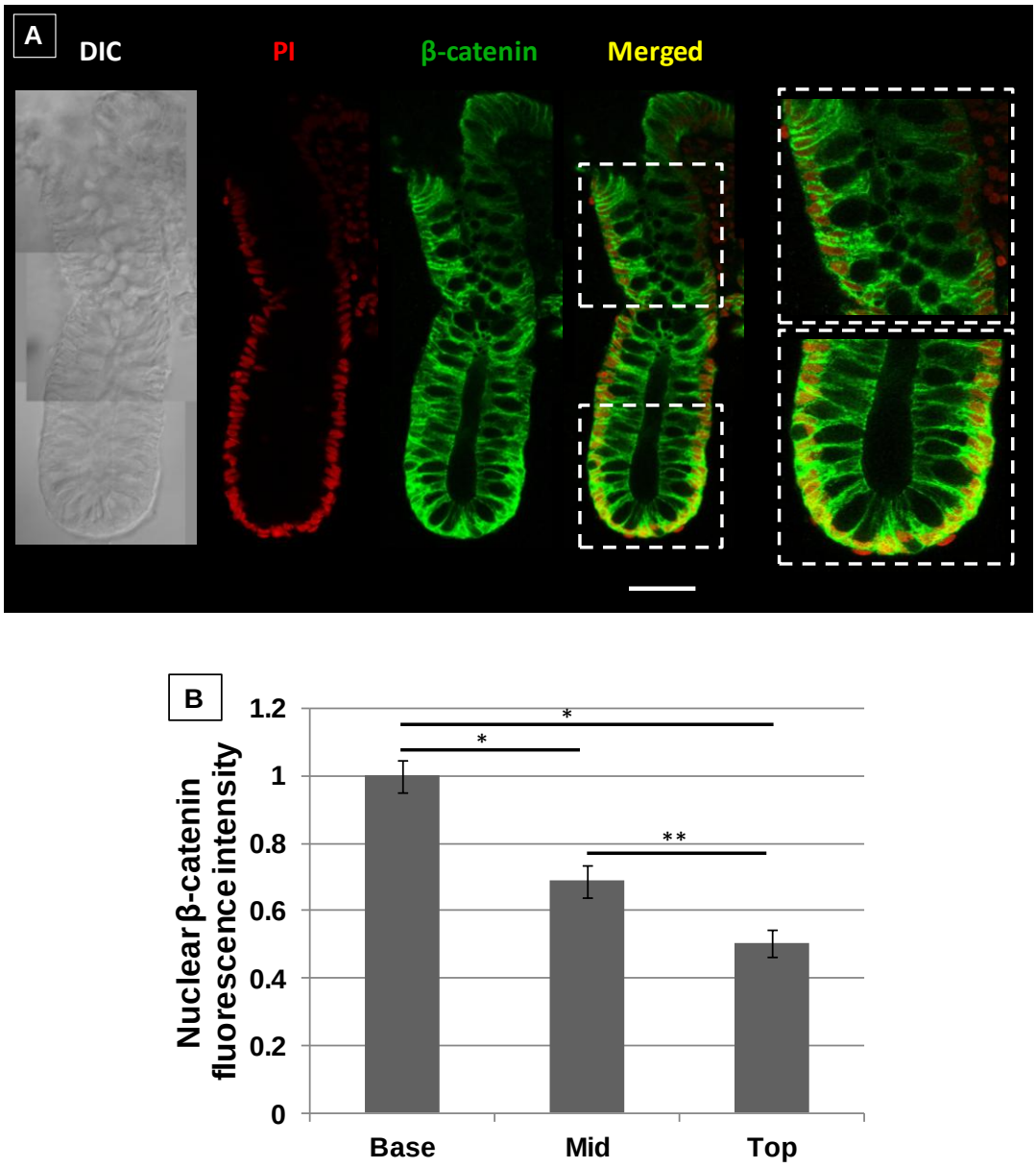


Figure 3.1Wnt signalling status in native colonic crypts. *A) Nuclear β-catenin is the hallmark of activated Wnt signalling. Scale bar on figure represents 50μm. B) Analysis of nuclear β-catenin along the crypt axis. Nuclear fluorescence intensity measurements from each region of the crypt were normalised to the crypt base. The fluorescence intensity of nuclear β-catenin predominates at the crypt base and diminishes progressively towards the top of the crypt. (*p=0.0, **p=0.012, N=123 crypts from 14 patients).*

The *in vivo* status of Wnt target genes axin II and c-Myc was also detected in colonic crypts using immunocytochemistry. Axin II is a negative regulator of the Wnt signalling pathway and down regulates β-catenin by facilitating its phosphorylation by GSK3β (245). Increased levels of axin II mRNA were found in human colon tumour samples and cell lines, indicating that axin II could be used as a marker of activation of Wnt signalling pathway (246). The oncogene c-Myc has also been identified by He *et al.* (247) as a Wnt signalling target gene. Rochlitz *et al.* (248) have shown that c-Myc is amplified in metastatic progression of colorectal cancer. Fig 3.2 shows that axin II expression is nuclear with higher expression at the crypt base and diminishes towards the top of the crypt and Fig 3.3 shows that c-Myc expression is also nuclear with higher expression at the crypt base.

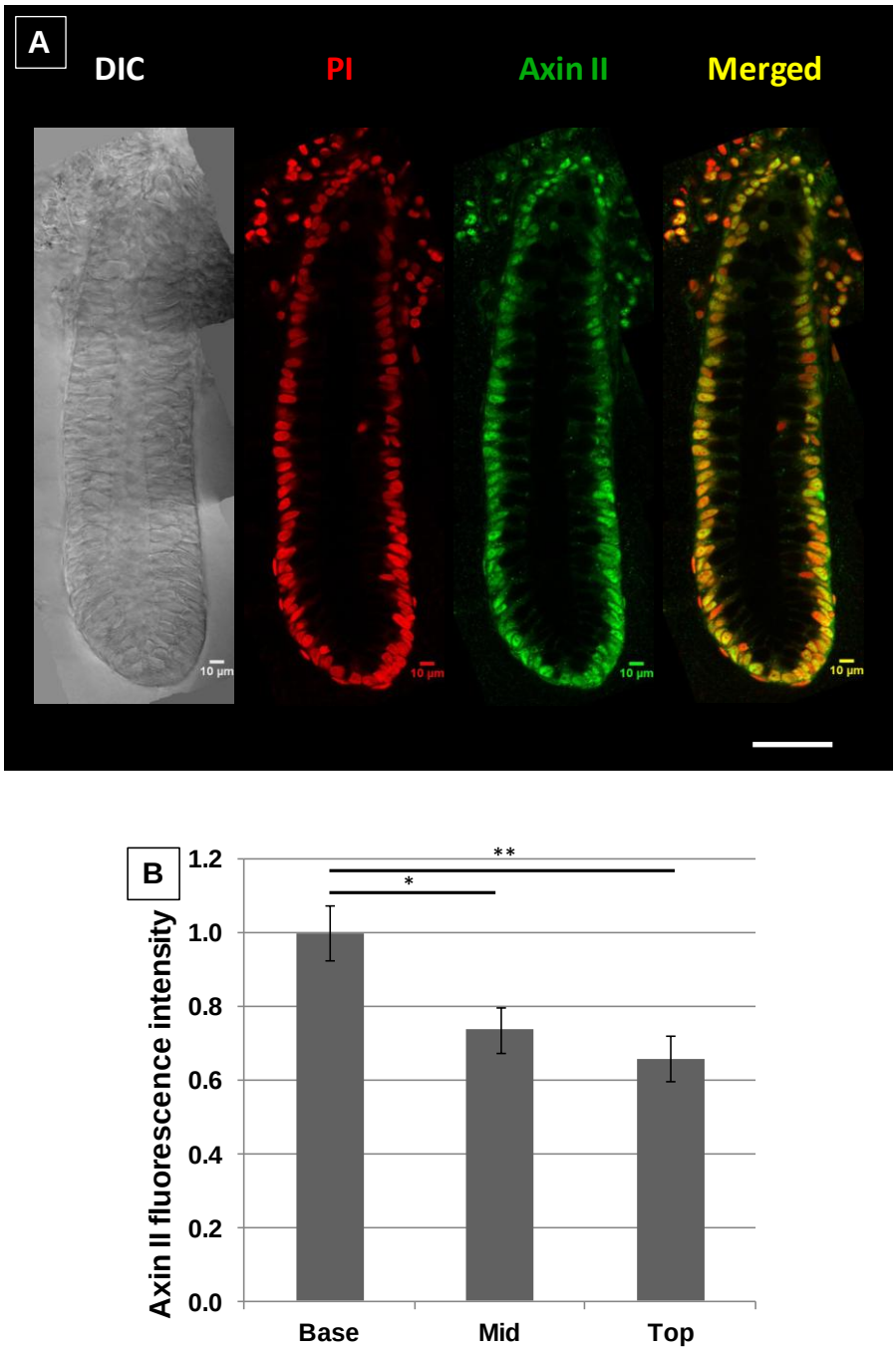
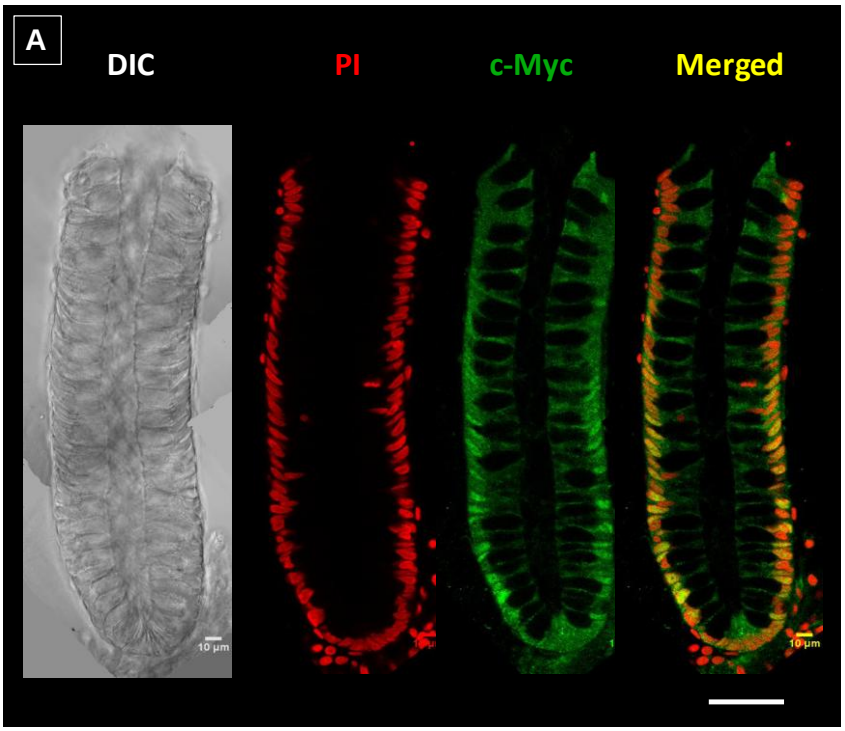


Figure 3.2 Expression of Wnt target gene axin II in native crypts. *A) Nuclear axin II is a marker of activated Wnt signalling. Scale bar represents 50µm. B) Analysis of axin II expression along the crypt axis. Nuclear fluorescence intensity measurements from each region of the crypt were normalised to the base of the crypt. (*p=0.016, **p=0.001, N=31 crypts from 9 patients).*



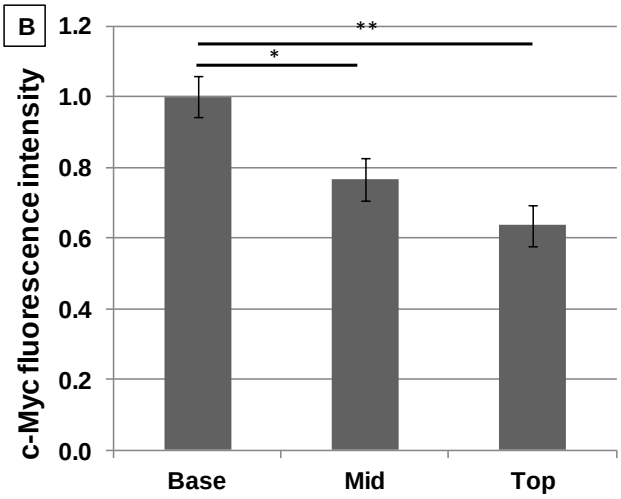


Figure 3.3 Expression of Wnt target gene c-Myc in native crypts. *A) c-Myc is a Wnt target gene demonstrating activated Wnt signalling. Scale bar represents 50µm. B) Analysis of c-Myc expression along the crypt axis. Nuclear fluorescence intensity measurements from each region of the crypt were normalised to the base of the crypt. Higher expression of c-Myc can be seen at the base of the crypt. (*p=0.016, **p=0.0, N=41 crypts from 13 patients).*

3.1.3 Functional role of Wnt signalling in human colonic crypt renewal

In order to investigate the functional influence of the Wnt signalling pathway on renewal of the human colonic epithelium we refined a native human colonic culture model. Using the signalling pathway profiles described in this chapter (Wnt) and the following chapter (BMP/TGFβ), and the conditions developed for intestinal organoid propagation (20) (220), the optimisation for the culture conditions was based on generating Wnt signals and suppressing BMP/TGFβ signals *ex vivo*. To determine the factors required to maintain near-native crypt length, topology, morphology and polarity, a range of growth factors in various combinations were tested (Fig 3.4).

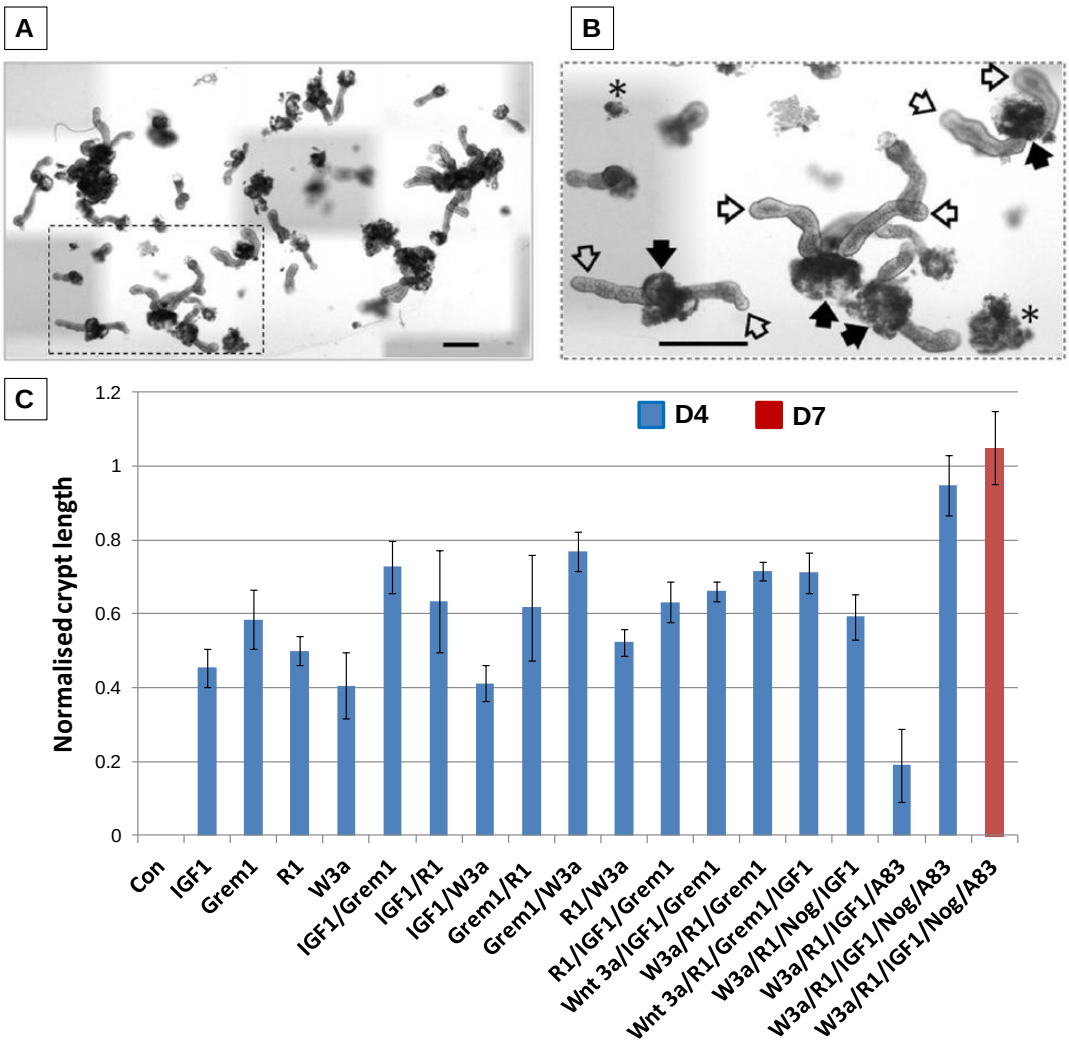


Figure 3.4 A combination of Wnt pathway activators and BMP/TGFβ pathway inhibitors is required for maintenance of native human colonic crypts ex vivo.

*A) Overview of human colonic crypts cultured within a matrigel droplet under optimised conditions. B) Enlargement view of cultured crypts representing a typical field of view (x4 objective lens). Example of crypt base and shedding domains are denoted by open and closed arrowheads respectively, * denotes dead crypt fragments. Scale bar represents 0.5mm. C) Quantification of crypt length at day 4 or 7 following culture in the presence of the indicated combination of recombinant human growth factors, recombinant human BMP binding protein and/or small molecule ALK 4/5/7 inhibitor: IGF-1 (50ng/ml), Gremlin-1(200ng/ml), Noggin (100ng/ml), Wnt3a (100ng/ml), R-Spondin-1 (500ng/ml), A83-01 (0.5μM). (N=130 crypts from 3 patients).*

Canonical Wnt pathway activation by Wnt3a and/or R-Spondin 1 was insufficient to maintain human colonic crypt length or viability beyond 4 days. However, in combination with the BMP antagonist Gremlin-1 or Noggin, the intestinotrophic factor IGF-1 and an ALK 4/5/7 inhibitor A83-01, crypts maintained their length and morphology for at least 7 days in culture, when the matrigel became unstable. Replacing IGF-1 with EGF promoted re-modelling of human colonic crypt morphology followed by multiple budding events characteristic of intestinal organoid growth (20) (Fig 3.5).

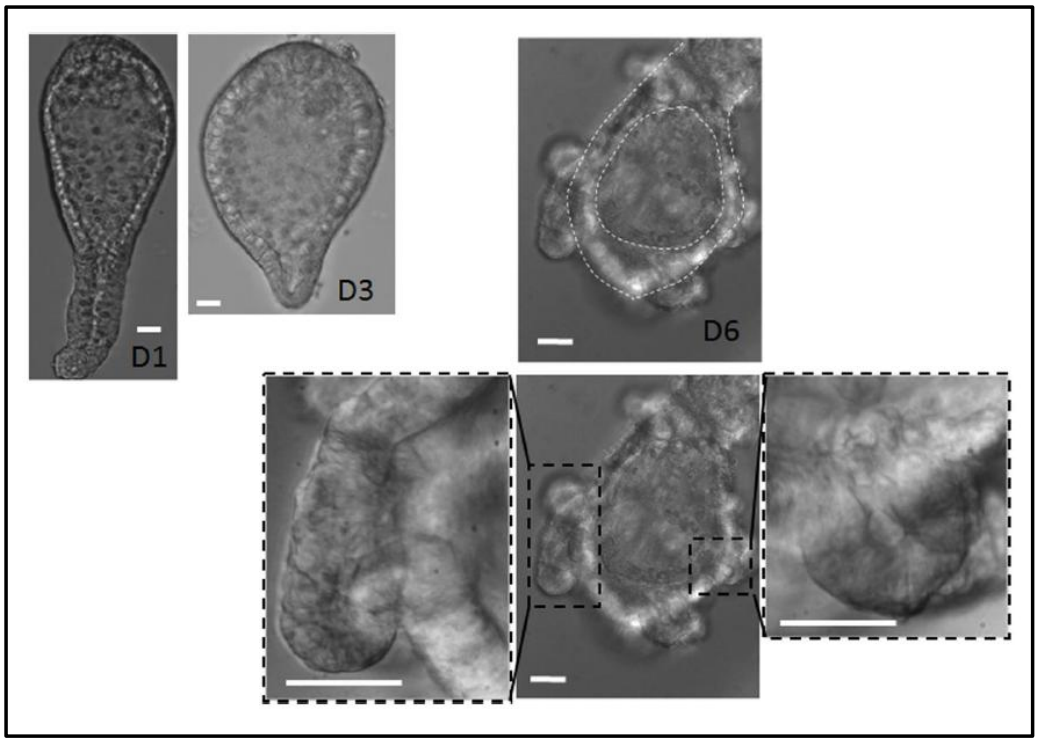


Figure 3.5 Effects of EGF on human colonic crypt morphology. *Replacement of IGF-1 with EGF in the colonic crypt culture media induced re-modelling of human colonic crypt morphology into typical budding organoid structures. Day 6 magnified image of new crypt buds. Scale bar represents 50μm.*

To determine activation of the Wnt signalling pathway and its role in maintaining crypt homeostasis, single crypts were liberated from biopsies removed from patients undergoing colonoscopy or surgery, placed into culture and treated with Wnt3a in the presence or absence of the Wnt antagonist, Dkk1. Dkk1 forms a ternary complex with LRP5/6 and Kremen which causes endocytosis of this complex and removal of LRP5/6 from the cell surface. The cultured crypts were then fixed and processed by immunocytochemistry for active β-catenin (de-phospho) (Fig 3.6). Nuclear β-catenin is the hallmark of activated Wnt signalling and there is a low level of β-catenin seen in the control group, which demonstrates endogenous levels of β-catenin *ex vivo* after 3 days in culture, stimulation with exogenous recombinant Wnt3a increased the levels of β-catenin. The antagonist Dkk1 prevented Wnt stimulated nuclear translocation of β-catenin. Analysis was carried out by measuring the nuclear fluorescence intensity of β-catenin and the measurements were normalised to the crypt base of the control group (Fig 3.6B).

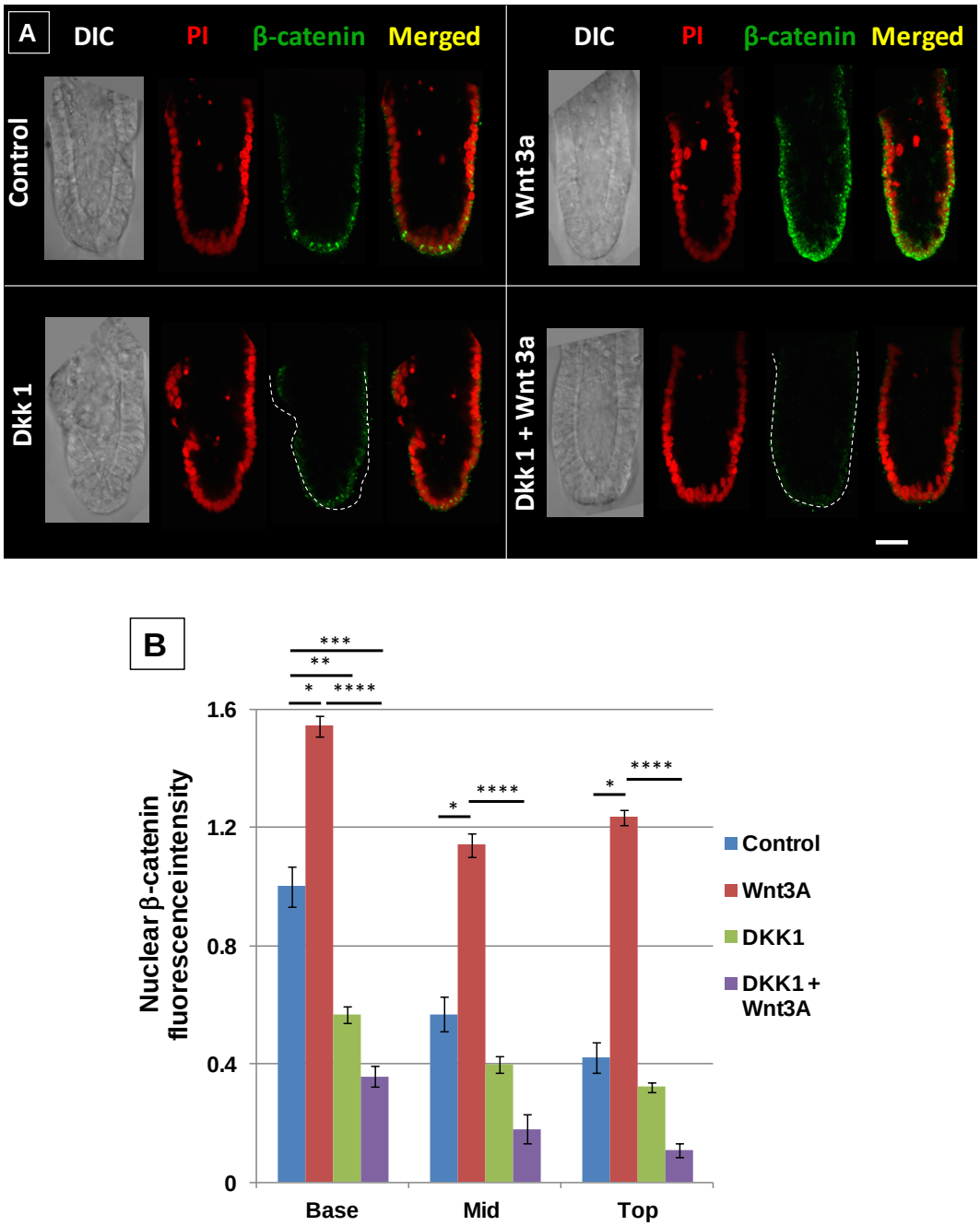


Figure 3.6 Dkk1 inhibits Wnt signalling. *A) Crypts were cultured in the presence of Wnt3a ligand and/or the Wnt antagonist Dkk1 for 3 days. Wnt3a stimulates de-phospho nuclear β-catenin levels which are inhibited by Wnt inhibitor Dkk1. B) Analysis of β-catenin in the different experimental groups. Nuclear fluorescence intensity measurements from each region of the crypt were normalised to the crypt base of the control group. (*p≤0.02 in all regions of crypt, **p=0.001, ***p=0.005, ****p≤0.007 in all regions of crypt, N=46 Crypts from 3 patients). Scale bar represents 50μm. Control media: IGF-1*

(50ng/ml), Noggin (100ng/ml), R-Spondin 1 (500ng/ml); Wnt3a (100ng/ml) and Dkk1 (800ng/ml) where indicated.

Another method of assessing Wnt signalling status in crypt cells is by using a Lenti TCF/LEF reporter which is a preparation of ready-to-transduce lentiviral particles which monitor the activity of Wnt signalling in cells. The Lenti TCF/LEF reporter is a preparation of replication incompetent, VSV-g pseudotyped lentivirus particles expressing the GFP gene under the control of a minimal (m)CMV promoter and tandem repeats of the TCF/LEF transcriptional response element (TRE) which are activated by the binding of β-catenin (244). To determine the status of Wnt signalling in cultured crypt, isolated crypts were transduced with Lenti TCF/LEF (TOP-GFP) reporter, cultured for 3 days in the different experimental conditions. Wnt signalling was stimulated by recombinant Wnt3a ligand and inhibited with IWP2. IWP2 prevents palmytoylation of Wnt proteins by Porcupine, a membrane bound O-acyltransferase, and so blocks Wnt secretion and activity. In parallel, some crypts were also transduced with Lenti-CMV-GFP which was used as a positive control for the TOP-GFP reporter. The cultured crypts were fixed after 3 days and processed by immunocytochemistry for GFP so that the signal could be amplified and visualised easier. Analysis was carried out by measuring fluorescence intensity and normalised to the base of the control group. There is a slight gradient along the crypt axis which reflects the status of β-catenin, with higher levels at the crypt base and diminishing towards the top. IWP2 inhibited endogenous Wnt signalling, which could be rescued by addition of Wnt3a ligand (Fig 3.7).

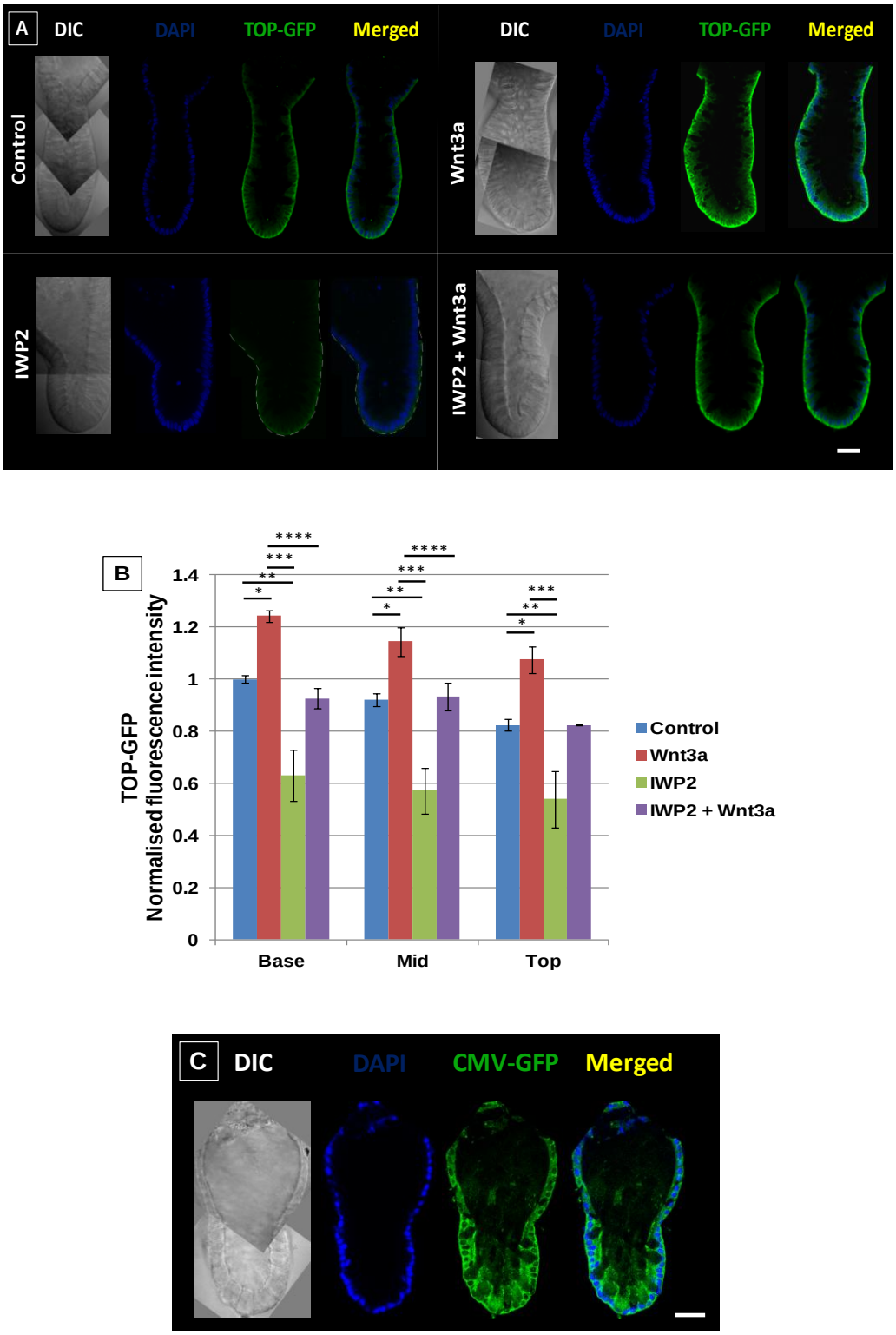
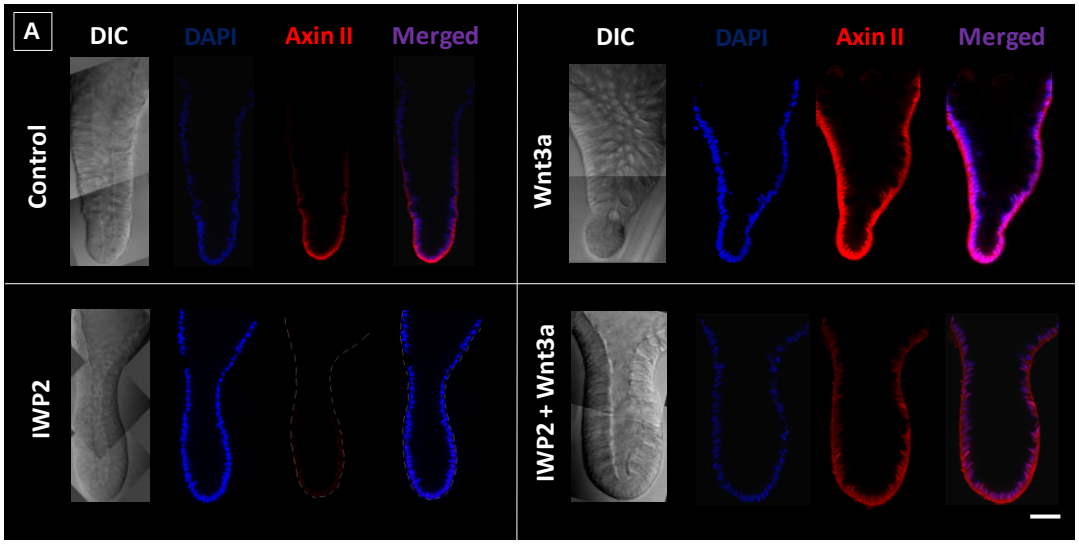


Figure 3.7 IWP2 inhibits endogenous Wnt signalling. *A) Cultured crypts were transduced with Lenti-TOP-GFP and cultured in the presence of Wnt3a ligand and/or the Wnt antagonist IWP2 for 3 days. IWP2 inhibits endogenous levels of Wnt signalling which can be rescued by adding exogenous Wnt3a ligand. Scale bar represents 50μm. B)*

*Analysis of TOP-GFP signal in the different experimental conditions. Fluorescence intensity measurements from each region of the crypt were normalised to the mean value at the base of the control group. (*p<0.05 between control and wnt3a in all regions of crypt, **p<0.05 between control and IWP2 in all regions of crypt, ***p<0.05 between wnt3a and IWP2 in all regions of crypt, ****p<0.05 between wnt3a and IWP2+Wnt3a in base and mid regions of crypt, N=21 crypts from 2 patients) Control media: IGF-1 (50ng/ml), Noggin (100ng/ml), R-Spondin 1 (500ng/ml); Wnt3a (100ng/ml), IWP2 (5μM) where indicated. C) In parallel, crypts were transduced with Lenti-CMV-GFP as a positive control. All cells within the crypt took up the lentivirus, demonstrating that the Lenti-TOP-GFP only labels cells that are active in Wnt Signalling.*

The effect of Wnt signalling inhibition with IWP2 was also determined on the Wnt target gene axin II. Isolated crypts were cultured for 3 days in media containing Wnt3a and/or IWP2. Crypts were then fixed and processed by immunocytochemistry for axin II (Fig 3.8). Analysis was carried out by measuring nuclear fluorescence intensity of axin II and measurements were normalised to the mean of the base of the crypt in the control group. IWP2 inhibited axin II expression which could be rescued by addition of exogenous Wnt3a.



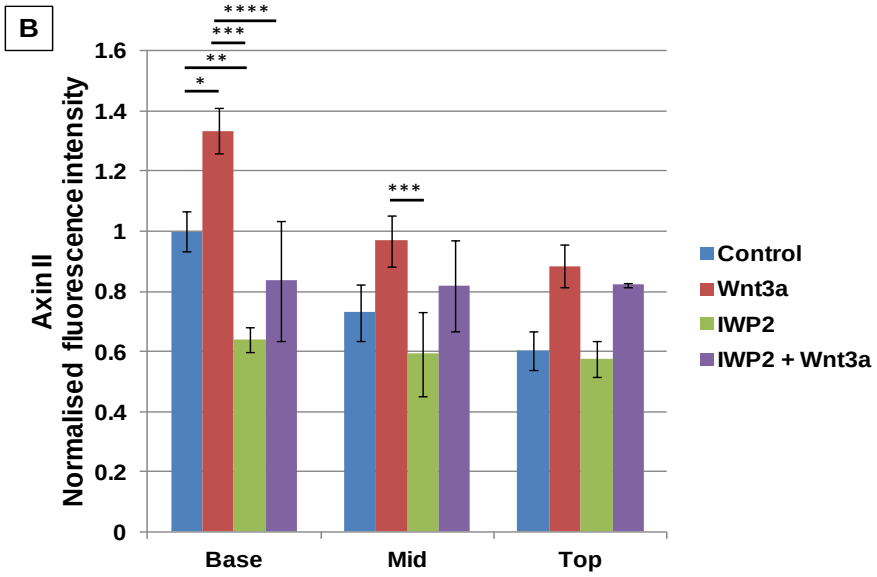
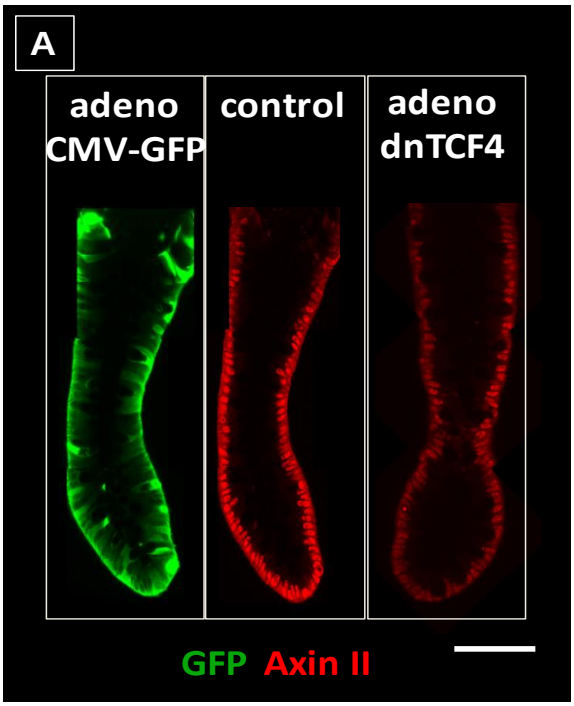


Figure 3.8 IWP2 inhibits Wnt target gene axin II expression. *A) Crypts were cultured in the presence of Wnt3a and/or the Wnt antagonist IWP2 for 3 days. Scale bar represents 50μm. B) Analysis of axin II expression in the different experimental conditions. Nuclear fluorescence intensity measurements from each region of the crypt were normalised to the mean value of the base of the crypt of the control group. (*p=0.037, **p=0.042, ***p≤0.026, ****p=0.038, N=21 crypts from 2 patients). Control media: IGF-1 (50ng/ml), Noggin (100ng/ml), R-Spondin 1 (500ng/ml); Wnt3a (100ng/ml), IWP2 (5μM) where indicated.*

Another approach to blocking Wnt signalling is by transducing with a dominant negative TCF4 construct. Cultured crypts were transduced with human dn-TCF4 adenovirus and placed into culture for 3 days. In parallel, crypts were also transduced with adeno-CMV-GFP as the positive control for the experiment. The adenovirus intake into the cells of the crypt was on average 65%. The effects of dn-TCF4 adenoviral inhibition on the Wnt target gene axin II expression was determined using immunocytochemitry. Axin II expression was significantly reduced upon transduction with dn-TCF4 (Fig 3.9).



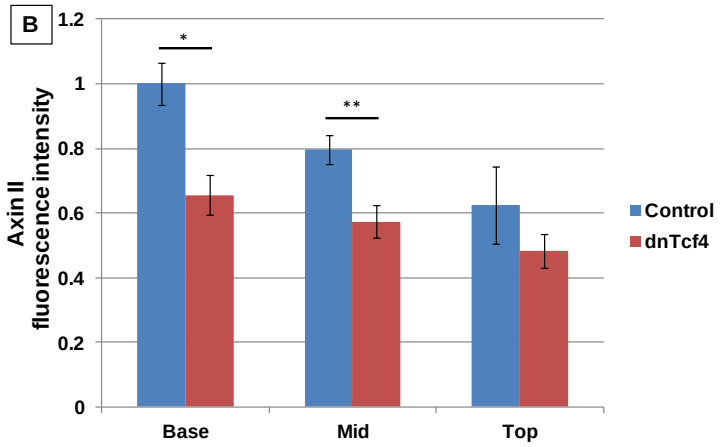


Figure 3.9 dnTCF4 inhibits Wnt target gene axin II expression. *A) Crypts were transduced with adenoviral dnTCF4 or CMV-GFP (as a positive control for adenoviral transduction) and placed into culture for 3 days. dnTCF4 significantly inhibited axin II expression. Scale bar represents 50μm. B) Analysis of axin II expression. Nuclear axin II immunofluorescence intensity measurements from each region of the crypt were normalised to the crypt baseof the control group. (*p=0.006, **p=0.011, N=10 crypts from 2 patients). Control media: IGF-1 (50ng/ml), Noggin (100ng/ml), R-Spondin 1 (500ng/ml), Wnt3a (100ng/ml).*

Crypt renewal is maintained by the proliferation of cells at the base of the crypts, be they stem or progenitor cells which then migrate up the crypt axis, differentiate and shed at the top of the crypt. The influence of Wnt signalling on crypt cell proliferation was studied by by the incorporation of BrdU, a thymidine analogue. Isolated crypts which had been cultured for two days in the different experimental groups had their media changed with that which contained BrdU for the next 24 hrs. Crypts were then fixed and processed by immunocytochemistry for BrdU (Fig 3.10). The number of BrdU positive cells was then manually counted on the equatorial plane of each crypt and the percentage of BrdU positive cells of the total number of cells was then calculated. Analysis shows that proliferation is increased by Wnt3a and Dkk1 prevents the rescue by exogenous Wnt3a ligand (Fig 3.10). R-Spondin 1 and Wnt3a however rescue the inhibitory effects of IWP2 (Fig 3.11).

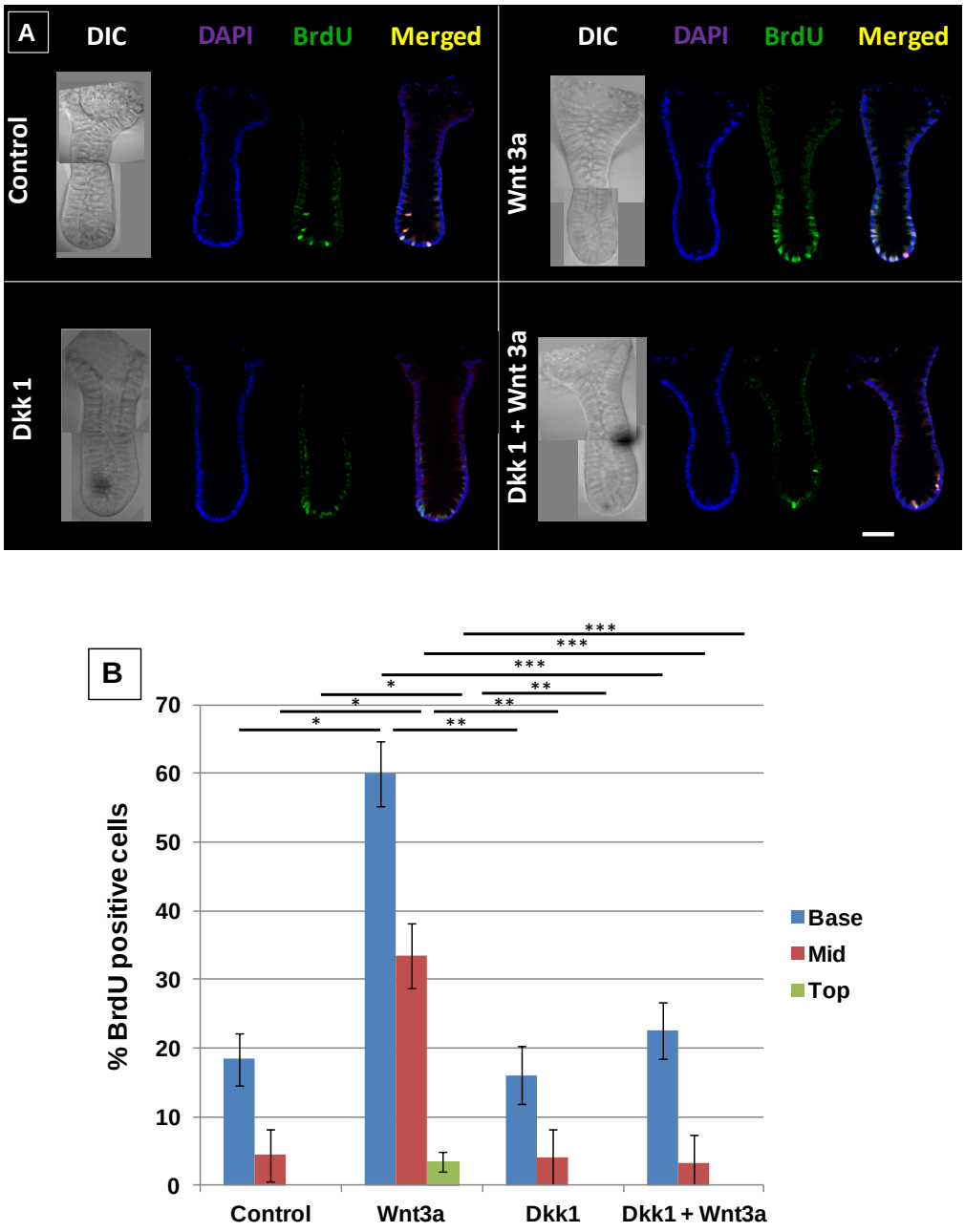


Figure 3.10 Wnt3a stimulates proliferation. *A) Crypts were cultured in Wnt3a ligand and/or the Wnt antagonist Dkk1 for 3 days. On day 2 of the experiment, BrdU, a marker of proliferation, was added to the media. Wnt3a stimulates crypt proliferation which is inhibited by Dkk1. There is a proliferation gradient along the crypt axis which is maintained by Wnt signalling. Scale bar represents 50μm. B) Percentage of BrdU-positive cells in the crypts of the different experimental conditions. (*p≤0.022 in all regions of crypt **p≤0.021 in all regions of crypt, ***p≤0.019 in all regions of crypt,*

N=23crypts from 2 patients). Control media: IGF-1 (50ng/ml), Noggin (100ng/ml), R-Spondin 1 (500ng/ml); Wnt3a (100ng/ml) and Dkk1 (800ng/ml) where indicated.

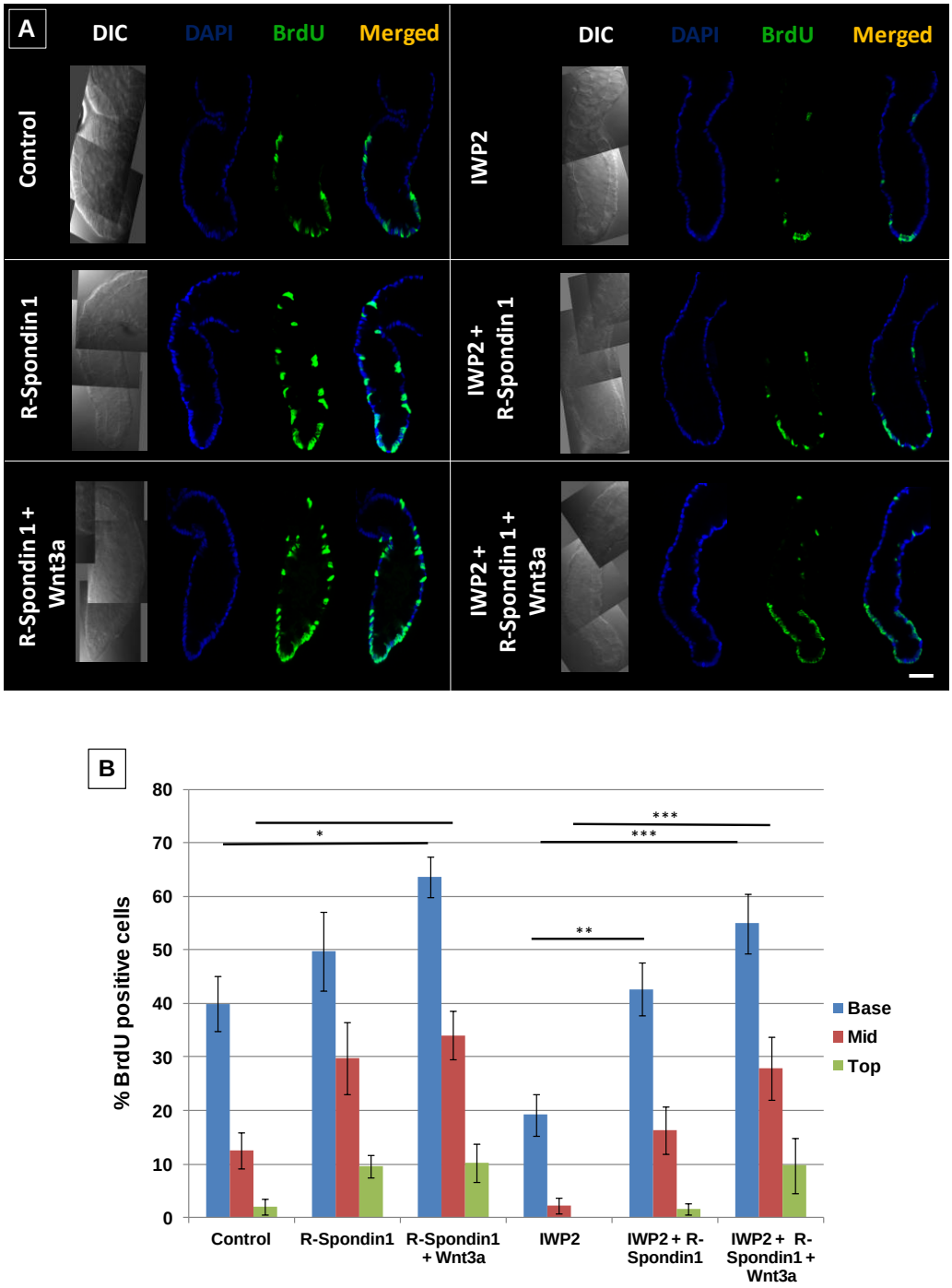


Figure 3.11 IWP2 inhibits proliferation. *A) Crypts were cultured in R-Spondin1, Wnt3a and/or IWP2 for 3 days. On day 2 of the experiment, BrdU was added to the media. Exogenous Wnt3a and R-Spondin 1 rescue the inhibitory effects of IWP2. Scale bar*

*represents 50μm. B) Percentage of BrdU positive cells in the crypts of the different experimental conditions. (*p≤0.018, **p=0.038, ***p≤0.01, N=36 crypts from 2 patients). Control media: IGF-1 (50ng/ml), Noggin (100ng/ml); R-Spondin 1 (500ng/ml), Wnt3a (100ng/ml) and IWP2 (5μM) where indicated.*

As mentioned previously, another method of inhibiting Wnt signalling is by transducing cells within the crypt with dnTCF4 adenovirus. Cultured crypts were transduced with dnTCF4 adenovirus, whilst in parallel the control group was transduced with CMV-GFP adenovirus. This parallel transduction was the positive control to demonstrate whether the adenovirus particles infected the cells. After culturing for 3 days, crypts were fixed and processed by immunocytochemitry for Ki67, a proliferation marker, and GFP, to amplify the signal (Fig 3.12). Percentage of proliferative cells was determined by counting the number of Ki67 positive cells against the total number of cells within the crypt. dnTCF4 significantly reduced proliferation at the base of the crypts.

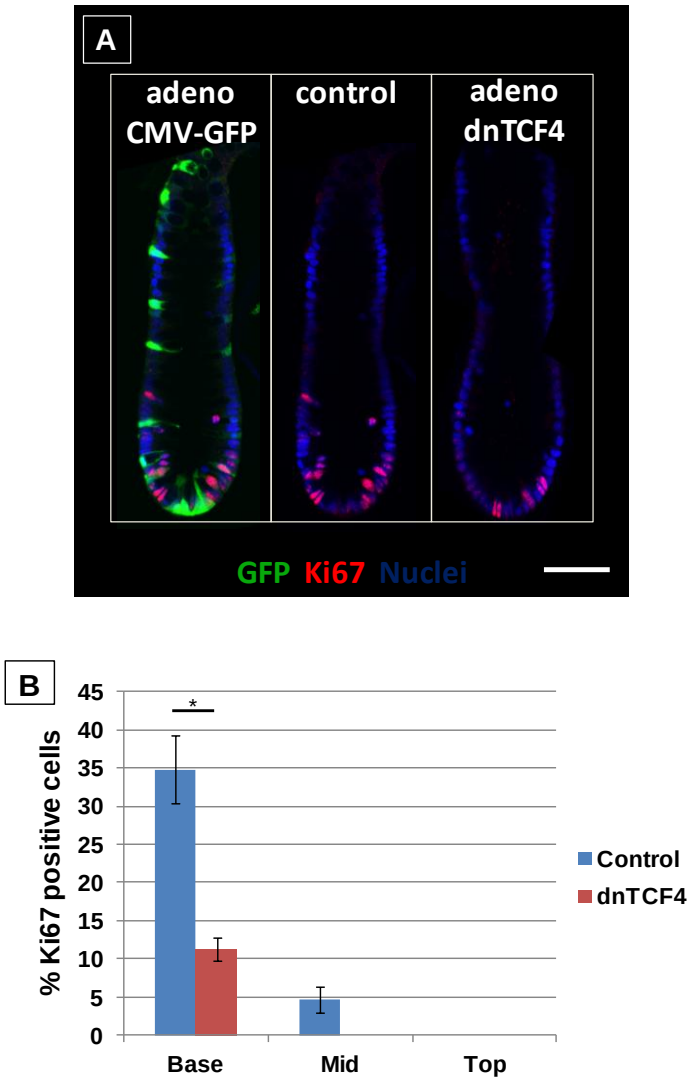
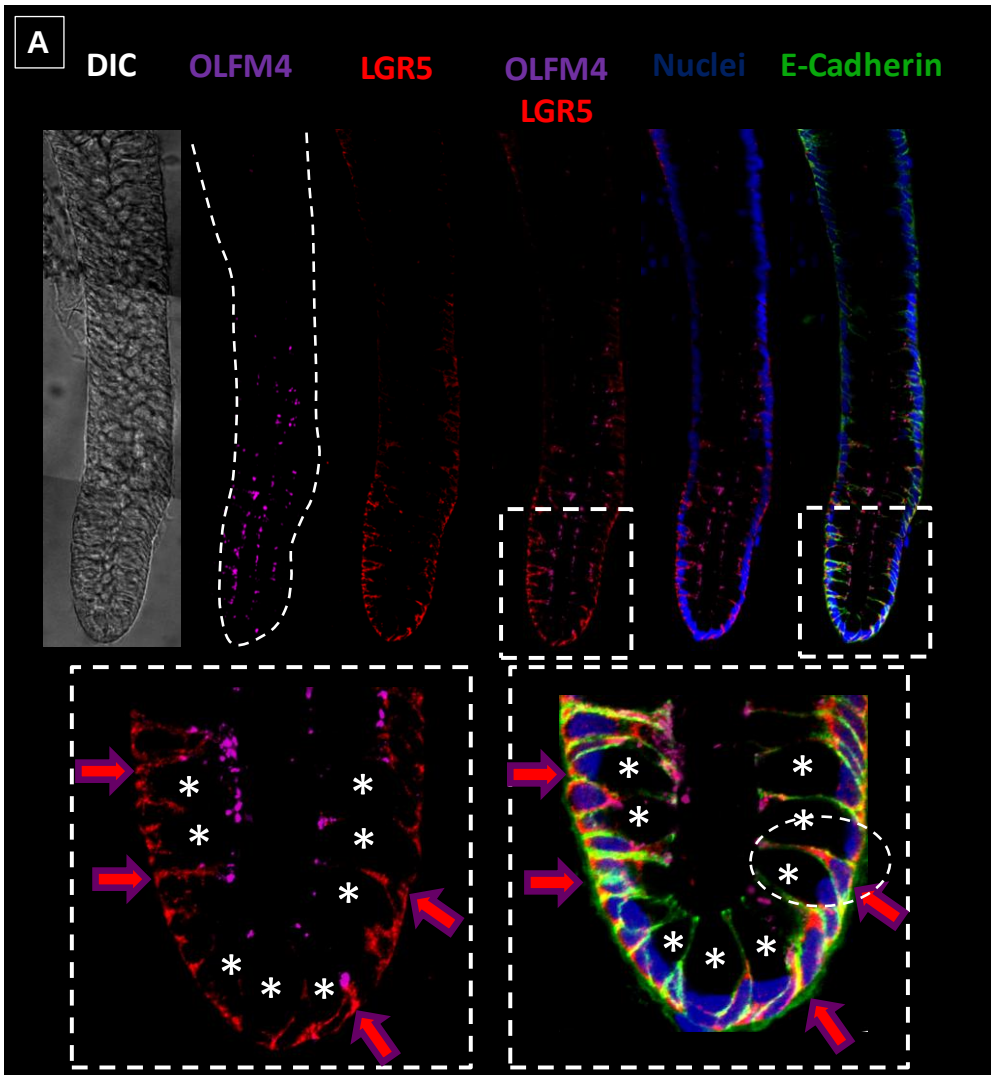


Figure 3.12 dnTCF4 inhibits proliferation. *A) Cultured crypts were transduced with adenoviral dnTCF4 and placed into culture for 3 days. In parallel, the control crypts were infected with CMV-GFP adenovirus. dnTCF4 inhibits proliferation, as determined by counting the percentage of Ki67- positive cells. Scale bar represents 50μm. B) Percentage of Ki67-positive cells in control and dnTCF4 crypts. dnTCF4 significantly inhibited proliferation at the base of the crypts.(*p=0.009, N=15 crypts from 2 patients). Control media: IGF-1 (50ng/ml), Noggin (100ng/ml), R-Spondin 1 (500ng/ml), Wnt3a (100ng/ml).*

3.1.4 Wnt signalling maintains stem/progenitor cells in human colonic crypts

Renewal of intestinal epithelium is driven by stem cells that reside at the base of the colonic crypts. The progeny of these stem cells differentiate into one of four lineages, enteroendocrine, goblet, enterocyte and tuft cells. This renewal is maintained by several signalling pathways, including Wnt, BMP, TGFβ and Notch. Wnt signalling plays a major role in proliferation of stem cells and in determining the fate of progeny of these stem cells. Lgr5, a Wnt target gene, has been identified by Barker *et al.* (4) as an intestinal stem cell marker. Van der Flier *et al.* (48) later identified that these $Lgr5^+$ stem cells were also enriched for Olfactomedin-4 (OLFM4) and could be used to identify the Lgr5-type crypt base columnar cells. Therefore, it would be informative to determine the status of these $Lgr5^+$ and $OLFM4^+$ stem cells within the human native microdissected crypts and whether Wnt signalling is involved in maintaining the stem cell population *ex vivo*. To demonstrate that both Lgr5 and OLFM4 labelled the same population of cells, colonic tissue biopsies were fixed , microdissected into single crypts and processed by immunocytochemistry for Lgr5 and OLFM4 (Fig 3.13). As can be seen, the $Lgr5^+$ and $OLFM4^+$ cells label the same population of cells at the base of the crypt. These cells are slender and almost triangular shaped, as indicated by the arrows. E-cadherin was used to label the cell membranes to make visualisation of these slender single cells easier. Lgr5 is a G-protein coupled receptor, and is expressed on the basal membrane of the cells. OLFM4 is a secreted molecule under the control of Notch signalling and is located in the apical cytoplasm and crypt lumen. There is a gradient of stem cells along the crypt-axis with about 30% of cells being $Lgr5^+/OLFM4^+$ at the base of the crypt, about 20% in the mid region and none at the top of the crypt (Fig 3.13B). Other differentiated cell types are found mostly in the mid and top regions of the crypt, with morphologically-identified goblet cells distributed fairly evenly across the three regions of the crypt. A 3D reconstructed movie of the base of the crypt can be seen in appendix: movie 1.



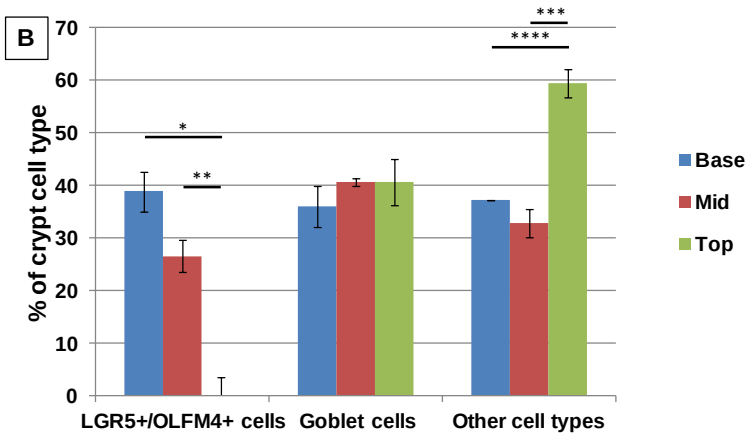


Figure 3.13 Status of Lgr5/OLFM4^{+} stem cells in native colonic crypts. *A) Biopsies removed from patients undergoing colonoscopy or surgery were fixed and microdisscted into individual crypts. The status of Lgr5 and OLFM4 stem cells was determined by*

*immunocytochemistry. E-cadherin was used to label cell membranes to clearly indicate single cells within the crypt. Lgr5^{+} and OLFM4^{+} cells labelled the same population of stem cells and resided mostly at the base of the crypt. In the mid region of the crypts there seemed to be a cut-off point where stem cells were no longer found. Arrows indicate cells that are positive for both Lgr5 and OLFM4 and label slender triangular shaped crypt base columnar cells (circled), * denotes goblet cells. B) Analysis of percentage of cell types in the colonic crypt. (*p=0.0, **p=0.002, ***p=0.005, ****p=0.011, N=4 crypts from 2 patients).*

To validate the Lgr5 antibody, *in situ hybridisation (ISH)* for Lgr5 mRNA was carried out by a fellow laboratory member (Alyson Parris). Native microdissected colonic crypts were processed by *ISH* for Lgr5 mRNA and on the same crypts immunocytochemistry was performed for OLFM4 protein. Fig 3.14 shows that Lgr5 mRNA is observed in a few cells that are restricted to the base of the crypt and these cells also co-label with OLFM4. This demonstrates that both Lgr5 antibody and probing for Lgr5 mRNA reveals a similar population of crypt base columnar cells that are restricted to the base of the crypt and co-localise with OLFM4. Therefore OLFM4 is used for most of the experiments within this project since it identifies the same population of intestinal stem cells as Lgr5.

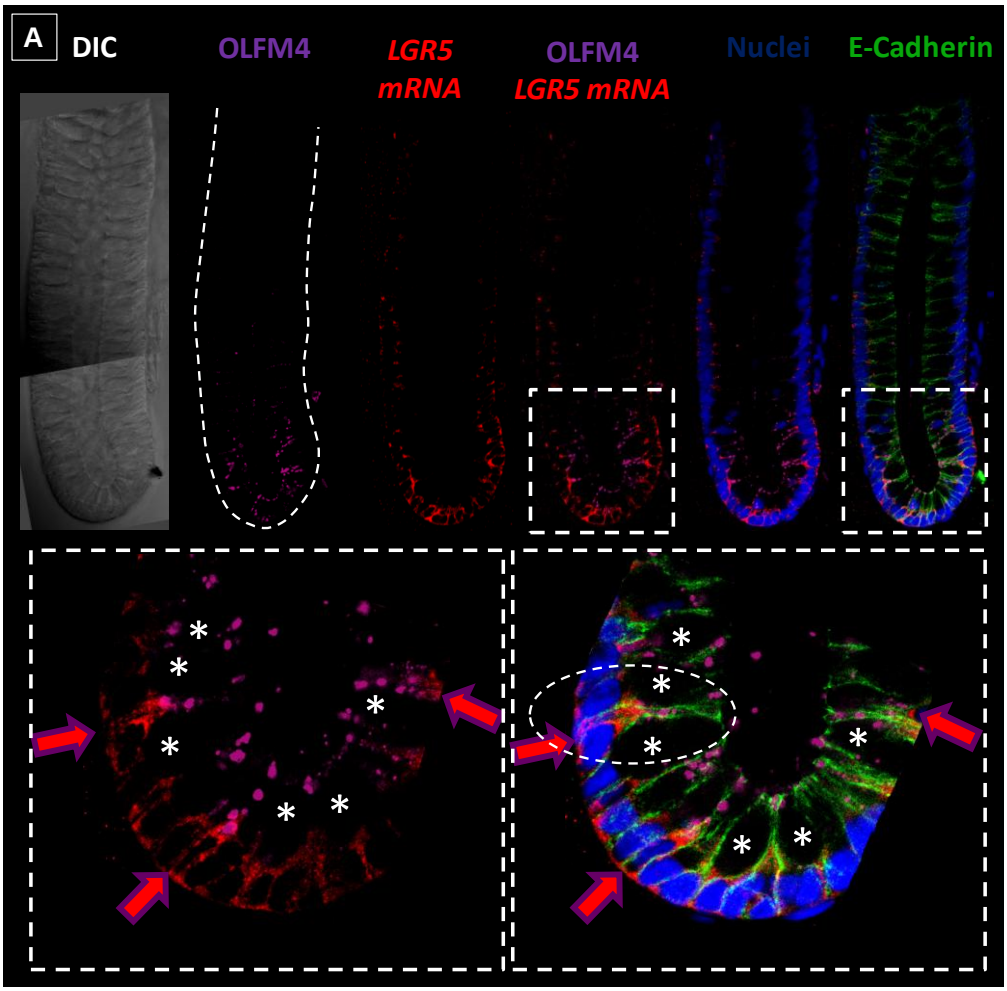


Figure 3.14 Status of OLFM4/Lgr5 mRNA stem cells in native colonic crypts. *A) Biopsies removed from patients undergoing colonoscopy or surgery were fixed and microdisscted into individual crypts. In situ hybridisation was performed to probe for Lgr5 mRNA and immunocytochemistry was used to label for OLFM4^{+} cells. E-cadherin was used to label cell membranes to clearly indicate single cells within the crypt. Lgr5^{+} and OLFM4^{+} cells labelled the same population of stem cells and resided mostly at the base of the crypt. Arrows indicate cells that are positive for both Lgr5 mRNA and OLFM4 and label slender triangular shaped crypt base columnar cells (circled), * denotes goblet cells.*

To demonstrate that these OLFM4$^+$ stem cells did not label other differentiated cells, native microdissected crypts were processed by immunocytochemistry for markers of differentiated cells in combination with OLFM4. Muc2 was used to stain for goblet cells, CroA for enteroendocrine cells, COX1 for Tuft cells and FABP to label enterocyte cells (Fig 3.15). The protein encoded by the Muc2 gene is secreted onto mucosal surfaces and is prominent in the gut where it is secreted from goblet cells and provides an insoluble mucous layer that protects the epithelium. Co-labelling of Muc2 and OLFM4 reveals that OLFM4 is not a maker of goblet cells. Two distinct populations of cells are labelled with the OLFM4$^+$ cells interspaced with goblet cells and mostly residing at the base of the crypt. Chromogranin A is a member of the granin family of neuroendocrine secretory proteins and is located in secretory vesicles of enteroendocrine cells. Double labelling of OLFM4 with Chromogranin A (CroA) shows that only a few cells within the crypts are CroA positive and these do not label the same cells as OLFM4. Tuft cells secrete opiods and produce enzymes that synthesise prostaglandins, therefore COX1 was used to label these cells. Immunocytochemistry revealed a distinct population of tuft cells dispersed along the crypt axis that do not co-label with OLFM4. The fatty acid-binding protein (FABP) is involved in lipid trafficking and metabolism and is highly expressed in enterocyte cells. Labelling of FABP with OLFM shows that these cell types do not label the same population of cells. FABP$^+$ cells are seen along the whole crypt axis whilst OLFM4$^+$ cells are restricted to the base of the crypt. Green arrows in Fig 3.15 indicate an example of a single OLFM4$^+$ cell, which red arrows indicate an example of the differentiated cells: goblet, enteroendocrine, tuft and enterocyte.

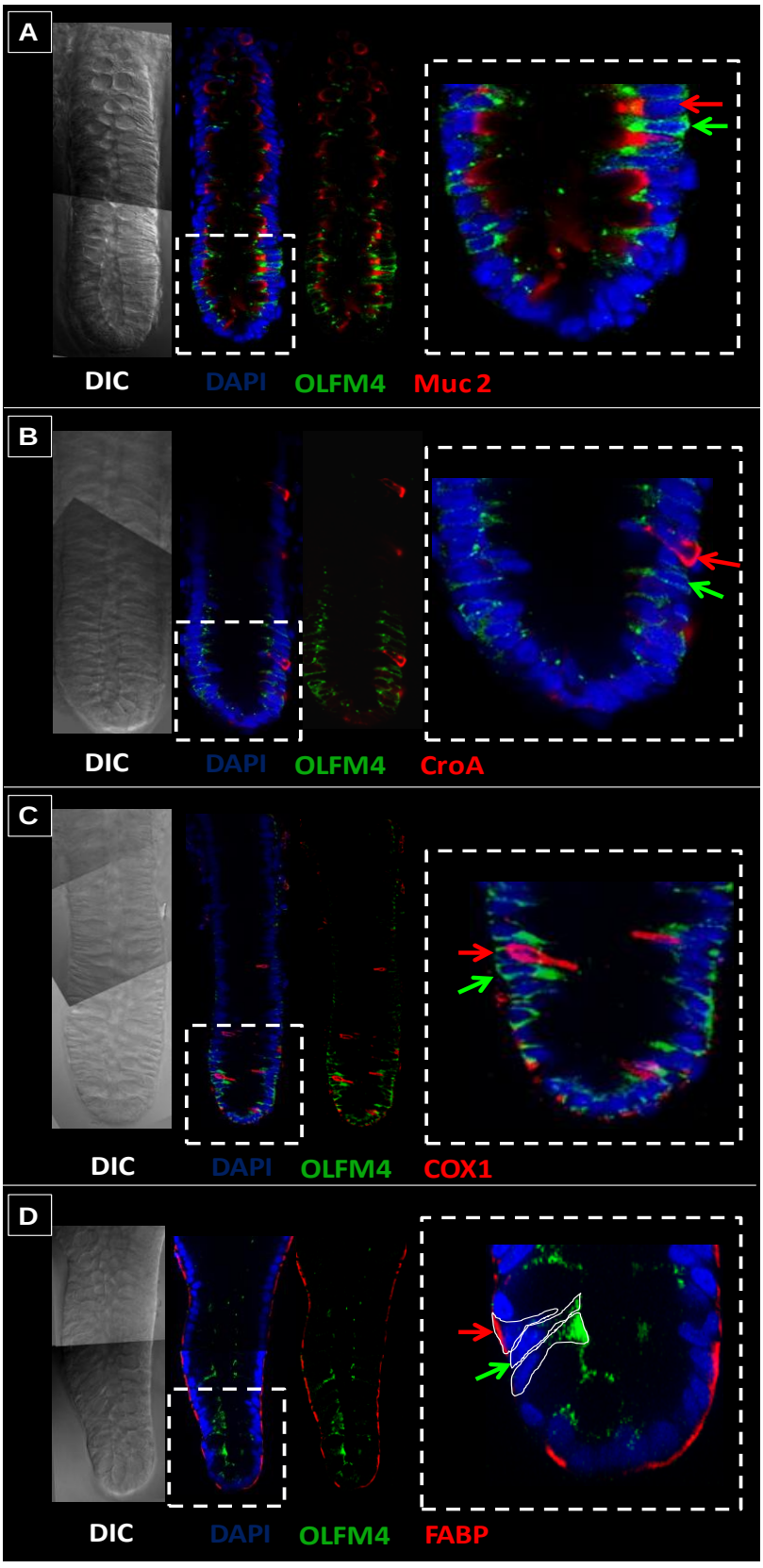


Figure 3.15 OLFM4 does not label differentiated cells. *A) Immunostaining for goblet cell marker Muc2 (red arrow) and OLFM4 (green arrow). B) Immunostaining for*

enteroendocrine cell marker CroA (red arrow) and OLFM4 (green arrow). C) Immunostaining for tuft cell marker COX1 (red arrow) and OLFM4 (green arrow). D) Immunostaining for enterocyte cell maker FABP (red arrow) and OLFM4 (green arrow).

Immunostaining for OLFM4 revealed that stem cells are restricted to the lower third of the crypt. To determine whether these cells are proliferating, native microdissected crypts were processed by immunocytochemistry for OLFM4 and the proliferation marker Ki67 (Fig 3.16). Analysis showed that OLFM4^{+} cells are restricted to the base of the crypt, about 30%, and only half of these cells are proliferating. Only 1% of OLFM4^{+} cells are proliferative in the mid region, and none at the top of the crypt.

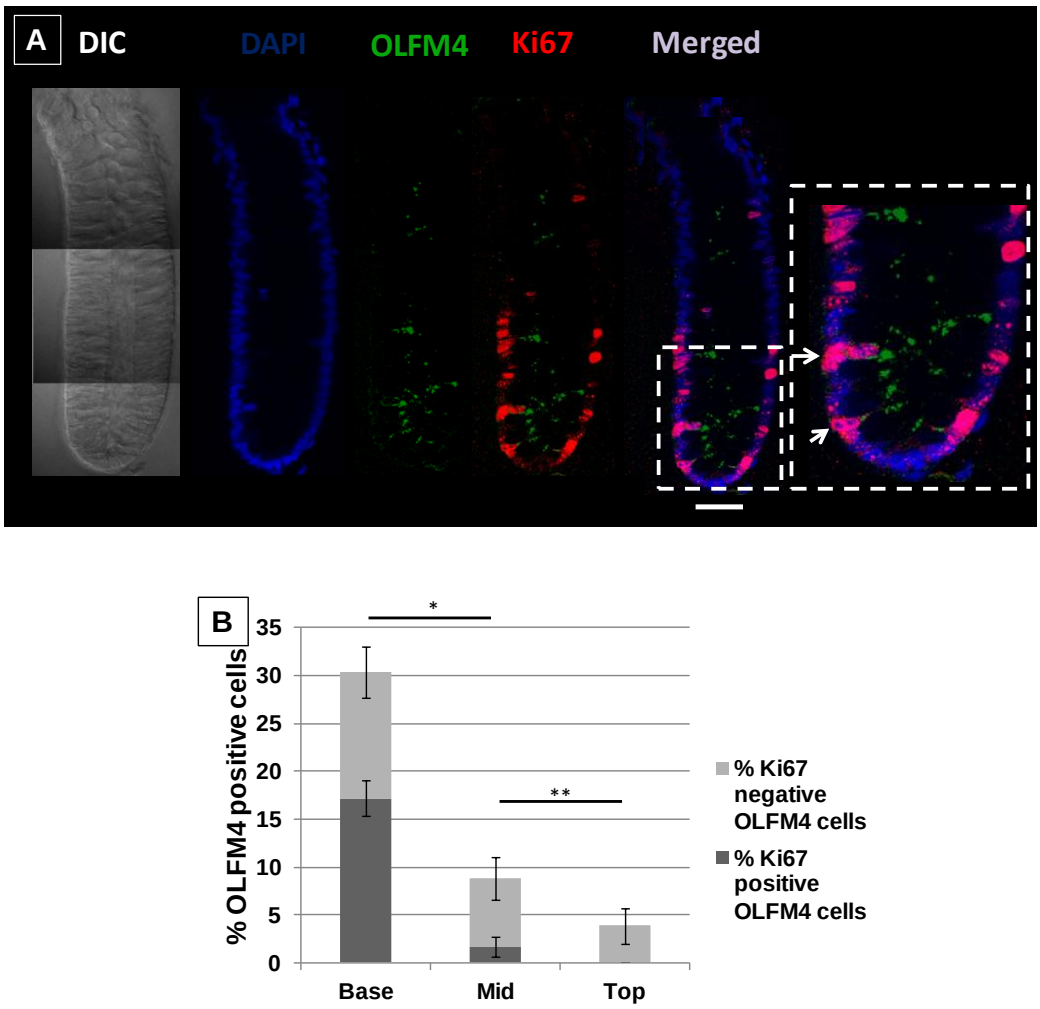


Figure 3.16 Proliferative status of OLFM4^{+} stem cells. *A) Native microdissected crypts were processed by immnocytochemistry for OLFM4 and Ki67. OLFM4^{+} cells were*

*restricted to the base of the crypt. B) Analysis shows that 30% of cells at the base of the crypt are OLFM4^{+}, with half of these undergoing proliferation at any given time. (*p=0.001,** p=0.0, N=18 crypts from 3 patients).*

Having determined the location of Lgr5/OLFM4^{+} stem cells in native colonic crypts and their proliferative status, whether these stem cells are maintained in culture *ex vivo* was demonstrated by labelling for Lgr5 and OLFM4 in crypts cultured for 3 days. Fig 3.17 shows that Lgr5/OLFM4 labels cells restricted to the base of the crypt and are maintained in culture.

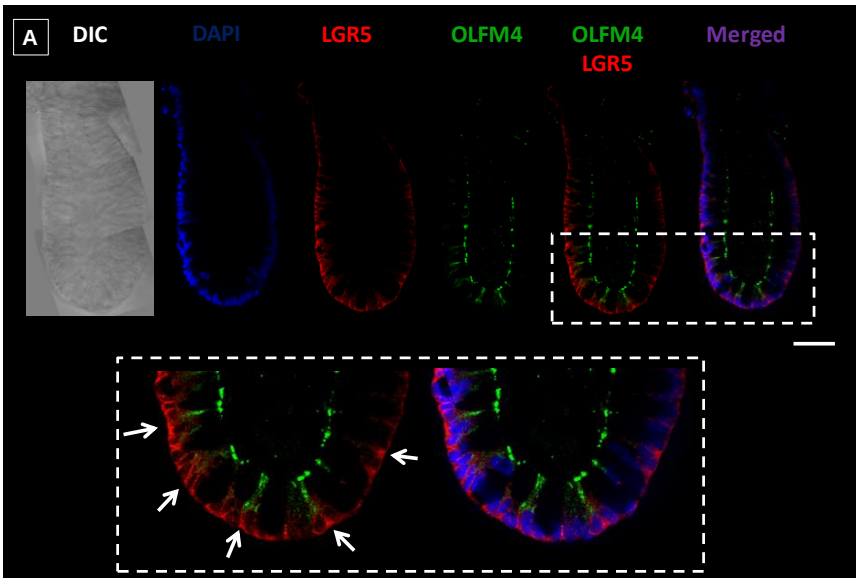


Figure 3.17 Lgr5/OLFM4^{+} stem cells are maintained in culture. *A) Cultured crypts were processed by immnocytochemistry for OLFM4 and Lgr5. Lgr5/OLFM4^{+} cells were restricted to the base of the crypt and labelled the same population of crypt base columnar cells.*

The functional effect of Wnt signalling on the stem cells was determined by culturing crypts in the presence of Wnt3a or the inhibitor IWP2 for 3 days. The crypts were then fixed and processed by immunocytochemistry for Lgr5 to determine the consequences of Wnt stimulation and inhibition on stem cell status (Fig 3.18). A gradient of Lgr5^{+} stem

cells was maintained in culture conditions reflecting the status of native microdissected crypts. IWP2 suppressed Lgr5^{+} stem cells, whilst exogenous Wnt3a rescued the effects.

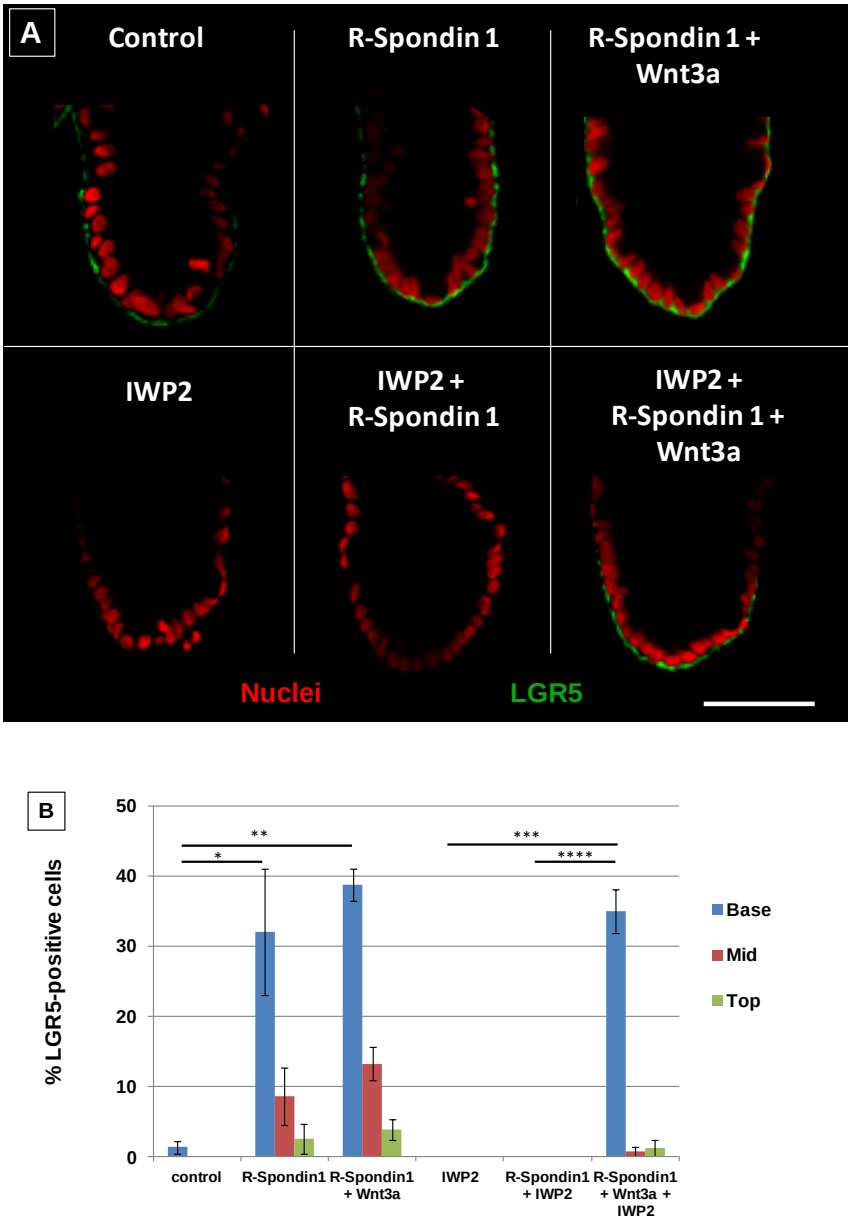


Figure 3.18 Wnt signals maintain Lgr5^{+} stem cells. *A) Crypt bases showing Lgr5 labelling. IWP2 suppressed Lgr5 but was rescued by exogenous Wnt3a. Scale bar represents 50μm. B) Percentage of Lgr5^{+} cells was calculated for the different culture conditions. * p=0.011, **p=0.0, *** p=0.001, **** p=0.002, (N=30 crypts from 3 patients). Control media: IGF-1 (50ng/ml), Noggin (100ng/ml); R-Spondin 1 (500ng/ml), Wnt3a (100ng/ml), IWP2 (5μM) where indicated.*

To determine whether OLFM4^{+} stem cells are still proliferating in culture after 3 days, crypts were cultured in Wnt3a or the Wnt inhibitor Dkk1. The crypts were then fixed and processed by immunocytochemistry for OLFM4 and proliferation marker BrdU. The percentage of OLFM4^{+} stem cells was calculated from the total number of cells for the three regions of the crypt, and the percentage of those stem cells that were also proliferating was also calculated. As can be seen in Fig 3.19, Wnt3a increases proliferation of stem cells whilst Dkk1 abolishes stem cells as well as Wnt3a stimulated stem cell proliferation.

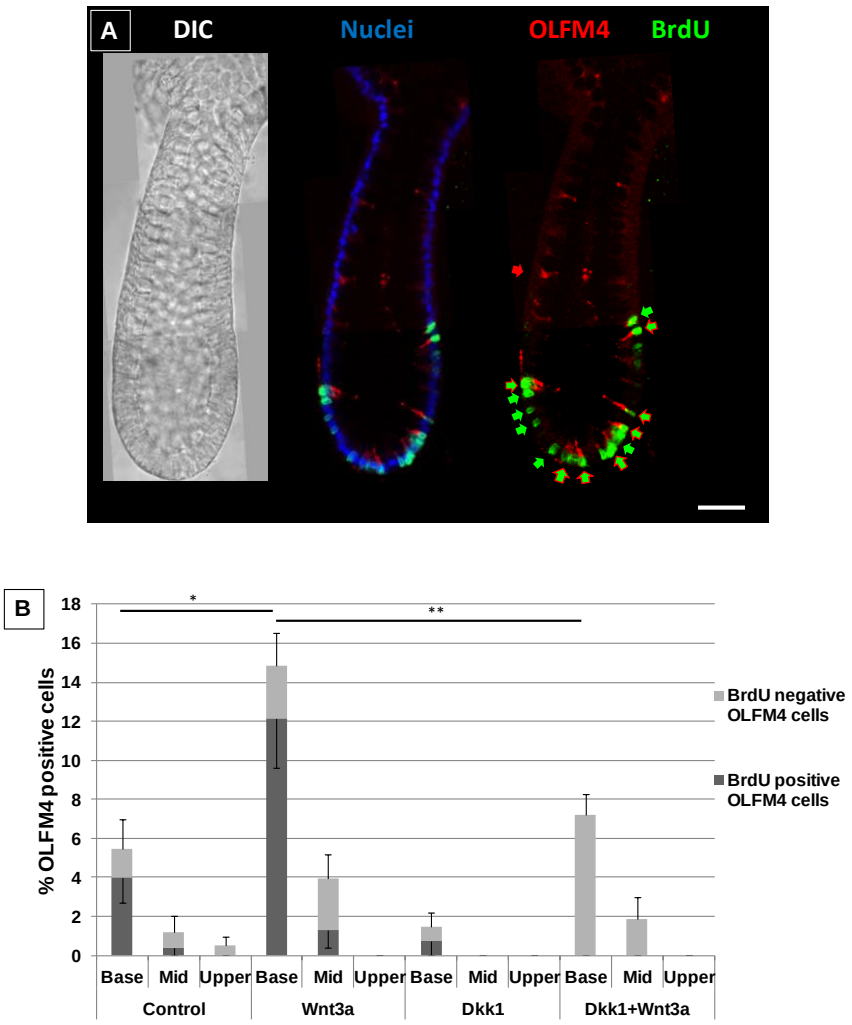


Figure 3.19 Dkk1 abolishes Wnt3a stimulated intestinal stem cell proliferation. *A) Crypts were cultured in Wnt3a or inhibited by Dkk1 and processed by immnocytochemistry for OLFM4. The proliferative status of OLFM4^{+} cells was stimulated by Wnt3a and*

*inhibited by Dkk1. Addition of Wnt3a to a Dkk1group does not rescue the proliferative status of OLFM4$^+$ stem cells. Scale bar represents 50μm. B) Percentage of OLFM4$^+$ cells and their proliferative status was calculated for all culture condition groups. (*p=0.012, **p=0.001, N=18 crypts from 2 patients). Control media: IGF-1 (50ng/ml), Noggin (100ng/ml), R-Spondin 1 (500ng/ml); Wnt3a (100ng/ml) and Dkk1 (800ng/ml) where indicated.*

The maintenance of OLFM4$^+$ stem cells can also be regulated by Notch signalling. Notch signalling is involved in lineage sprecification, with its activation resulting in goblet cell depletion and Notch inhibition resulting in increase of goblet cells (Fig 1.11). OLFM4 has also been shown to be under the control of Notch signalling. The effects of Notch inhibition on the number of both OLFM4$^+$ stem cells and goblet cells was determined by culturing crypts either in Wnt3a or DBZ, an inhibitor of Notch signalling. After 3 days, crypts were fixed, processed by immunocytochemistry and labelled for OLFM4 and Muc2, a marker of goblet cells. The percentages of both OLFM4+ cells and goblet cells was then calculated from the total number of cells and plotted in Fig 3.20 B and C. Inhibition of Notch signalling with DBZ caused a significant increase in the number of goblet cells within the colonic crypts after 3 days in culture. Concurrently, DBZ caused a dramatic loss of OLFM4$^+$ stem cells, possibly due to the conversion of those stem cells to goblet cells.

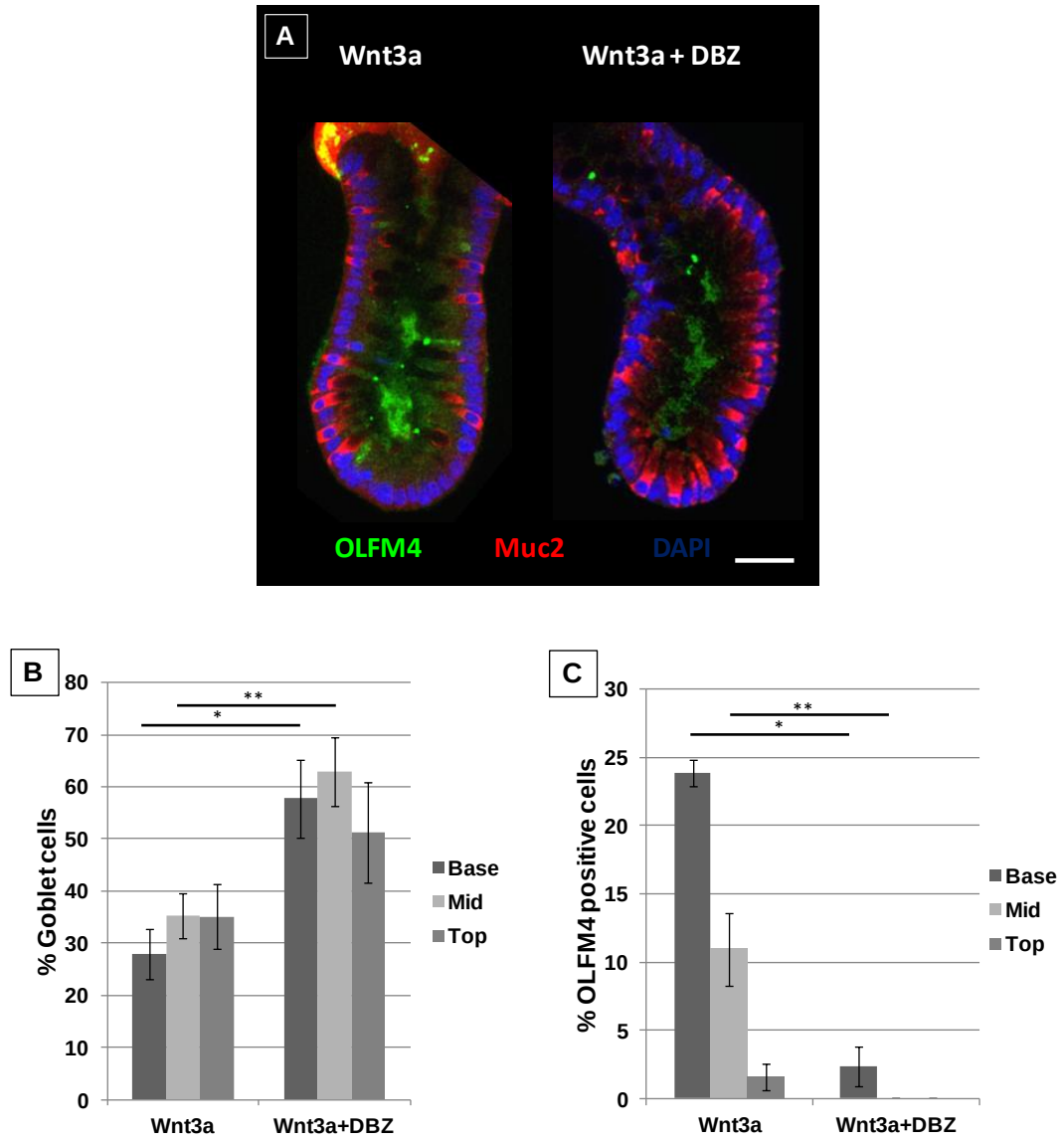


Figure 3.20 Notch inhibition promotes conversion to goblet cells and loss of OLFM4^{+} stem cells. *A) Crypts were cultured in Wnt3a or DBZ and processed by immnocytochemistry for OLFM4 and Muc2. The percentage of goblet cells significantly increased with inhibition of Notch signalling whilst percentage of OLFM4^{+} cells was siginificantly lost. Scale bar represents 50µm. B) (*p=0.011, ** p=0.009, N=11 crypts from 3 patients). C) (*p=0.0, **p=0.005 between N=7 crypts from 2 patients). Control media: IGF-1 (50ng/ml), Noggin (100ng/ml), R-Spondin 1 (500ng/ml), Wnt3a (100ng/ml; DBZ (10mM) where indicated.*

3.1.5 Wnt signalling maintains colonic crypt renewal

Crypt renewal is maintained by a fine balance between stem cell proliferation, cell migration and differentiation, and cell shedding at the top of the colonic crypt. Wnt signalling, along with BMP/TGFβ signalling are tight regulators of these processes. The influence of Wnt signals on crypt length and morphology was investigated by culturing crypts in Wnt3a or the Wnt inhibitor Dkk1 for 3 days (Fig 3.21). The contribution of R-Spondin 1 and Wnt3a to the maintanance of crypt length is suppressed by Dkk1.

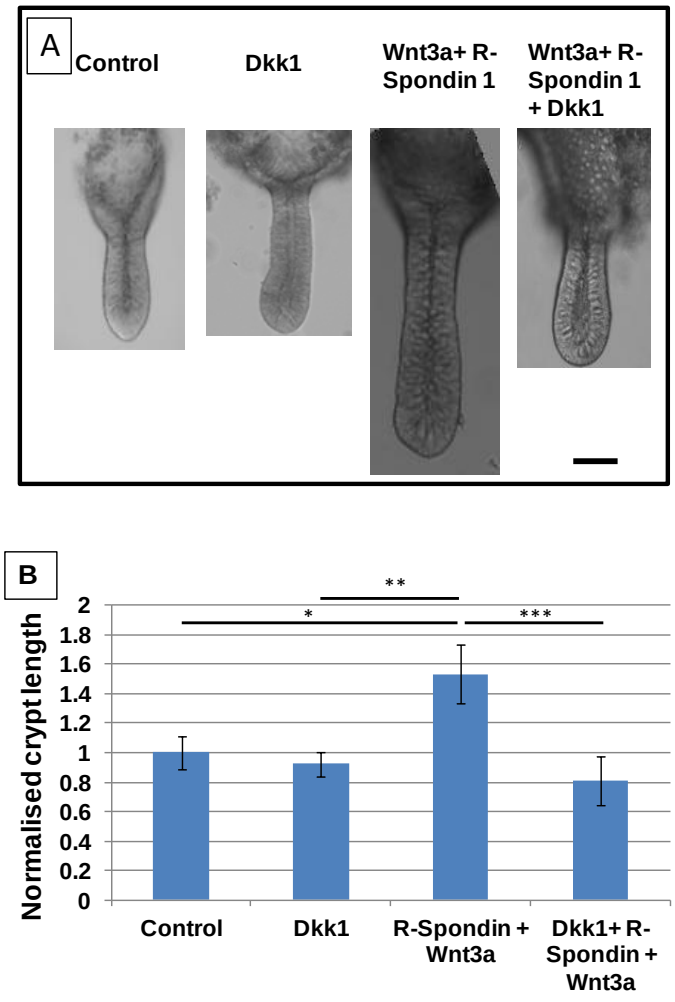


Figure 3.21 Inhibition of Wnt signals compromises human colonic crypt morphology. *A) Crypts were cultured either in the presence of Wnt3a and R-Spondin 1 or the Wnt inhibitor Dkk1. B) Dkk1 suppressed the contribution of Wnt3a and R-Spondin1 to the maintenance of crypt length. (*p=0.049, **p=0.009, ***p=0.043, N=50 crypts from 2*

patients). Control media: IGF-1 (50ng/ml), Noggin (100ng/ml); R-Spondin 1 (500ng/ml), Wnt3a (100ng/ml), Dkk1 (800ng/ml) where indicated.

To explore the link between crypt cell proliferation and migration and the contribution of Wnt signals, crypts were cultured under conditions that imposed different levels of proliferation and were observed under time-lapse microscope. Crypt length and crypt cell proliferation were increased by Wnt3a, but crypt cell migration rate stayed constant even under culture conditions with less proliferative potential (Fig 3.22).

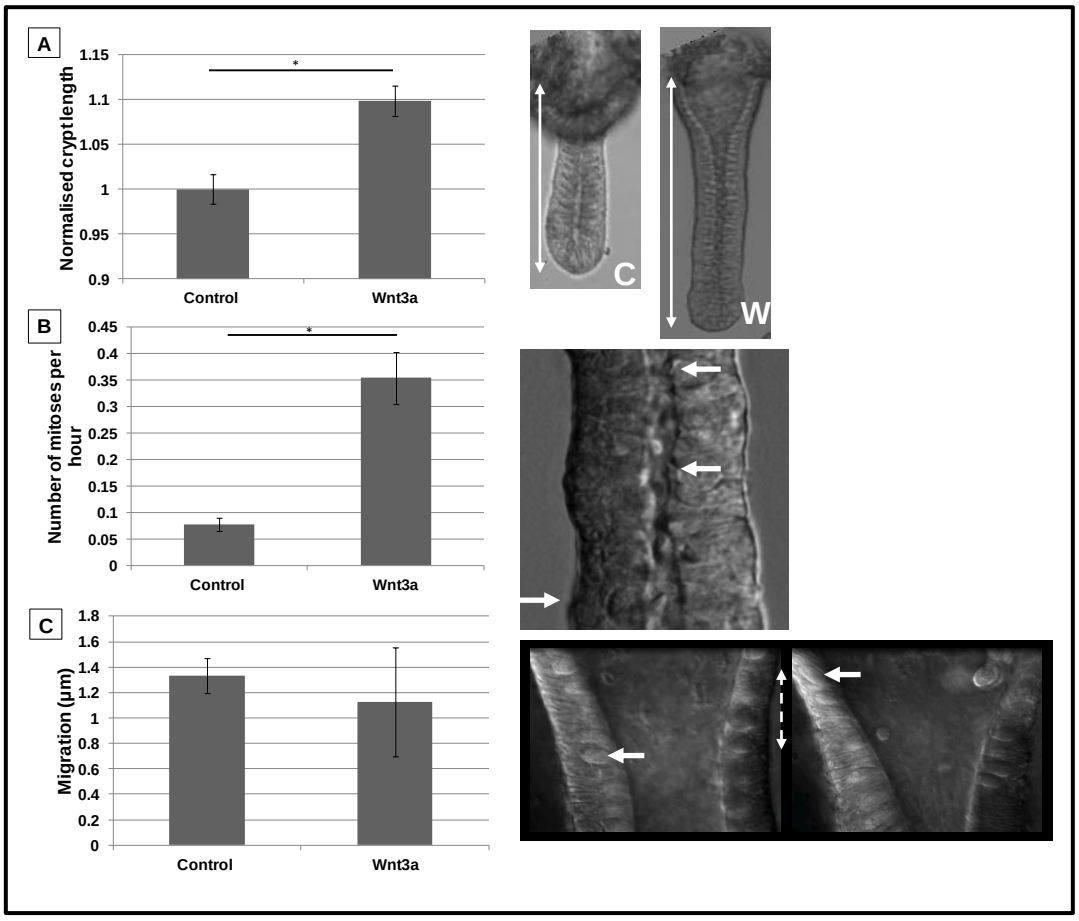


Fig 3.22 Wnt signalling maintains crypt cell renewal. *A) Wnt3a increases crypt length in culture compared to control, *p=0.0. B) Wnt3a stimulates cell mitosis, *p=0.0. C) No significant difference in cell migration between the control and Wnt3a groups. Control media: IGF-1 (50ng/ml), Noggin (100ng/ml), R-Spondin 1 (500ng/ml), Wnt3a (100ng/ml) where indicated.*

Crypt cell shedding was observed by labelling of 'live' cultured crypts with live/dead fluorophores Calcein-AM and Propidium Iodide and immunolabelling fixed crypts for activated caspase-3 (Fig 3.23). Cell shedding was observed in the upper region of the crypt. Crypt cell proliferation in the lower half of the crypt is required to maintain a steady-state cell population by replenishing cells shed from the upper surface.

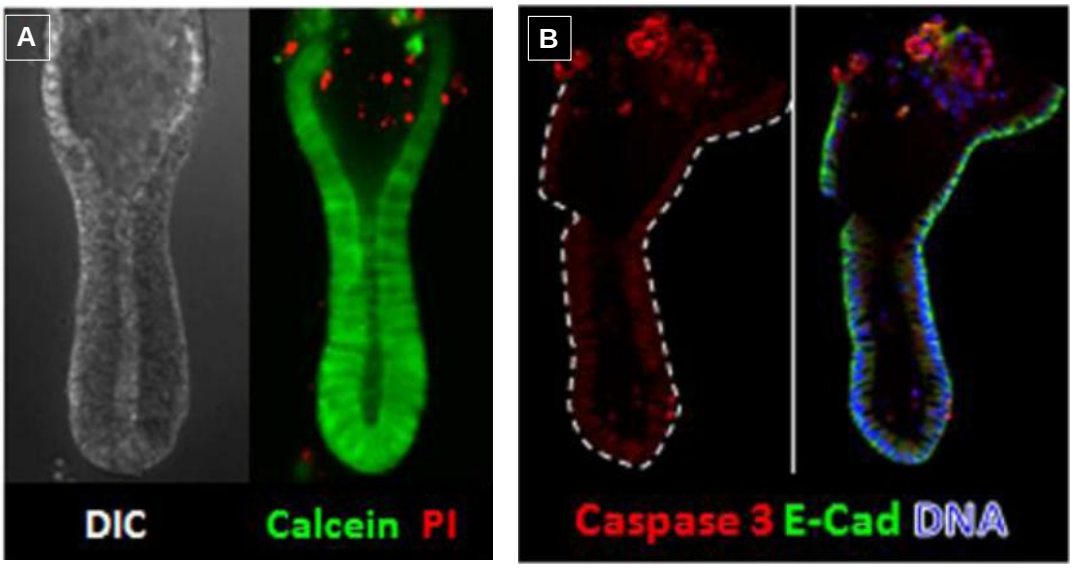


Fig 3.23 Crypt cell shedding localised to the upper crypt region. *A) Hierarchy of Calcein-AM labelled 'live' cells (green) and PI-positive dead cells (red). B) Cells at the crypt opening are positive for activated caspase-3.*

3.1.6 Discussion

The processes by which the intestinal epithelium renews itself has been well described in the mouse, but the molecular and cellular mechanisms that govern tissue renewal in the human colonic epithelium are less well understood. To gain a more detailed understanding of the processes of tissue renewal in health and disease, an *ex vivo* human tissue culture model is required that is amenable to bio-imaging and functional genomic approaches. Recently there has been the development of intestinal organoid culture systems that are predominantly composed of immature stem/progenitor cells that can be induced to differentiate by withdrawl of Wnt stimulation (20) (220). Complementary to these organoid culture systems, we have developed a culture model of near-native human colonic crypts that maintains its cellular hierarchy and morphogenic gradients *ex vivo.* We found that addition of EGF to the culture media led to remodelling of human colonic crypt morphology with the development of multiple budding events that are characteristic of intestinal organoid growth (Fig 3.5). Testing various combinations of growth factors in our culture media, with the aim of maintaining crypt length and morphology, we found that Wnt pathway activation with Wnt3a and R-Spondin 1, as well as BMP antagonist Noggin, the intestinotrophic factor IGF-1 and an ALK 4/5/7 inhibitor A83-01 was required to maintain crypt length and morphology for at least 7 days in culture (Fig 3.4).

The role of Wnt signalling in regulating the renewal of human colonic epithelium was also explored. The status of Wnt signalling components was investigated in native microdissected crypts and it was found that β-catenin labelled the basal and lateral membranes (Fig 3.1), similar to that found by Anderson *et al.* (249), as well as in the nucleus, the hallmark of activated Wnt signalling. Nuclear β-catenin predominated at the base of the crypts and its expression was reduced along the crypt axis with significantly lower levels at the top of the crypt. The two target genes axin II and c-Myc were also found to label in the nucleus and have higher expression at the base of the crypt and lower further up the crypt axis (Fig 3.2 and Fig 3.3). This means that activation of Wnt signalling

leads to accumulation of nuclear β-catenin which then activates oncogenes such as c-Myc which are involved in cell proliferation. Stem cells have been found to reside at the base of the crypt, therefore activation of Wnt signalling means that these stem cells are activated to self-renew and maintain progeny further up the crypt axis. Gregorrief *et al*. (142) and Holcombe *et al*. (127) who found that Wnt ligands such as Wnt3, Wnt9b and Wnt5a predominated at the base of the crypt along with the LRP5/6 coreceptors. Previous work in our laboratory has shown that human colonic crypts have *Wnt1, Wnt2, Wnt2b, Wnt3, Wnt3a, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7b* and *Wnt11* mRNA (21). Farin *et al*. (25) have recently demonstrated that deletion of Paneth cell derived Wnt3 in the mouse intestinal epithelium showed no effect *in vivo* but was required for growth and sustainability in organoid *in vitro* culture. Co-culturing of mesenchymal derived Wnt2b ligand restored the growth of organoids, thus demonstrating a compensatory mechanism in the stem cell niche that safeguards against stem cell dysfunction.

The important role of the Wnt signalling pathway in maintaining crypt homeostasis was shown by Kuhnert *et al*. (2) who demonstrated that adenoviral expression of Dkk1, a Wnt antagonist, resulted in inhibition of proliferation and loss of crypts in the mouse colon and small intestine. Dkk1 inhibition of Wnt signalling and proliferation was also observed in this study. Dkk1 was found to inhibit expression of β-catenin even in the presence of Wnt3a ligand which otherwise stimulated β-catenin (Fig 3.6). Dkk1 also prevents rescue of proliferation with exogenous Wnt3a ligand (Fig 3.10). Pinto *et al*. (3) have also found that transgenic expression of Dkk1 results in greatly reduced epithelial proliferation and an absence of nuclear β-catenin as well as inhibition of c-Myc expression. To corroborate these findings Koch *et al*. (150) also found that reduced expression of Dkk1 increased proliferation of epithelial cells and increased transcriptional activity of β-catenin.

Chen *et al*. (250) have found that IWP2, a small molecule that inhibits the activity of Porcupine, a membrane-bound acyltransferase that is essential in the production of Wnt proteins, blocked the phosphorylation of the LRP6 receptor and Dvl2 as well as β-catenin

accumulation. In this study, IWP2 was found to inhibit activity of Wnt signalling and proliferation of cells. The Lenti-TCF/LEF GFP reporter is a preparation of ready-to-transduce lentiviral particles which monitor the activity of Wnt signalling in cells through the binding of β-catenin to a (m)CMV promoter and tandem repeats of the TCF/LEF transcriptional response element. It was found that IWP2 inhibited endogenous levels of Wnt signalling but this could be rescued by addition of Wnt3a ligand (Fig 3.7). The effect of IWP2 on the Wnt target gene axin II was also investigated. It was found that IWP2 significantly inhibited expression of axin II, even in the presence of Wnt3a ligand (Fig 3.8). Covey *et al*. (251) have found that knockdown of Porcupine with IWP2 also inhibits proliferation and growth of a number of epithelial cancer cell lines. In our human colonic crypts, IWP2 was found to significantly inhibit proliferation of cells through the inhibition of Wnt protein production. Exogenous addition of Wnt3a ligand overcame this inhibition and significantly maintained the level of proliferation in cultured crypts.

Disruption of the Wnt signalling pathway via β-catenin/TCF4 activity has been shown to induce a rapid G1 arrest and blockage of a genetic programme that is physiologically active in the proliferative compartment of colonic crypts and induce an intestinal differentiation programme instead (140). Korinek *et al.* (252) have also shown that mice deficient for the TCF4 transcription factor completely lack proliferative cells in the fetal small intestinal epithelium suggesting that β-catenin/TCF4 signalling is essential for maintaining the proliferative/undifferentiated state of intestinal epithelial cells. In isolated crypts it is possible to interfere with β-catenin signalling by transfection of a dominant negative version of TCF4. In this study, we disrupted β-catenin/TCF4 activity by use of a dnTCF4 adenovirus on isolated colonic crypts. Proliferation of cells was significantly inhibited with dnTCF4 (Fig 3.12) as well as the Wnt target gene axin II (Fig3.9). In their study of the expression pattern of Wnt signalling components, Gregorrief *et al*. (142) found unexpectedly that TCF4 was abundant in differentiated cells on the surface epithelium and that expression dimished in the lower half of the crypts. Barker *et al*. (47) also found high

expression of TCF4 at the top of the crypt. Angus-Hill *et al.* (164) suggest that TCF4 acts as a tumour suppressor by modulation proliferation. They show that loss of TCF4 early in development and in adult colon results in increased cell proliferation and leads to colon tumourigenesis. Their model suggests that low levels of TCF4 favor normal intestinal cell proliferation whilst high levels of TCF4 protein epithelial cell differentiation.

It has recently been demonstrated that the Wnt signalling pathway can also be activated by the R-Spondin family of secreted ligands. The CR domain of the R-Spondin proteins is primarily responsible for activation of Wnt signalling. Deletion of one or two furin-like motifs of the CR domain of R-Spondin abolishes its activity to activate canonical Wnt signalling (253) (254). Nam *et al.* (151), Binnerts *et al*. (255) and Wei *et al.* (256) have all demonstrated the requirement of LRP5/6 receptors to transmit R-Spondin activation of the canonical Wnt signalling pathway. R-Spondin 1 renders the cells more sensitive to low levels of Wnt ligand by modulating the LRP6 receptor by interacting with Kremen 1 and antagonising Dkk1. This interaction interferes with LRP6 internalisation which results in increased LRP6 levels at the cell surface. In this study IWP2 was used to inhibit Wnt signalling and lead to significantly reduced cell proliferation (Fig 3.11), and addition of R-Spondin 1 to the culture media led to an increase in proliferation, possibly by rendering the cells more sensitive to any Wnt ligands that were not inhibited by IWP2. This result is similar to that obtained by Kim *et el.* (257) who found that R-Spondin 1 induced a dramatic increase in proliferation of intestinal crypt epithelial cells. R-Spondin 1 has also been shown to have strong mitogenic activity on $Lgr5^+$ cells of the intestinal crypts (67) and hair follicles (258) by supporting survival and proliferation *in vitro* through activation of Wnt signalling. Our investigations revealed that R-Spondin 1, especially in combination with Wnt3a, significantly maintained $Lgr5^+$ stem cells in culture and rescued the inhibitory effects of IWP2 (Fig 3.18). Several studies have also shown that R-Spondin proteins are also the ligands for Lgr4, 5 and 6 receptors. Carmon *et al.* (6) demonstrate that Lgr4 and Lgr5 bind to R-Spondins with high affinity and mediate the potentiation of

Wnt signalling by enhancing Wnt-induced LRP6 phosphorylation. More recently they report that following costimulation with the ligands R-spondin1 and Wnt3a, Lgr5 interacts and forms a supercomplex with the Wnt coreceptors LRP6 and Fzd5 which is rapidly internalized and then degraded through a dynamin- and clathrin-dependent pathway. Deletion of the C-terminal tail of Lgr5 maintained its ability to interact with LRP6, yet this Lgr5 mutant exhibits increased signaling activity and a decreased rate of endocytosis in response to R-spondin1 compared to the wild-type receptor. Their study therefore provides direct evidence that Lgr5 becomes part of the Wnt signaling complex at the membrane level to enhance Wnt signalling (259). Ruffner *et al*. (260) also identified Lgr4 as a cognate receptor of R-Spondin. They found that depletion of Lgr4 completely abolished R-Spondin-induced β-catenin signalling and that loss of Lgr4 could be compensated by overexpression of Lgr5, suggesting that Lgr4 and Lgr5 are functional homologs. They further demonstrated that R-Spondin binds to the extracellular domain of Lgr4 and Lgr5, and that overexpression of Lgr4 strongly sensitizes cells to R-Spondin-activated β-catenin signalling.

Lineage tracing (4), propagation of self-renewing intestinal organoids (67) and transplantation assays (68) have defined Lgr5 as a marker of proliferative intestinal stem cells. Barker *et al*. (65) also identified $Lgr5^+$ stem cells at the base of pyloric glands of the stomach which lineage tracing experiments revealed gave rise to all differentiated stomach epithelial cells. Wnt signalling is also involved in hair follicle stem cell biology, and since Lgr5 is a Wnt target gene, it is no surprise that Jaks *et al*. (258) found *Lgr5* expression in the hair follicle at sites where hair growth is initiated. Again, lineage tracing showed that $Lgr5^+$ stem cells generated all hair follicle lineages. All the tissues where Lgr5 has been identified as a stem cell marker share the characteristics of rapidly proliferating epithelia and the stem cell niche allows unidirectional displacement if daughter cells (152). These studies were carried out on mice, which although informative, do not reveal the status of Lgr5 in human tissue. In our study, the expression of Lgr5 was investigated on native

human microdissected crypts and on isolated crypts that were kept in culture *ex vivo*. Immunocytochemistry on native microdissected crypts revealed Lgr5 expression on slender crypt base columnar cells at the base of the crypt (Fig 3.13). Analysis of the percentage of $Lgr5^+$ cells versus other differentiated cell types revealed that around 30% of $Lgr5^+$ stem cells reside at the base of the crypt and decreasing along the crypt axis, with none found at the top of the crypt. Conversly, the other cell lineages although also found to be about 30% at the base of the crypt, increase along the crypt axis. *In situ hybridisation* for *Lgr5* mRNA also revealed these $Lgr5^+$ stem cells to reside at the base of the crypt with none found at the top (Fig 3.14). In the mouse small intestine, $Lgr5^+$ stem cells are found interspersed between Paneth cells which have been shown to provide stem cell niche factors such as Wnt3a (34). The colon lacks Paneth cells, so we found the $Lgr5^+$ cells to be interspersed between goblet cells. Some of these goblet cells could secrete niche factors that still support the $Lgr5^+$ stem cells. Rothenberg *et al*. (35) have recently found goblet cells that contained a distinct cKit/$CD117^+$ crypt base subpopulation that expressed Dll1, Dll4 and EGF which are also found in Paneth cells that are also marked by cKit. They found that these $cKit^+$ goblet cells were interdigitated with $Lgr5^+$ stem cells. Along with the detection of $Lgr5^+$ stem cells in native microdissected crypts, we found that isolated cultured colonic crypts also maintained $Lgr5^+$ stem cell expression when cultured in Wnt3a and R-Spondin 1.

Differential gene expression profile for Lgr5 stem cells and their daughter cells revealed that OLFM4 was also enriched in the $Lgr5^+$ cells (48). Using *in situ hybridisation*, Van Der Flier *et al*. (48) found *OLFM4* expression in the small intestine and colon in crypt base columnar cells that were also positive for Lgr5. OLFM4 has also been found to be highly expressed in a subset of cells in colorectal carcinomas with its expression much higher in these tumour cells than in wild-type crypt base columnar cells. In our study we investigated whether OLFM4 does indeed label the same population of cells as Lgr5. Immunocytochemistry performed on native microdissected crypts showed that indeed

OLFM4 and Lgr5 mark the same population of crypt base columnar cells (Fig 3.13 and 3.14). Co-labelling with E-cadherin to visualise single cells easier revealed that these OLFM4/Lgr5^{+} cells are slender, almost triangular shaped and reside at the crypt base. OLFM4 is a secreted molecule so as well as intracellular OLFM4 being observed, there was some OLFM4 protein at the apical pole of the cells and some in the lumen that was flushed out of the crypt as the cell makes more protein. OLFM4 has been shown to be upregulated in inflammatory bowel disease mucosa and secreted into the mucous as a protective role by binding defensins in the mucous (75). We also show that OLFM4, just like Lgr5 is maintained in isolated cultured crypts (Fig 3.17) and the double-labelling of OLFM4 and Lgr5 in the same population of cells confirms the results seen in native human crypts. To demonstrate that these OLFM4^{+} stem cells are not in fact a lineage of differentiated cell of the colonic crypt, native microdissected crypts were processed by immunocytochemistry and double labelled with a known marker of the four differentiated cell types (Fig 3.15). Double labelling of OLFM4 with Muc2, CroA, Cox1 and FABP revealed that the OLFM4^{+} cells are a distict population of cells that only label Lgr5^{+} crypt base columnar cells. OLFM4 has also been shown to be under the control of Notch signalling (261). Notch signalling is involved in lineage specification, with its activation resulting in goblet cell depletion and inhibition resulting in increase of goblet cells. We found that inhibiting Notch signalling with DBZ resulted in a significant increase in the number of goblet cells and a dramatic loss of OLFM4^{+} stem cells (Fig 3.20). This result was also found by VanDussen *et al*. (261) who showed that Notch inhibition led to differentiation of epithelial progenitors into secretory cell types and induced a rapid crypt base columnar cell loss (OLFM4^{+}) with reduced proliferation, apoptotic cell death and reduced efficiency of organoid initiation.

Crypt base columnar stem cells have been shown to be rapid-cycling with Lgr5^{+} cells having an average cycling time of one day (4). Since OLFM4 labels the same population of cells as Lgr5, we found that colabelling of OLFM4 with the proliferation marker Ki67

showed that approximately half of total OLFM4$^+$ cells were undergoing proliferation (Fig 3.16). In cultured crypts this proliferative status of OLFM4$^+$ stem cells was increased by Wnt signalling but inhibited with Dkk1 (Fig 3.19). Takashima *et al.* (262) show that activation of Wnt signalling with R-Spondin 1 increased the number of OLFM4$^+$ stem cells in crypts as well as an increased number of Ki67$^+$ cycling cells. R-Spondin 1 also enhanced repopulation of OLFM4$^+$ stem cells after mucosal injury with bone marrow transplantation.

The role of Wnt signalling on cell renewal was investigated by culturing crypts in Wnt pathway activators (Wnt3a and R-Spondin 1) and measuring crypt length after several days in culture (Fig 3.21) or monitoring the cultured crypts for 24 hrs by real-time time-lapse microscopy (Fig 3.22). Crypt length was maintained by Wnt signals and this was due to maintainance of cell proliferation, as measured by counting number of cell mitoses per hour. Although Wnt3a may have maintained proliferation compared to control, no difference was made to the rate of migration of cells within the crypt. Crypt cell shedding was observed by labelling of 'live' cultured crypts Calcein-AM and Propidium Iodide and immunolabelling fixed crypts for activated caspase-3 (Fig 3.23). We observed that cell shedding occurred in the upper region of the crypt. This suggests that crypt cell proliferation in the lower half of the crypt is required to maintain a steady-state cell population by replenishing cells shed from the upper surface, but does not appear to drive crypt cell migration.

In this chapter, the role of Wnt signalling in regulating intestinal stem cell status and tissue renewal was demonstrated. Exogenous Wnt ligand was required for human colonic crypt culture and the maintanance of Wnt signalling gradient sustained the hierarchy of tissue renewal for at least 7 days. Cultured crypts exhibited basal levels of Wnt signal activation, stem cell marker expression and cell proliferation, all of which were abolished by IWP2, an inhibitor of Wnt ligand secretion. A number of Lgr5$^+$/OLFM4$^+$ stem cells were

identified at the crypt base that were interspersed between goblet-like cells and were maintained in culture by Wnt signals (Fig 3.24).

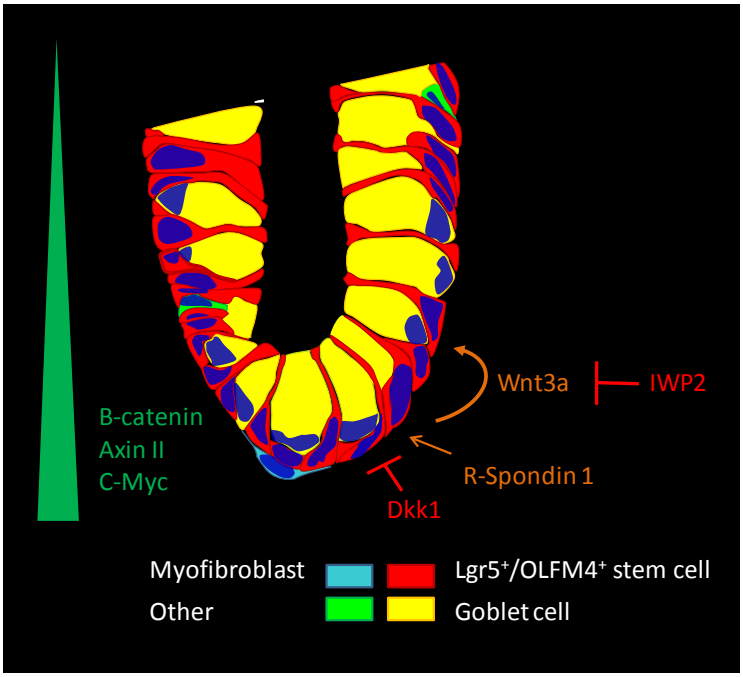


Fig 3.24 Wnt signals maintain intestinal stem-cell driven tissue renewal. *Wnt signalling predominates at the crypt base. Wnt signals maintain stem cells and cell proliferation. Wnt signalling inhibitors IWP2 and Dkk1 supress expression of Wnt target genes, stem cell status and cell proliferation.*

3.2 BMP/TGFβ signalling regulated tissue renewal

3.2.1 Introduction

Along with the Wnt signalling pathway, BMP and TGFβ signalling pathways have been implicated in regulating the hierarchy of intestinal tissue renewal. An analaysis of gene expression patterns of normal human colonic crypt bases and tops has revealed expression of signalling pathway activators and inhibitors that are predicted to establish morphogenic gradients along the human colonic colonic crypt-axis (74). In mice, it has been shown that BMP/TGFβ signals predominate at the top of the crypt where they are thought to influence crypt cell positioning, differentiation and apoptosis. The reciprocal inhibition of Wnt and BMP/TGFβ pathways appear to maintain the hierarchy of tissue renewal along the crypt-axis. Migration, differentiation and compartmentalisation along the crypt-axis has been shown to be controlled by TGFβ and Wnt gradients, with TGFβ controlling cell polarisation proteins and Wnt controlling the expression of EphB sorting receptors (109).

Patients with mutations in the BMP signalling pathway develop juvenile polyposis which increases the risk of developing cancer and loss of SMAD4 and pSMAD1,5,8 is also associated with progression to adenocarcinomas. Loss of TGFβ receptors in intestinal cells has been shown to promote the invasion and malignant transformation of tumours that were initiated by APC mutation (207). Thus Wnt signalling deregulation and BMP/TGFβ signalling inactivation cooperate to drive the initiation and progression of intestinal cancers.

A detailed knowledge of the role of BMP/TGFβ signalling pathways in stem cell-driven tissue renewal in the human colonic epithelium is still lacking. Therefore, using our near-native human colonic culture model, the role of BMP/TGFβ pathways on Wnt gene expression, stem cell maintaince and cell proliferation will be investigated in this chapter.

3.2.2 Functional role of BMP signalling in human colonic crypts

To investigate the BMP signalling status in human colonic crypts, colonic biopsies were fixed and microdissected into individual crypts. Immunocytochemistry was performed on the microdissected crypts to label for p-SMAD1,5,8, a marker for the *in vivo* signalling status of BMP signalling. Nuclear immunofluorescence intensity was measured and normalised to the base of the crypt (Fig 3.25). Analysis revealed that there is low p-SMAD1,5,8 activation at the base of the crypt, but increases along the crypt-axis with higher levels in the upper region of the crypt.

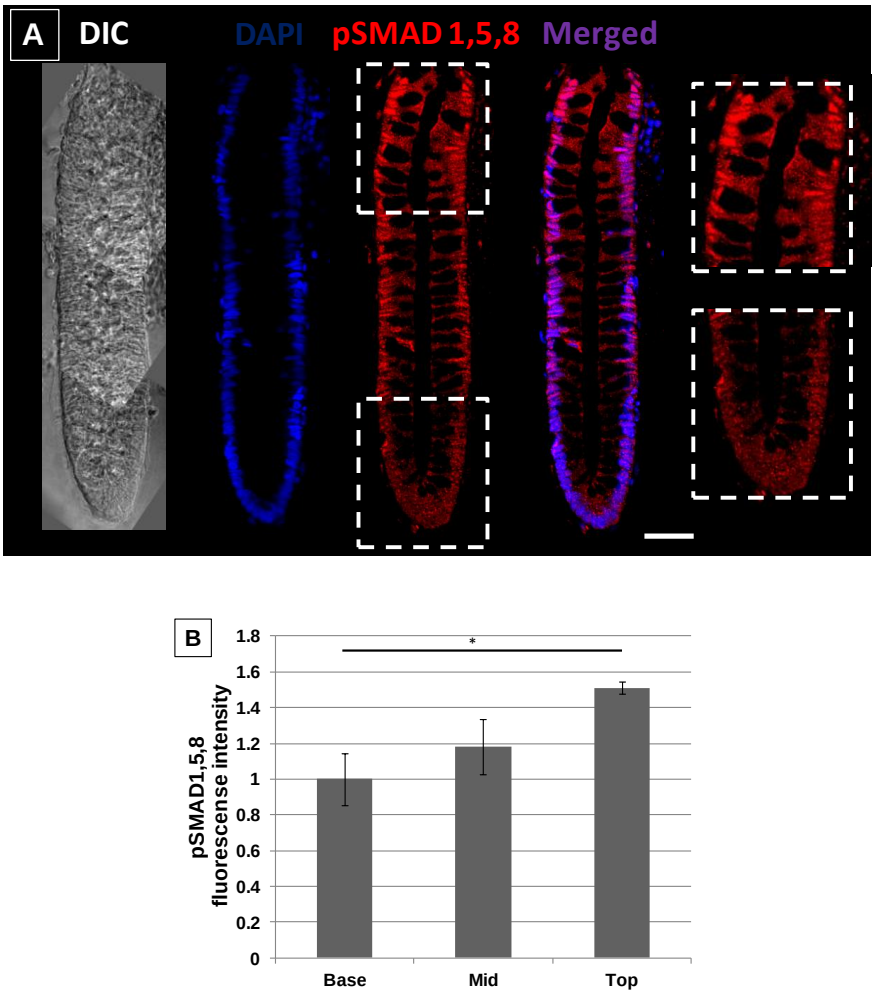


Figure 3.25 BMP signalling status in vivo. *A) Nuclear p-SMAD1,5,8 is the hallmark of activated BMP signalling. Biopsies were fixed, microdissected into single crypts,*

*processed by immunocytochemistry and labelled for p-SMAD1,5,8. Scale bar represents 50μm. B) Analysis of nuclear p-SMAD1,5,8 along the crypt axis. Nuclear fluorescence intensity measurements from each region of the crypt were normalised to the base of the crypt. (*p=0.027, N=3 crypts from 2 patients).*

In the mouse, it has been suggested by He *et al*. (8) that BMP signalling inhibits intestinal stem cell self-renewal though activation of PTEN which leads to the suppression of Wnt signalling. It is only by overcoming this suppression with transient expression of noggin, a BMP antagonist, which leads to Akt activation that enhances nuclear β-catenin activity and promoting stem cell self-renewal and proliferation. Therefore the effect of noggin on BMP signalling was determined by culturing crypts with noggin and/or BMP4 for 3 days. These crypts were then fixed and processed by immnocytochemistry for pSMAD1,5,8 and nuclear fluorescence intensity of pSMAD1,5,8 measured and normalised to the crypt base of the control group (Fig 3.26). BMP4 significantly increased levels of nuclear pSMAD1,5,8, which was suppressed by noggin. Noggin also suppressed endogenous levels of pSMAD1,5,8 suggesting there is a low level of BMP signalling in cultured crypts.

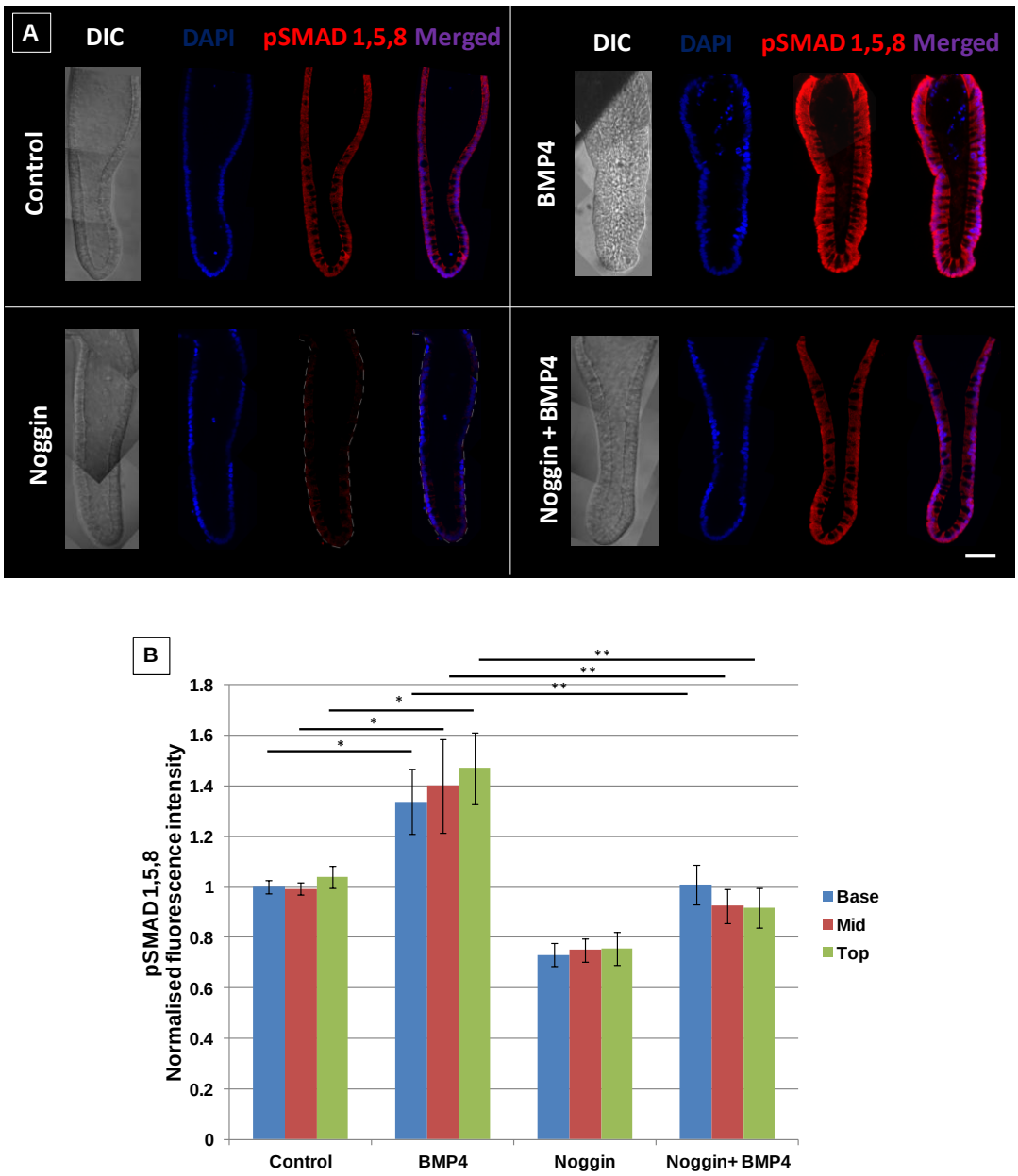


Figure 3.26 Noggin suppresses BMP signalling. *A) Crypts were cultured in BMP4 and/or the BMP antagonist noggin for 3 days. Crypts were processed by immunocytochemistry and labelled for pSMAD1,5,8. Scale bar represents 50μm. B) Analysis of nuclear pSMAD1,5,8 along the crypt axis. Nuclear fluorescence intensity measurements from each region of the crypt were normalised to the crypt base of the control group. BMP4 increased levels of pSMAD1,5,8 which was suppressed by addition of noggin. (*p<0.05 in all regions of crypt, **p<0.05 in all regions of crypt, N=25 crypts*

from 2 patients). Control media: IGF-1 (50ng/ml), R-Spondin 1 (500ng/ml), Wnt3a (100ng/ml); Noggin (100ng/ml) and BMP4 (100ng/ml) where indicated.

Since BMP signalling is involved in cell differentiation and suppression of stem cell proliferation, it has the reciprocal function of Wnt signalling. Therefore, to determine the effect of BMP signalling and its inhibition on Wnt target genes, crypts were cultured in BMP4 or the BMP antagonist noggin for 3 days, processed by immunocytochemistry and labelled for β-catenin (Fig 3.27) or axin II (Fig 3.28). Noggin significantly increased levels of β-catenin probably by inhibiting levels of endogenous BMP signals. BMP4 also inhibited the Wnt target gene axin II which was rescued by addition of noggin. Noggin increased levels of axin II probably by inhibiting the endogenous levels of BMP signals.

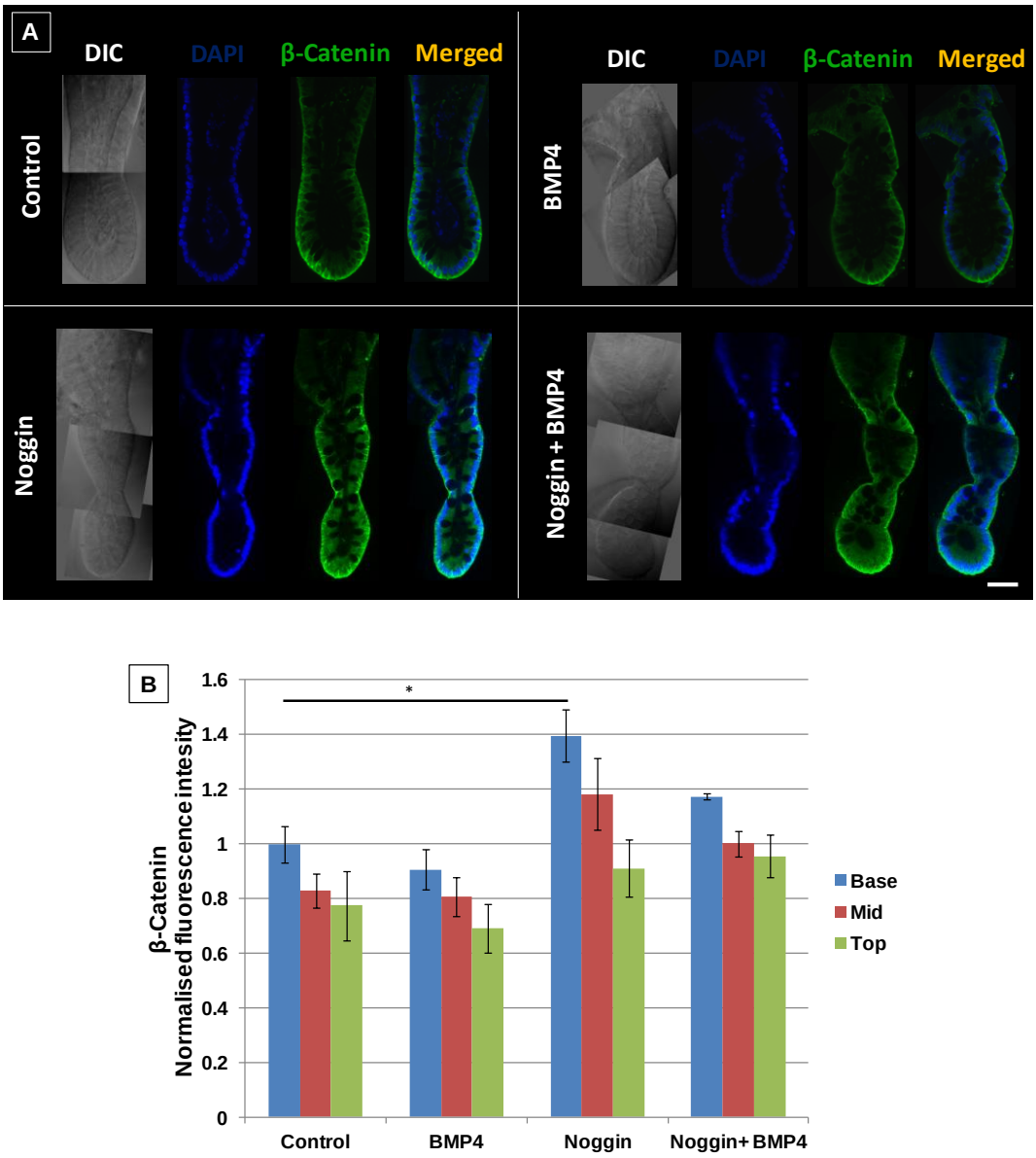


Figure 3.27 Noggin inhibits BMP signalling. *A) Crypts were cultured in BMP4 and/or the BMP antagonist noggin for 3 days. Crypts were processed by immunocytochemistry and labelled for β-catenin. Scale bar represents 50μm. B) Analysis of nuclear β-catenin along the crypt axis. Nuclear fluorescence intensity measurements from each region of the crypt were normalised to the crypt base of the control group. Noggin increased levels of β-catenin probably by suppressing levels of endogenous BMP signals. (*p=0.049, N=8 crypts from 2patients). Control media: IGF-1 (50ng/ml), R-Spondin 1 (500ng/ml), Wnt3a (100ng/ml); Noggin (100ng/ml) and BMP4 (100ng/ml) where indicated.*

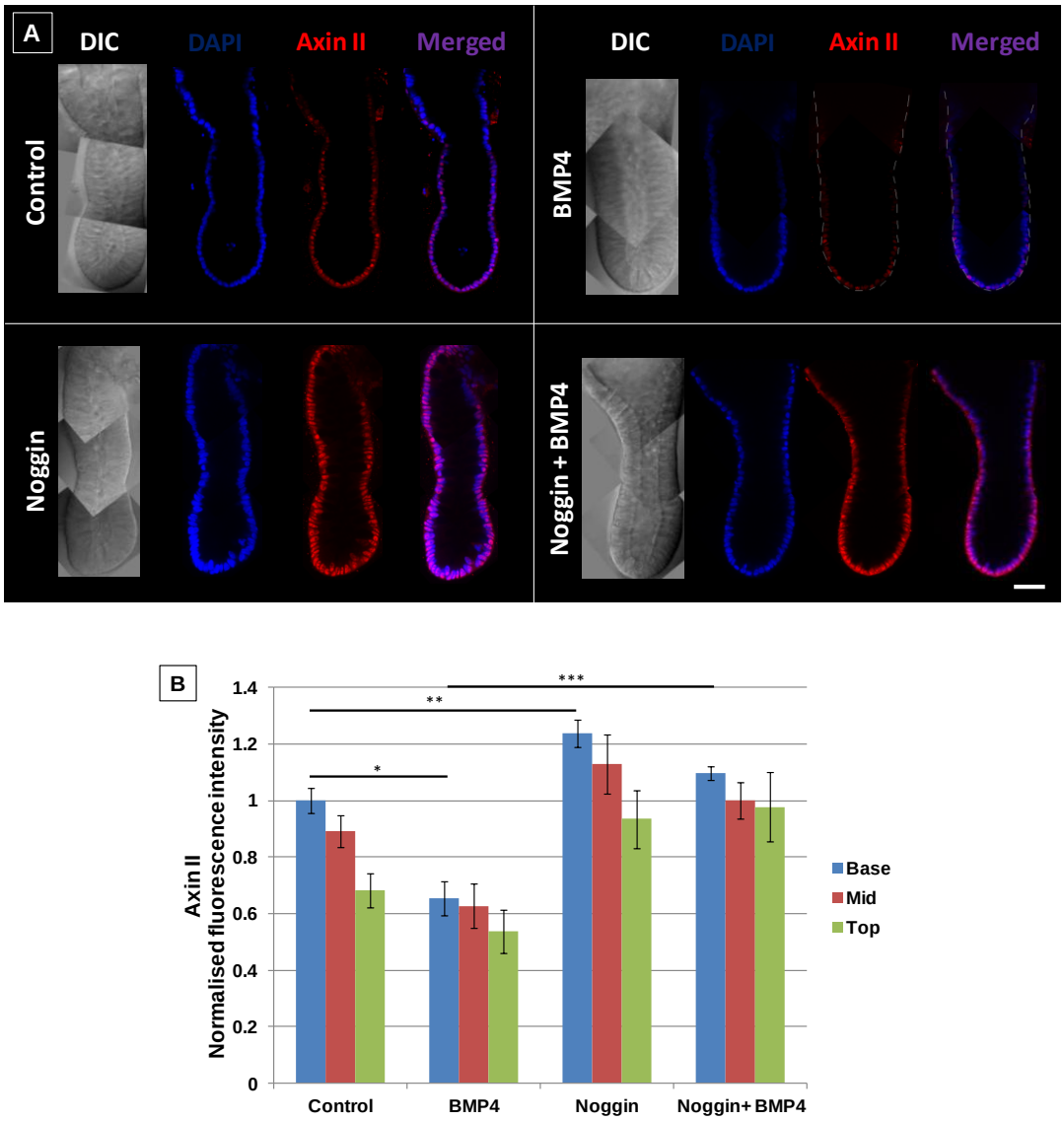


Figure 3.28 BMP4 inhibits Wnt signalling. *A) Crypts were cultured in BMP4 and/or the BMP antagonist noggin for 3 days. Crypts were processed by immunocytochemistry and labelled for axin II. Scale bar represents 50μm. B) Analysis of nuclear axin II along the crypt axis. Nuclear fluorescence intensity measurements from each region of the crypt were normalised to the crypt base of the control group. BMP4 inhibited axin II whilst noggin increased levels of axin II probably by suppressing levels of endogenous BMP signals. The inhibitory effects of exogenous BMP4 were rescued by addition of noggin. (*p=0.002, **p=0.017, ***p=0.042, N=18 crypts from 2 patients). Control media: IGF-1 (50ng/ml), R-Spondin 1 (500ng/ml), Wnt3a (100ng/ml); Noggin (100ng/ml) and BMP4 (100ng/ml) where indicated.*

3.2.3 Role of BMP signalling in crypt renewal

The levels of pSMAD1,5,8 were found to be low at the base of the crypt and high in the top region in a reciprocal manner to that of Wnt target genes β-catenin and axin II. This suggests that BMP pathway activation may favour cell cycle withdrawl. BMP signals are kept low at the base of the crypt by BMP antagonists such as noggin. To investigate the effects of BMP signalling on stem cells, crypts were cultured either in BMP4 and/or noggin for 3 days. Crypts were then processed by immunocytochemistry and labelled for the stem cell marker OLFM4 (Fig 3.29). Noggin increased the number of $OLFM4^{+}$ stem cells that were otherwise inhibited by endogenous levels of BMP signals (control). Addition of exogenous BMP4 completely abolished the stem cells in the crypts, but this effect was rescued by the addition of noggin.

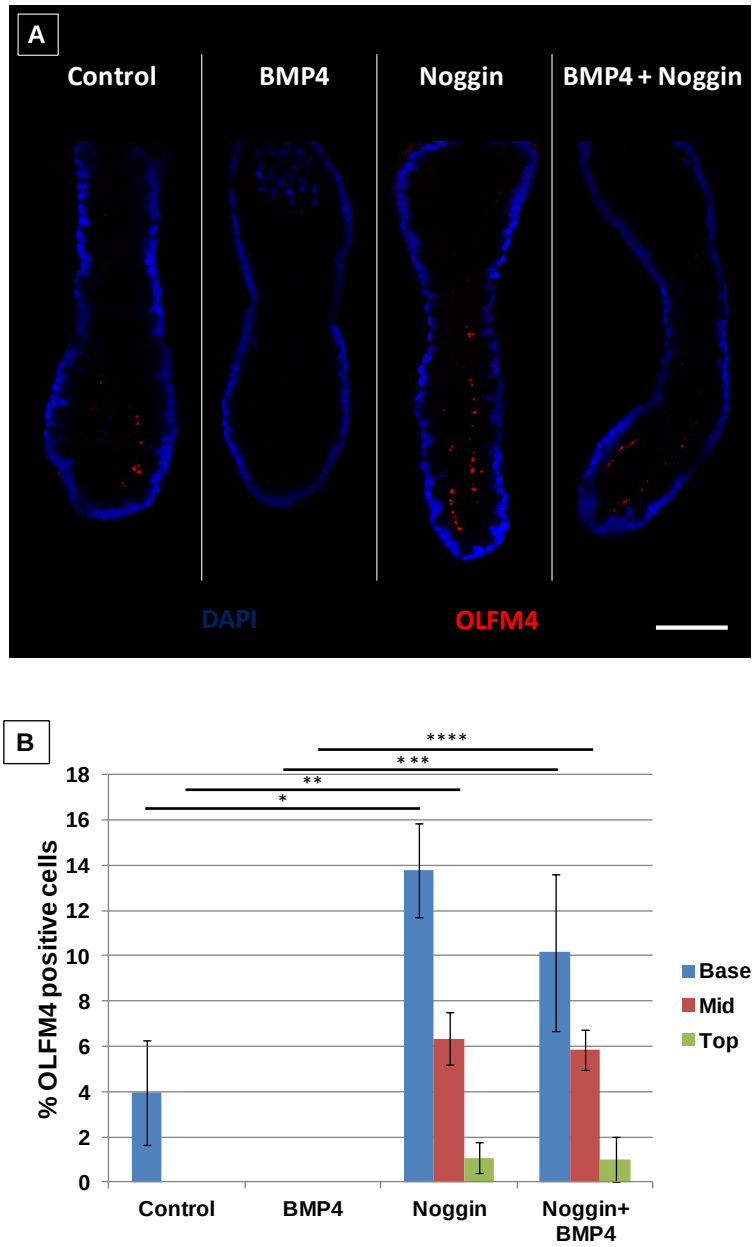
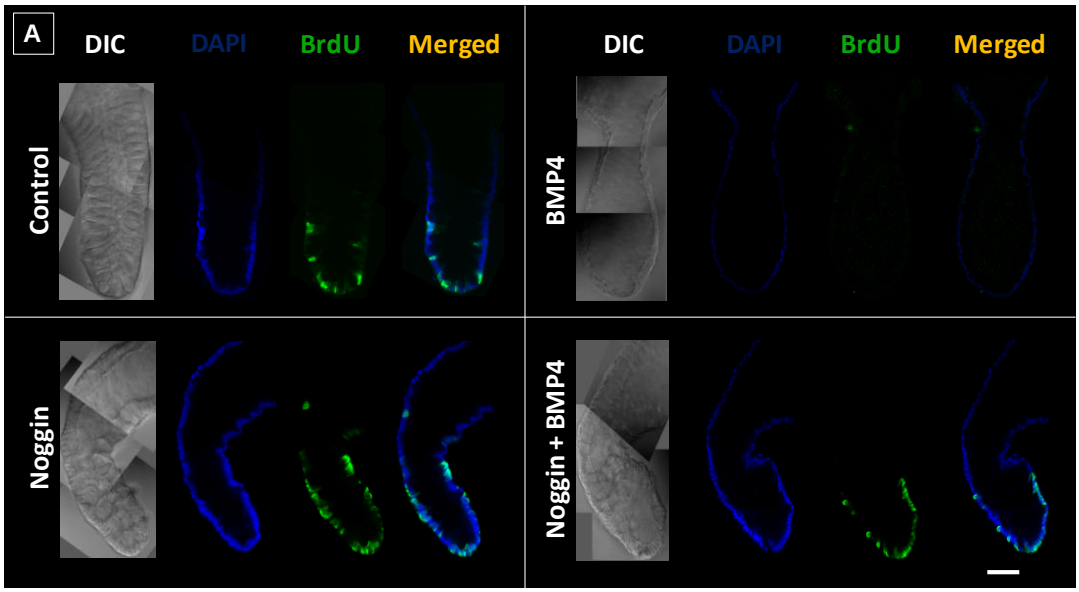


Figure 3.29 BMP4 inhibits OLFM4^{+} stem cells. *A) Crypts were cultured in BMP4 and/or the BMP antagonist noggin for 3 days. Crypts were processed by immunocytochemistry and labelled for OLFM4. Scale bar represents 50μm. B) Noggin increases number of OLFM4^{+} stem cells which otherwise diminish after 3 days in culture possibly due to endogenous BMP signals. BMP4 completely abolishes OLFM4^{+} stem cells but this inhibitory effect is rescued by noggin. (*p=0.039, **p=0.0, *** p=0.041, ****p=0.001, N=17 crypts from 2 patients). Control media: IGF-1 (50ng/ml), R-Spondin 1 (500ng/ml), Wnt3a (100ng/ml); Noggin (100ng/ml) and BMP4 (100ng/ml) where indicated.*

To determine the effects of BMP signalling on crypt cell proliferation, isolated crypts were cultured either in BMP4 or noggin for 3 days. On day 2-3 the proliferation marker BrdU was added to the media for 24 hrs. Crypts were then fixed, processed by immunocytochemistry and labelled for BrdU (Fig 3.30). Noggin significantly increased crypt proliferation compared to control, whilst BMP4 completely abolished proliferation after 3 days in culture. The low proliferation rate in the control crypt could be due to the endogenous levels of BMP signals that accumulate after several days in culture, which inhibit Wnt signalling and therefore proliferation. The inhibitory effects of BMP4 however were rescued by the addition of noggin.



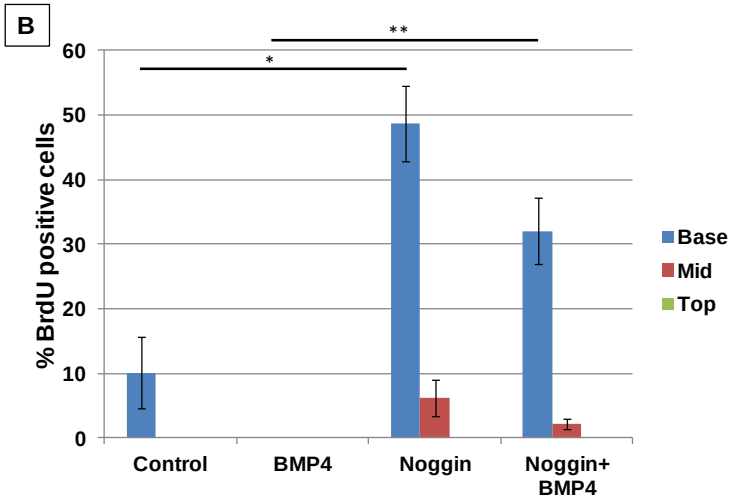


Figure 3.30 BMP4 inhibits proliferation. *A) Crypts were cultured in BMP4 and/or the BMP antagonist noggin for 3 days. Crypts were processed by immunocytochemistry and labelled for BrdU. Scale bar represents 50μm. B) Noggin increases proliferation of cells which otherwise diminish after 3 days in culture possibly due to endogenous BMP signals. BMP4 completely abolishes proliferaion but this inhibitory effect is rescued by noggin. (*p=0.000, **p=0.003, N=17 crypts from 2 patients). Control media: IGF-1 (50ng/ml), R-Spondin 1 (500ng/ml), Wnt3a (100ng/ml); Noggin (100ng/ml) and BMP4 (100ng/ml) where indicated.*

This increase of proliferation as well as number of stem cells with noggin suggests that renewal of the colonic crypt is maintained in culture over several days. The effect of inhibited proliferation due to BMP4 or the increase of proliferation and number of stem cells can be determined by looking at the morphology and length of crypts after those 3 days in culture. Fig 3.31 shows the morphology of crypts in the different culture conditions after 3 days. BMP4 inhibits proliferation and therefore the overall length on the crypt is shortened compared to control crypts. Addition of noggin means that BMP signals are inhibited and Wnt signalling can be transactivated and lead to activation of stem cell self-renewal and promotion of proliferation. Crypt length and morphology is therefore maintained. The crypts are longer compared to control crypts due to endogenous levels of BMP signals that accumulate when noggin is not present leading to crypt shortening over time due to reduced proliferation and self-renewal and stem cells. Noggin is a potent inhibitor of BMP signalling, so as can be seen it rescues the effects caused by BMP4 when both are added to the culture media.

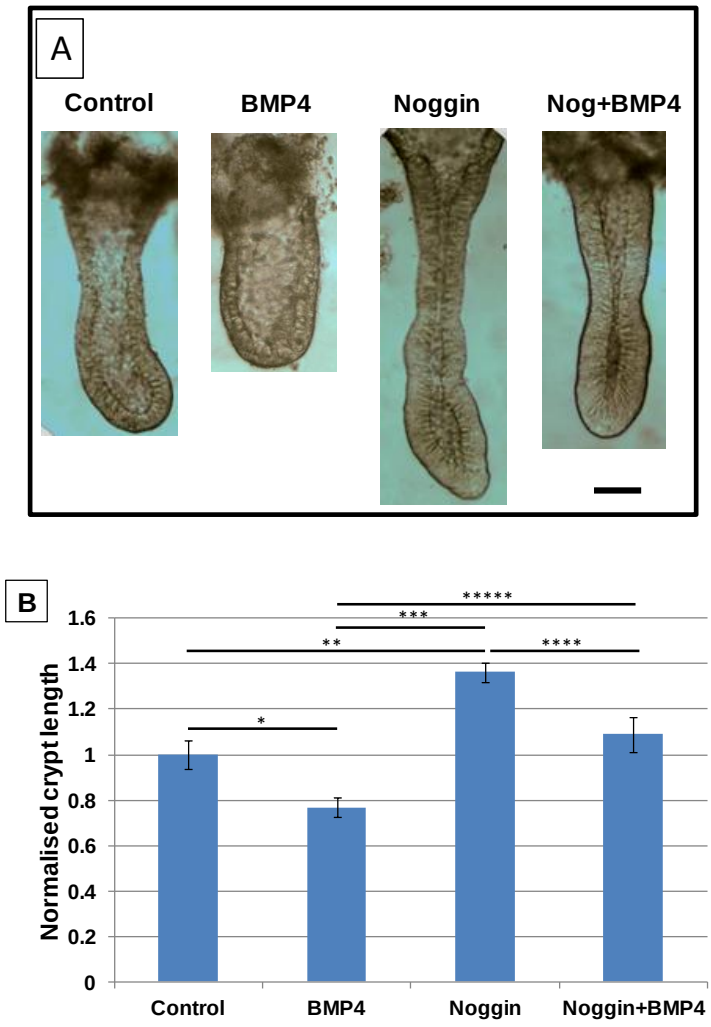
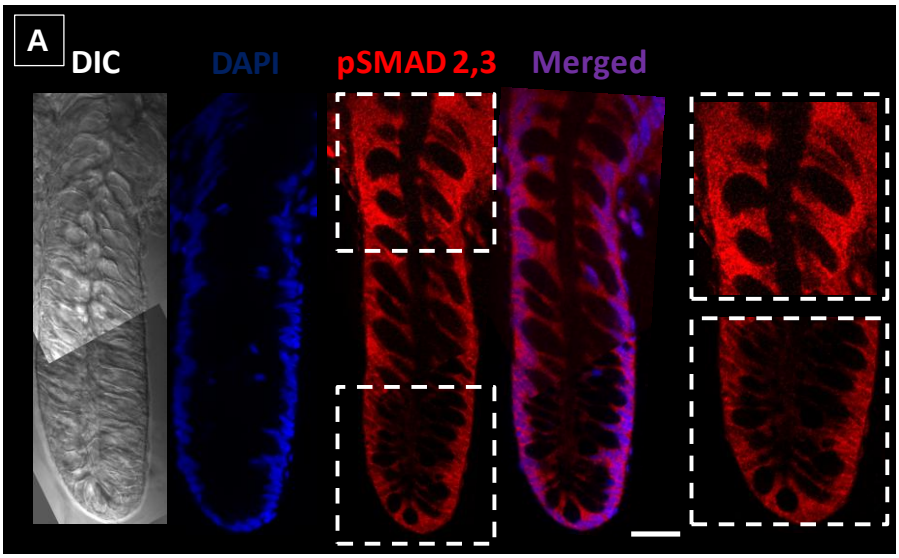


Figure 3.31 Noggin maintains crypt morphology and length. *A) Crypts were cultured in BMP4 and/or the BMP antagonist noggin for 3 days. Crypt length was measured on day 3 of the experiment and normalised against the control crypts. Scale bar represents 50μm. B) Noggin increases crypt length and morphology, whilst BMP4 leads to crypt shortening. Noggin also rescues the detrimental effects of BMP4. (*p=0.041, **p=0.001, ***p=0.0, ****p=0.016, *****p=0.004, N=14 crypts from 2 patients). Control media: IGF-1 (50ng/ml), R-Spondin 1 (500ng/ml), Wnt3a (100ng/ml); Noggin (100ng/ml) and BMP4 (100ng/ml) where indicated.*

3.2.4 Functional role of TGFβ/Activin signalling in human colonic crypts

TGFβ signalling is involved in cell differentiation and apoptosis by maintaining a reciprocal gradient with Wnt signalling to ensure cells within the crypt are kept in the correct compartment for proliferation, migration and then shedding at the top. In the mouse small intestine, the presence of TGFβ signals and the absence of Wnt signalling in the villus compartment results in rapid cell cycle arrest and differentiation, with SMAD4 and TCF4 being the dominant switch between the proliferative progenitor and the differentiated epithelial cells (109). To determine the status of TGFβ signalling in human colonic crypts, biopsies were removed from patients undergoing colonoscopies and immediately fixed. Biopsies were then microdissected into single crypts, processed by immnocytochemistry and labelled for pSMAD2,3 (Fig 3.32). Although no gradient can be seen along the crypt axis, there is a slightly higher level of pSMAD2,3 at the top of the crypt compared to the base.



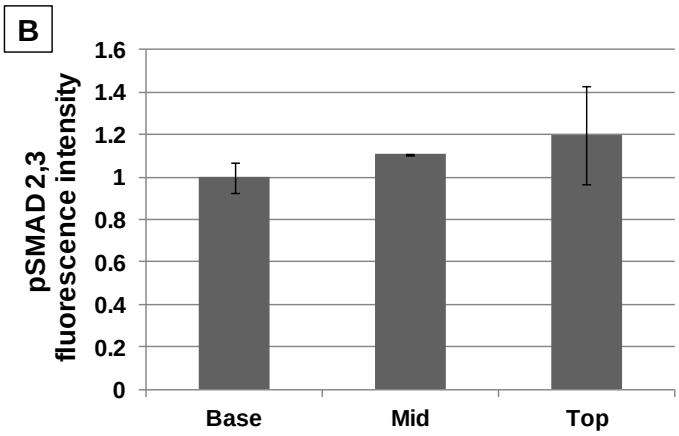
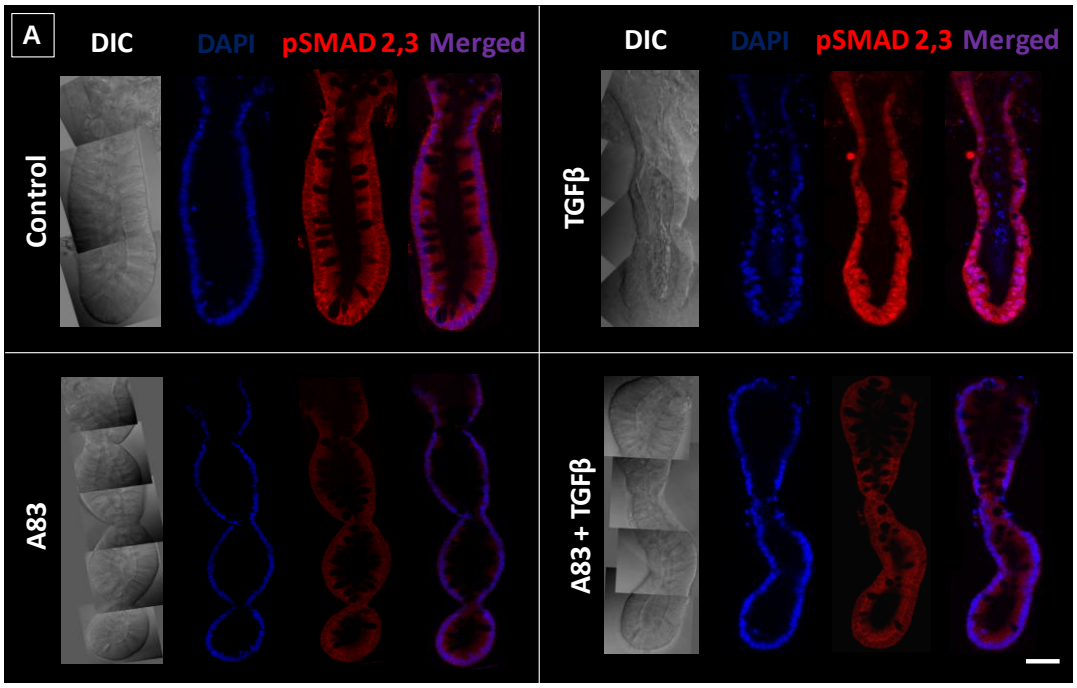


Figure 3.32 TGFβ signalling status in vivo. *A) Nuclear pSMAD2,3 is the hallmark of activated TGFβ signalling. Biopsies were fixed immediately following removal from the patient, microdissected into single crypts, processed by immunocytochemistry and labelled for pSMAD2,3. Scale bar represents 50μm. B) Analysis of nuclear pSMAD2,3 along the crypt axis. Nuclear fluorescence intensity measurements from each region of the crypt were normalised to the crypt base. No gradient is observed along the crypt axis. (N=4 crypts from 2 patients).*

To investigate the consequences of TGFβ signalling activation and inhibition on colonic crypts, isolated crypts were cultured either in TGFβ or the Alk receptor 4/5/7 inhibitor A83-01 for 3 days. A83-01 has been found to inhibit SMAD signalling and epithelial-to-mesenchymal transition by TGFβ (263). Fig 3.33 shows that TGFβ activates the TGFβ signalling pathway by increasing levels of pSMAD2,3 within the cells. The control crypts seem to have a basal level of pSMAD2,3 which could account for the reason that over time the crypts shorten in length and have decreased proliferation and stem cell number (data shown later in chapter). Addition of A8-01 reduces the level of pSMAD2,3 activation and this is maintained even when TGFβ is present.



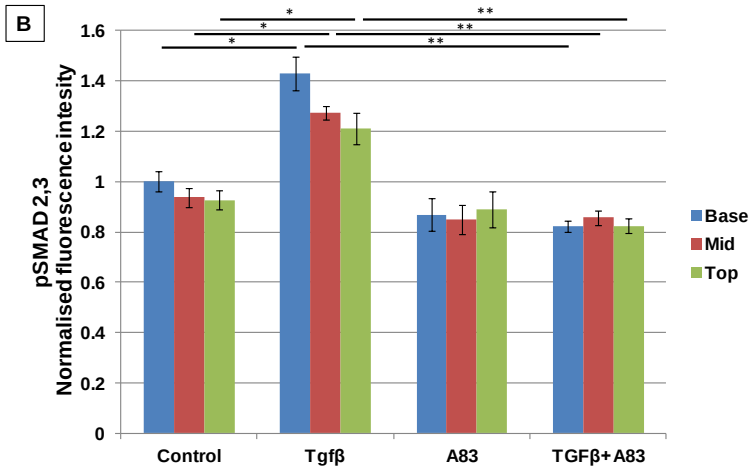


Figure 3.33 A83 inhibits TGFβ/SMAD signalling. *A) Crypts were cultured in TGFβ and/or A83-01 for 3 days. Crypts were then processed by immunocytochemistry and labelled for pSMAD2,3. Scale bar represents 50μm. B) Analysis of nuclear pSMAD2,3 along the crypt axis. Nuclear fluorescence intensity measurements from each region of the crypt were normalised to the crypt base of the control group. TGFβ significantly increased levels of pSMAD2,3. (*p=0.0, **p=0.0, N=22 crypts from 2 patients). Control media: IGF-1 (50ng/ml), R-Spondin 1 (500ng/ml), Wnt3a (100ng/ml), Noggin (100ng/ml); TGFβ (20ng/ml) and A83-01 (0.5μM) where indicated.*

As with BMP signalling, TGFβ signalling has the reciprocal effect on proliferation and stem cell activation to that of Wnt signalling. The transactivating effect of TGFβ on Wnt signalling can be determined by looking at expression of the Wnt target gene axin II. Isolated crypts were cultured either in the presence of TGFβ or the Alk 4/5/7 inhibitor A83-01, processed by immunocytochemistry and labelled for axin II (Fig 3.34). Analysis revealed that TGFβ significantly decreased axin II expression whilst A83-01 increased axin II expression presumably by inhibiting any endogenous TGFβ ligands. A83-01 also rescued the inhibitory effects of TGFβ by restoring axin II expression to that of the control group.

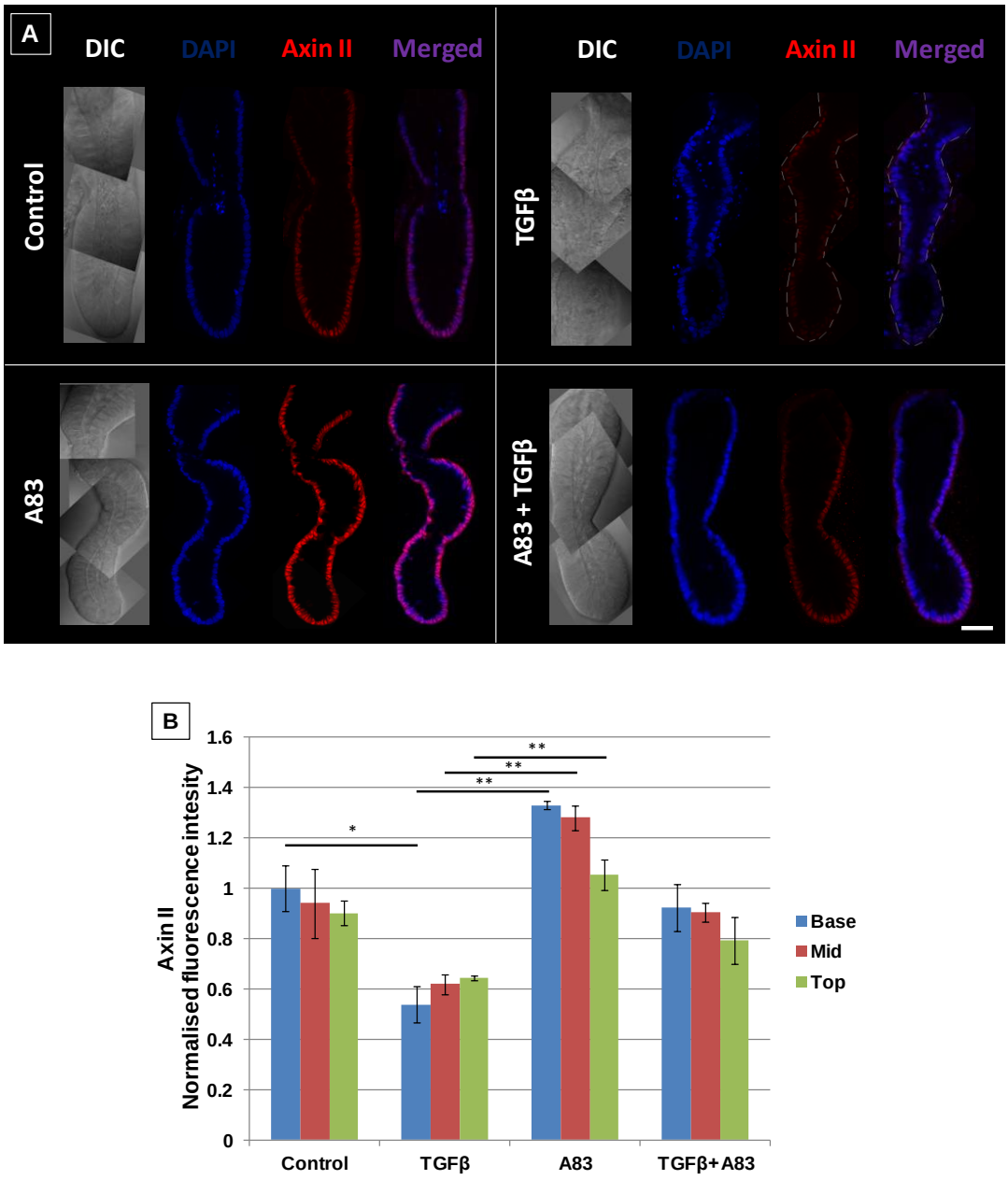
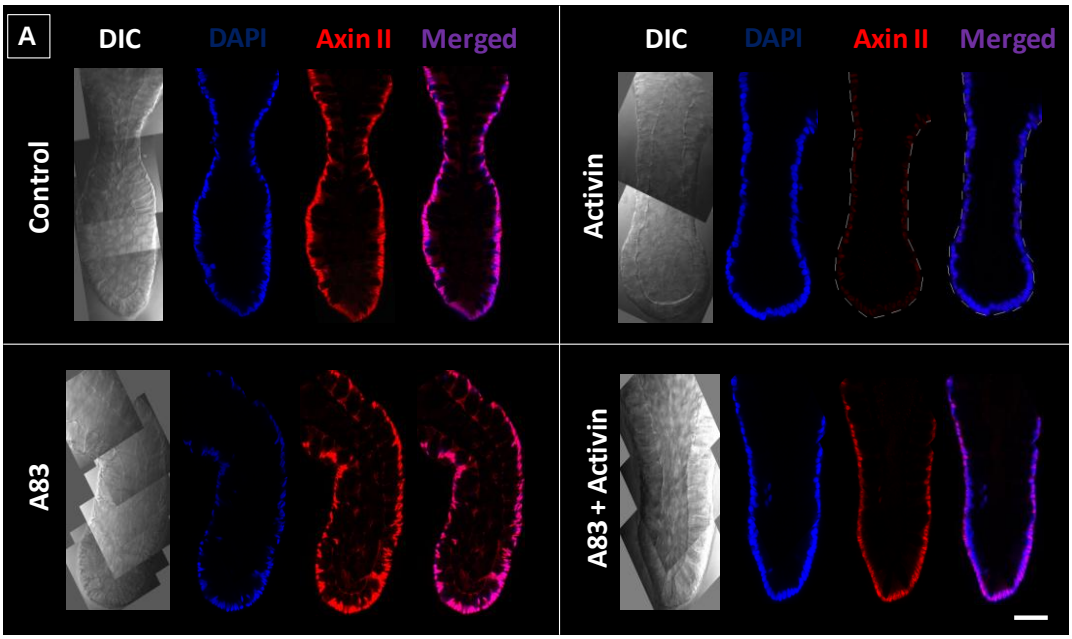


Figure 3.34 TGFβ inhibits Wnt target gene axin II. *A) Crypts were cultured in TGFβ and/or A83-01 for 3 days. Crypts were then processed by immunocytochemistry and labelled for axin II. Scale bar represents 50μm. B) Analysis of nuclear axin II along the crypt axis. Nuclear fluorescence intensity measurements from each region of the crypt were normalised to the crypt base of the control group. TGFβ significantly reduced axin II expression. (*p=0.039, **p≤0.031 in all regions of crypts N=8 crypts from 1 patient). Control media: IGF-1 (50ng/ml), R-Spondin 1 (500ng/ml), Wnt3a (100ng/ml), Noggin (100ng/ml); TGFβ (20ng/ml) and A83-01 (0.5μM) where indicated.*

Another ligand and member of the TGFβ signalling family is activin, which also has a role in cell differentiation and apoptosis. It binds to a type II activin receptor which activates and phosphorylates the type I activin receptor. SMAD 2 and 3 are then phosphorylated and activated and bind with SMAD 4 which translocates to the nucleus. To reaffirm the effect of TGFβ signalling on the inhibition of Wnt signalling, the effects of activin on Wnt target gene axin II expression was investigated. Isolated crypts were cultured either in the presence of activin or A83-01 for 3 days, processed by immunocytochemistry and labelled for axin II (Fig 3.35). Like the TGFβ ligand, activin significantly suppressed axin II expression and A83-01 rescued the inhibitory effects of activin.



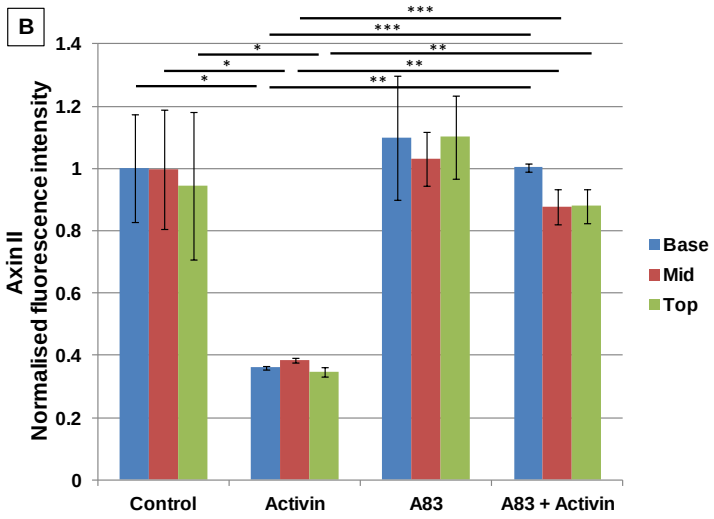
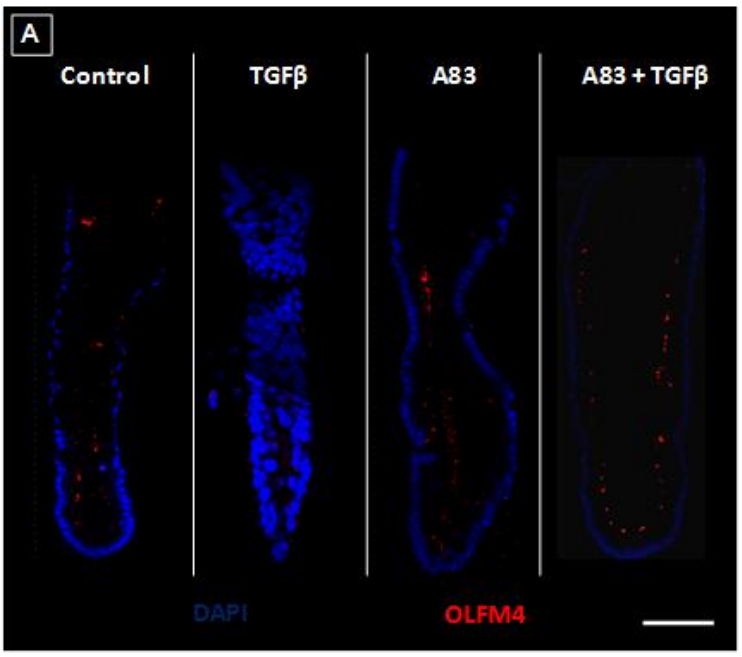


Figure 3.35 Activin inhibits Wnt target gene axin II. *A) Crypts were cultured in activin and/or the Alk 4/5/7 inhibitor A83-01 for 3 days. Crypts were then processed by immunocytochemistry and labelled for axin II. Scale bar represents 50μm. B) Analysis of nuclear axin II along the crypt axis. Nuclear fluorescence intensity measurements from each region of the crypt were normalised to the crypt base of the control group. Activin significantly reduced axin II expression whilst A83 rescued the inhibitory effects of activin. (*p<0.05 in all regions of crypt, **p<0.05 in all regions of crypt, ***p<0.05, N=9 crypts from 1 patient). Control media: IGF-1 (50ng/ml), R-Spondin 1 (500ng/ml), Wnt3a (100ng/ml), Noggin (100ng/ml); TGFβ (20ng/ml) and A83-01 (0.5μM) where indicated.*

3.2.5 Role of TGFβ/Activin signalling in crypt renewal

As mentioned before, TGFβ signalling has an opposing role to Wnt signalling by inhibiting stem cell self-renewal and proliferation of cells and promoting cell differentiation and apoptosis as well as epithelial-to-mesenchymal transition. To investigate the consequences of TGFβ signalling on maintenance of stem cells in culture, isolated crypts were cultured in TGFβ ligand or A83-01 for 3 days, processed by immunocytochemistry and labelled for OLFM4 (Fig 3.36). A83-01 increased the number of $OLFM4^{+}$ stem cells, whilst TGFβ completely abolished all the stem cells in culture. TGFβ (and BMP4) also suppressed Lgr5 immunolabelling (Fig 3.37).



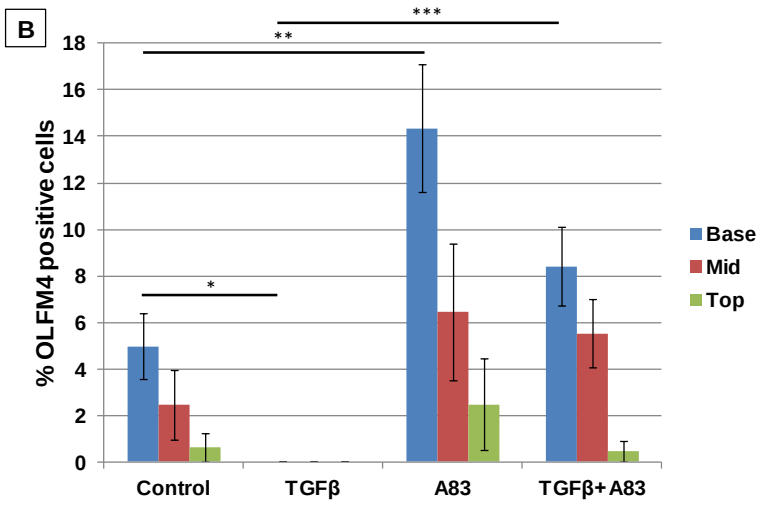


Figure 3.36 TGFβ abolishes OLFM4$^+$ stem cells. *A) Crypts were cultured in TGFβ and/or A83-01 for 3 days. Crypts were then processed by immunocytochemistry and labelled for OLFM4. Scale bar represents 50μm. B) Percentage of OLFM4$^+$ stem cells. A83-01 maintains number of OLFM4$^+$ stem cells whilst TGFβ completely abolished OLFM4$^+$ stem cells in culture. A83-01 also rescued the inhibitory effects of TGFβ on the maintenance of stem cells. (*p=0.023, **p=0.013, ***p=0.003, N=1 crypts from 2 patients). Control media: IGF-1 (50ng/ml), R-Spondin 1 (500ng/ml), Wnt3a (100ng/ml), Noggin (100ng/ml); TGFβ (20ng/ml) and A83-01 (0.5μM) where indicated.*

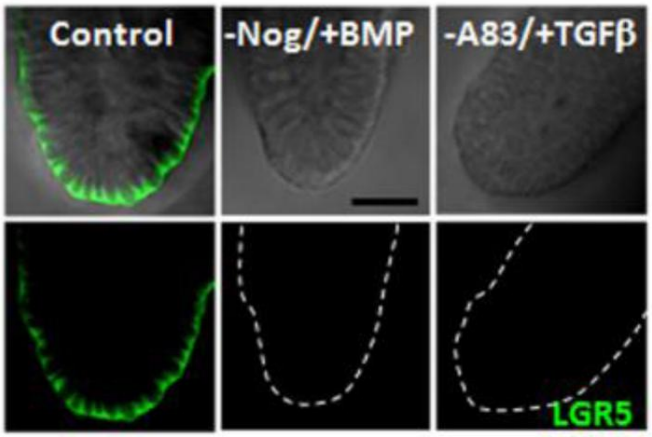


Figure 3.37 TGFβ pathway activation or BMP pathway activation suppresses Lgr5 immunolabelling. *Crypts were cultured either in TGFβ or BMP4 for 3 days. Crypts were then processed by immunocytochemistry and labelled for Lgr5. Scale bar represents 50μm. (N=4 crypts from 2 patients). Control media: IGF-1 (50ng/ml), R-Spondin 1 (500ng/ml), Wnt3a (100ng/ml); BMP4 (100ng/ml), TGFβ (20ng/ml) where indicated.*

To investigate the consequences of TGFβ signalling on proliferation of cells in culture, isolated crypts were culture in TGFβ ligand or A83-01 for 3 days, processed by immunocytochemistry and labelled for BrdU (Fig 3.38). A83-01 significantly increased proliferation of cells in culture whilst TGFβ completely abolished proliferation. The crypts in TGFβ also started to lose their polarity and overall morphology, possibly due to the TGFβ activating epithelial-to-mesenchymal transition. A83-01 significantly rescued the effects of TGFβ on both proliferation and morphology. The control crypts have significantly lower proliferation of cells compared to the A83 group possibly due to the endogenous levels of TGFβ ligands that accumulate in culture which would inevitably inhibit Wnt ligands that promote proliferation.

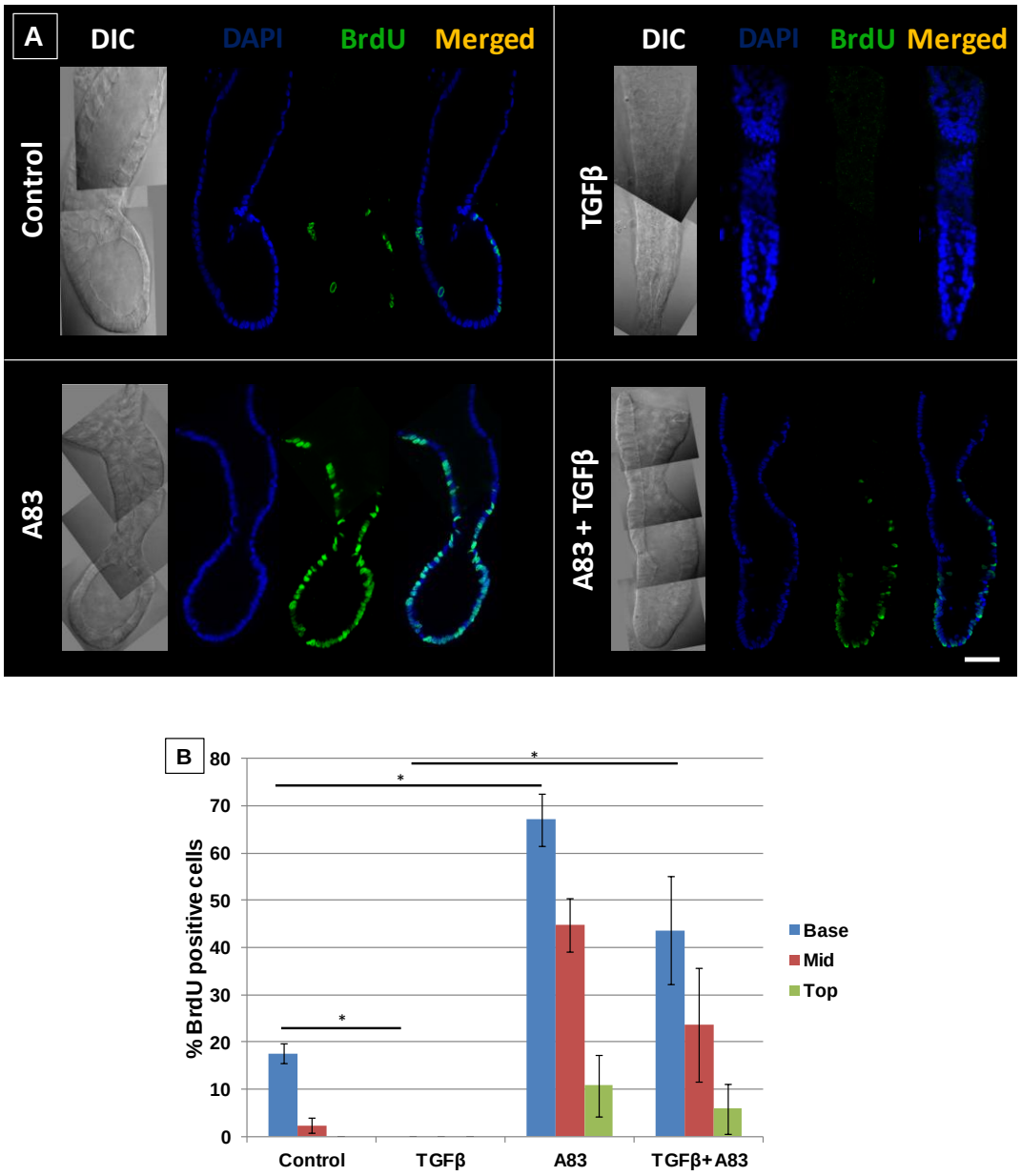


Figure 3.38 TGFβ abolishes proliferation. *A) Crypts were cultured in TGFβ and/or A83-01 for 3 days. Crypts were then processed by immunocytochemistry and labelled for BrdU. Scale bar represents 50μm. B) Percentage of BrdU^{+} proliferating cells. A83-01 increased proliferation of cells whilst TGFβ completely abolished it. A83-01 also significantly rescued the inhibitory effects of TGFβ. (*p=0.0, N=21 crypts from 2 patients). Control media: IGF-1 (50ng/ml), R-Spondin 1 (500ng/ml), Wnt3a (100ng/ml), Noggin (100ng/ml); TGFβ (20ng/ml) and A83-01 (0.5μM) where indicated.*

To demonstrate that TGFβ ligand is responsible for the inhibition effects on proliferation of cells and that A83-01 targets the TGFβ signalling pathway, the effects of TGFβ were inhibited by addition of an antibody against TGFβ. This was done by culturing the crypts with TGFβ antibody to inhibit any endogenous TGFβ ligands. A83-01 was used as a positive control to show that a similar level of inhibition of TGFβ can be achieved by another inhibitor of TGFβ signalling. COX2 antibody was used as another control to demonstrate that only the specific antibody against TGFβ will block the effects of TGFβ and not the experimental protocol. Fig 3.39 shows the percentage of proliferating cells is almost the same in both the anti-TGFβ and A83 groups which are both significantly higher than control, thus confirming that it is endogenous TGFβ ligand that has the inhibitory effect on proliferation. The control and COX2 also have similar percentage of proliferation showing that the experimental protocol of antibody blocking has no effect on proliferation itself.

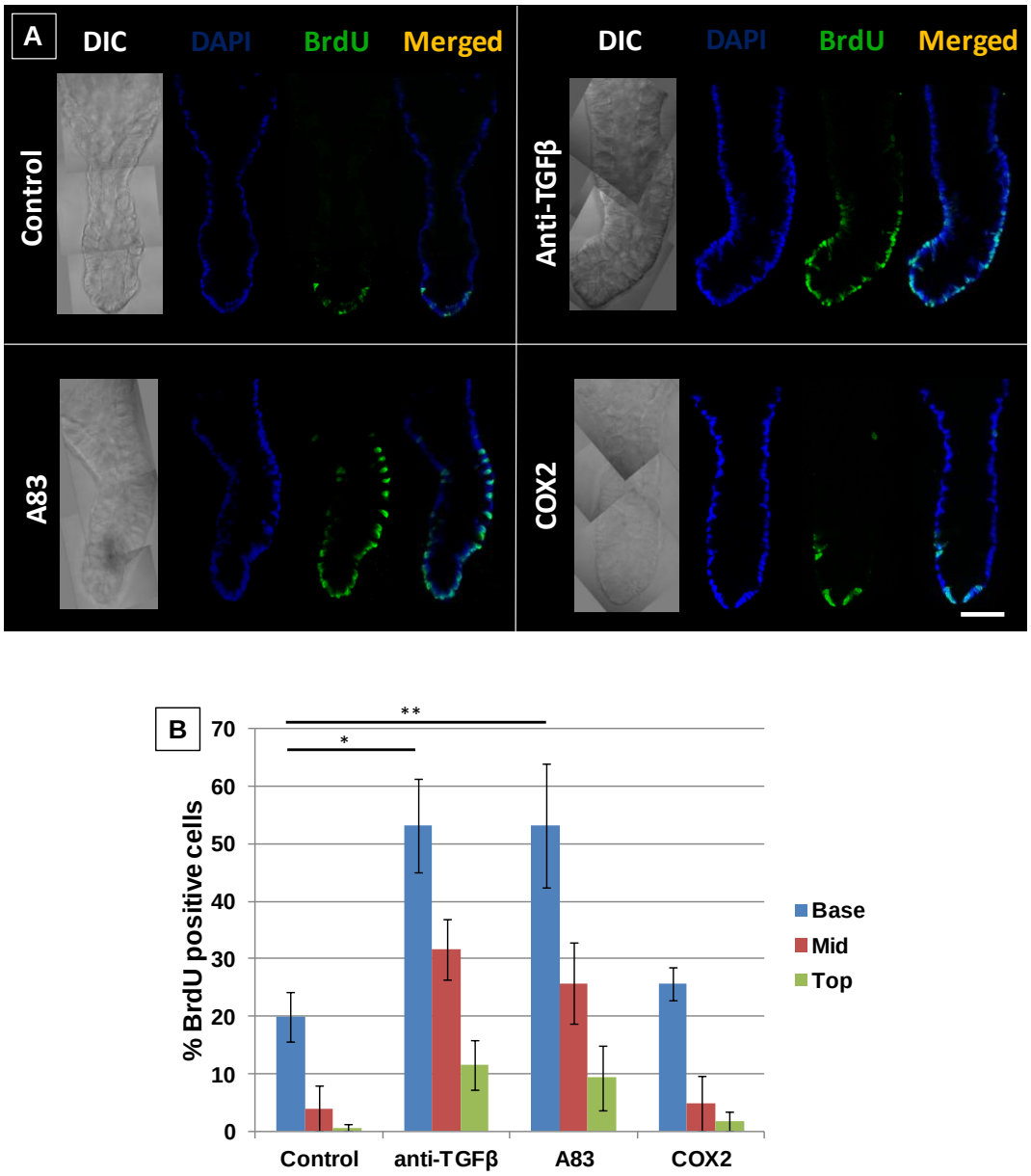


Figure 3.39 anti-TGFβ maintains proliferation. *A) Crypts were cultured either in TGFβ blocking antibody or the Alk inhibitor 4/5/7 A83-01 for 3 days. Crypts were then processed by immunocytochemistry and labelled for BrdU. Scale bar represents 50μm. B) Percentage of BrdU^{+} proliferating cells. Anti-TGFβ and A83 significantly maintained proliferation of cells compared to control and COX2 (antibody control). (*p=0.045, **p=0.032, N=17 crypts from 1 patient). Control media: IGF-1 (50ng/ml), R-Spondin 1 (500ng/ml), Wnt3a (100ng/ml), Noggin (100ng/ml); A83-01 (0.5μM), anti-TGFβ monoclonal antibody (10μg/ml), anti-COX2 antibody (10μg/ml) where indicated.*

As mentioned before, another agonist of TGFβ signalling is activin, therefore its effects on proliferation were investigated. Crypts were cultured either in activin or A83-01 for 3 days, processed by immunocytochemistry and labelled for BrdU. Fig 3.40 shows that activin inhibited proliferation of cells, whilst A83-01 significantly increased proliferation. A83-01 also rescued the inhibitory effects of activin.

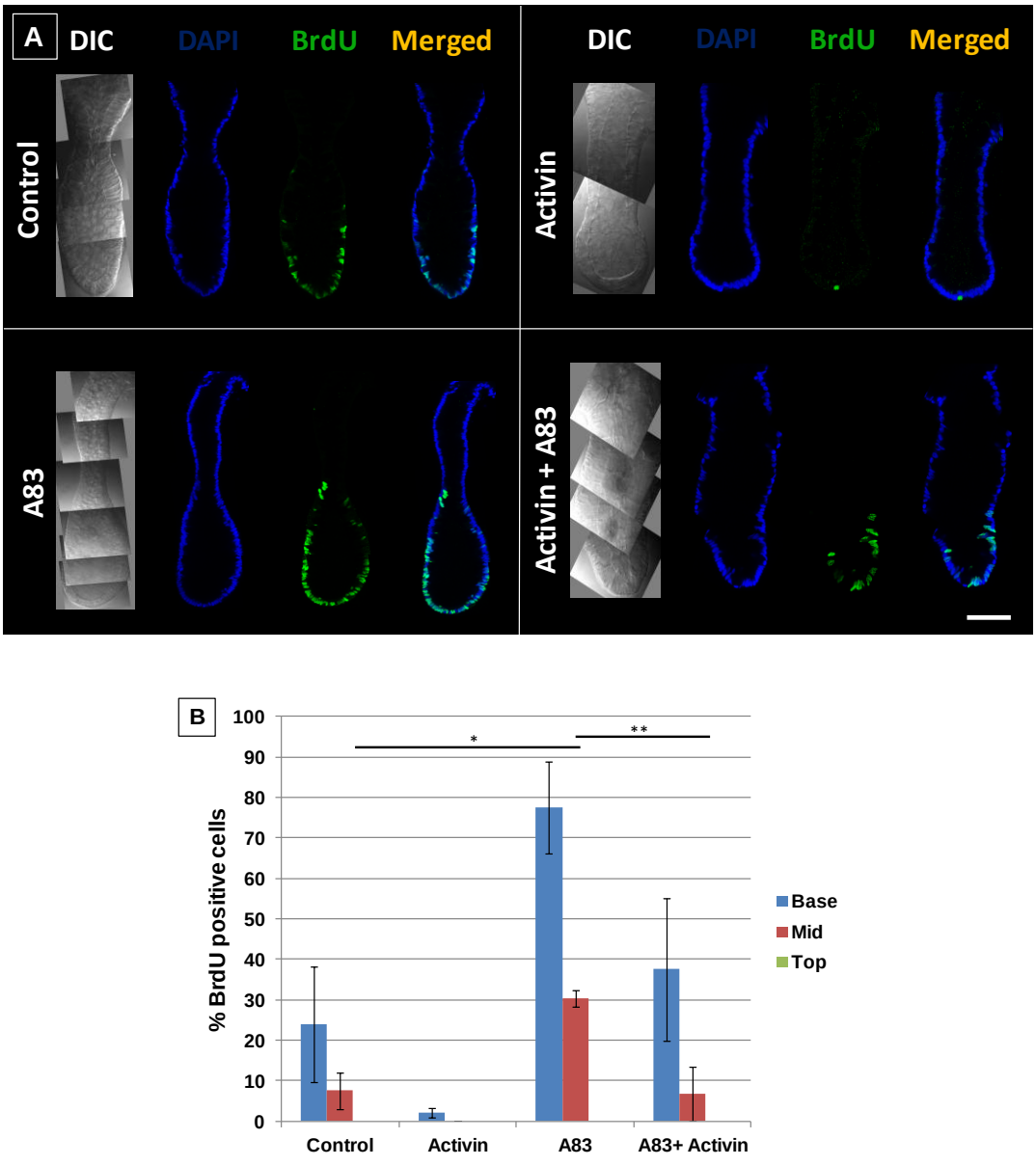


Figure 3.40 Activin inhibits proliferation. *A)Ccrypts were cultured in activin and/or A83-01 for 3 days. Crypts were then processed by immunocytochemistry and labelled for BrdU. Scale bar represents 50μm. B) Percentage of BrdU^{+} proliferating cells. Activin*

*abolished proliferation whilst A83 increased proliferation of cells. (*p=0.027, **p=0.023, N=9 crypts from 1 patient). Control media: IGF-1 (50ng/ml), R-Spondin 1 (500ng/ml), Wnt3a (100ng/ml), Noggin (100ng/ml); A83-01 (0.5μM), Activin (50ng/ml), where indicated.*

Having established that ligands of TGFβ signalling, both TGFβ and activin abolish proliferation of cells within the crypts, it was also noted that TGFβ induced loss of morphology possibly though initiating epithelial-to-mesenchymal transition. Loss of proliferation along with loss of stem cells means that no self-renewal of stem cells occurs and further proliferation is also inhibited which leads to the homeostasis of crypt renewal being imbalanced. This means that if cells are still being shed at the top, yet not being replaced, the crypts will shorten in length and eventually die. The effects of TGFβ signalling on morphology and crypt length was investigated by culturing isolated crypts either in TGFβ, A83-01 or both for 3 days and images of the crypts being taken on day 3. Crypt length was then measured for all crypts and the lengths normalised to the control group. Fig 3.41 shows that TGFβ caused the crypts to shorten and the cells to lose their morphology. A83-01 by comparison increased crypt length and also rescued the effects of TGFβ.

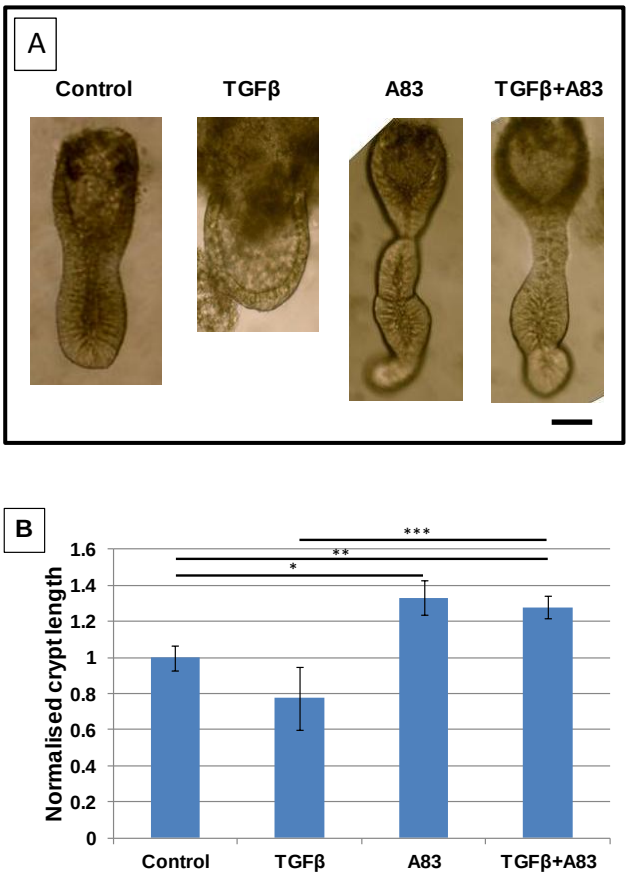


Figure 3.41 A83 maintains crypt morphology and length. *A) Crypts were cultured in TGFβ and/or the TGFβ antagonist A83-01 for 3 days. Crypt length was measured on day 3 of the experiment and normalised against the control crypts. Scale bar represents 50μm. B) A83-01 increasess crypt length and morphology, whilst TGFβ leads to crypt shortening and loss of morphology. A83-01 rescues the detrimental effects of TGFβ. (*p=0.041, **p=0.027, ***p=0.008, N=18 crypts from 2 patients). Control media: IGF-1 (50ng/ml), R-Spondin 1 (500ng/ml), Wnt3a (100ng/ml), Noggin (100ng/ml); A83-01 (0.5μM), TGFβ (20ng/ml), where indicated.*

Real-time time-lapse microscopy was carried out to determine the effects of TGFβ pathway inhibition on crypt length, proliferation and migration (see appendix: movie 2). Inhibition of TGFβ pathway with A83-01 significantly increased crypt length and proliferation (number of mitoses per hour), whilst crypt cell migration stayed constant (Fig 3.42). Under these conditions, crypt cell proliferation in the lower half of the crypt is

required to maintain a steady state crypt cell population, as measured by crypt length, by replenishing cells shed from the upper surface, but does not appear to drive crypt cell migration.

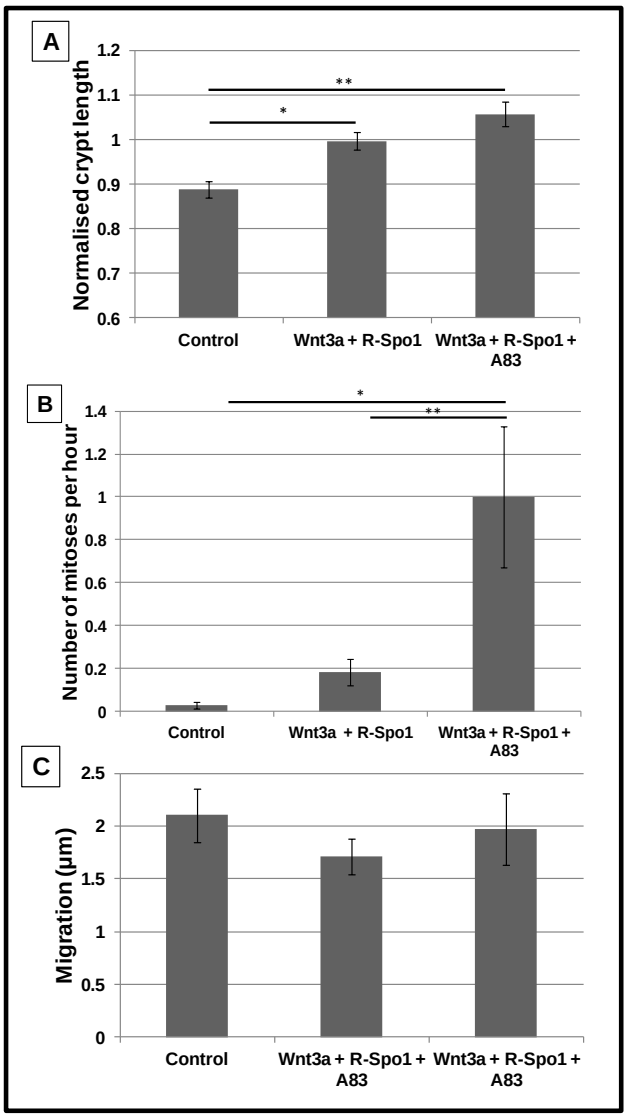


Fig 3.42 Inhibition of TGFβ signalling maintains crypt cell renewal. *A) Wnt signals along with Alk 4/5/7 inhibitor A83-01 increased crypt length (*p=0.009, **p=0.0). B) A83-01 increased proliferation of cells (*p=0.002, **p=0.007). C) Crypt cell migration rate stayed constant. Control media: IGF-1 (50ng/ml), Noggin (100ng/ml); R-Spondin 1 (500ng/ml), Wnt3a (100ng/ml) and A83-01 (0.5µM) where indicated.*

3.2.6 Discussion

Along with Wnt signalling, BMP and TGFβ signalling are involved in maintaining crypt renewal and hierarchy along the crypt axis. In the previous chapter Wnt signalling was found to predominate at the base of the crypts and have a role in maintaining stem cell renewal. However, BMP and TGFβ signalling have a proposed role in cell differentiation and apoptosis and have been found to predominate at the top of the crypt. Kosinski *et al.* (74) have found BMP ligands such as BMP1, BMP2, BMP5 and BMP7 to be highly expressed in colon tops whilst the BMP antagonists such as gremlin and Chordin-like 1 to be expressed at the base of crypts. He *et al.* (8) found BMP4 in the intravillus and intercrypt mesenchymal cells whilst the BMP antagonist noggin was expressed in the submucosal region adjacent to the crypt base. They also found pSMAD1,5,8, the hallmark of activated BMP signalling, in both villi and intestinal stem cells. Auclair *et al*. (108) also found pSMAD1,5,8 immunostaining in villus epithelial cells as well as crypt-villus junction and to a lesser extent at the bottom of the crypt near the stem and Paneth cell region. In our study we found that pSMAD1,5,8 activation was predominantly at the top of the crypt but there was low level of activation in the stem cell region of the crypt too (Fig 3.25). He *et al.* (8) suggest that transient expression of noggin in the stem cells overrides the BMP signal to release β-catenin inhibition by PTEN which then activates the stem cells for proliferation. This balance between BMP and Wnt signals inhibits aberrent proliferation of stem cells to prevent crypt fission and increase in crypt number. The effects of noggin on pSMAD1,5,8 levels were investigated in the present study which demonstrated that noggin suppressed endogenous pSMAD1,5,8 levels as well as rescuing the inhibitory effects of exogenous BMP4 ligand (Fig 3.26). Haramis *et al*. (7) also found that pSMAD1,5,8 was almost entirely absent when BMP signalling was inhibited by transgenic expression of noggin. Farral *et al*. (166) have found that as well as high levels of Wnt and Notch ligands, high BMP4 expression was also observed in their tumour spheroids and in adenomas. In the mouse adenomas, pSMAD1,5,8 activation was consistently strongest in regions of high BMP4 expression and overlapped with enhanced

β-catenin levels which were detected throughout the adenoma. These regions also had low expression levels of Wnt target gene CD133. These spheroids are normally cultured in the presence of noggin to inhibit BMP signalling and avoid cell differentiation. However, culturing them in the absence of noggin, induced an increase in pSMAD1,5,8 levels as well as enterocyte cell maker FABP2 and a reduction in stem cell markers and Wnt target genes CD44, CD133 and Lgr5. Addition of recombinant BMP4 led to the spheroids displaying a 'dimpling' effect in their surface and attenuated cell growth rates. Farral *et al*. (166) suggest that high Wnt, Notch and BMP ligands in the cultured tumour spheroids replicate the growth factors in a tumour microenvironment and that autocrine secretion of BMP signals induce the tumour cells to differentiate.

BMP signalling has been shown to counterbalance intestinal stem cell self-renewal through the suppression of β-catenin signalling and induce differentiation in the villus (8). This suppression of β-catenin was also observed in our study (Fig 3.27). We found that BMP4 ligand slightly suppressed endogenous levels of β-catenin, whilst the BMP antagonist noggin significantly increased levels of nuclear β-catenin. This suggests that within the culture system the epithelial cells secrete low levels of BMP ligands which are not suppressed by antagonists such as noggin and gremlin, which are secreted by the mesenchymal cells (74). Our isolated cultured crypts lack the mesenchymal cells that normally surround the crypt, therefore only exogenous BMP antagonists such as recombinant noggin will suppress levels of pSMAD1,5,8 (Fig 3.26) and stimulate β-catenin levels by inhibiting BMPs. BMP4 also significantly inhibited the Wnt target gene axin II, which was rescued by addition of noggin (Fig 3.28). Auclair *et al.* (108) however found that loss of epithelial BMP signalling does not effect β-catenin nuclear expression or modulates the Wnt target gene c-Myc.

As mentioned before, Farral *et al*. (166) found that BMP4 suppressed expression of stem cell markers such as Lgr5 and CD133 in their tumour spheroids and a similar result was seen in our study. BMP4 completely abolished the number of $OLFM4^{+}$ stem cells in crypts

after 3 days in culture (Fig 3.29). However, this inhibition was significantly rescued by addition of noggin, even when BMP4 ligand was present. BMP4 also suppressed expression of Lgr5 (Fig 3.37). Lombardo *et al.* (264) found that BMP4 induced terminal differentiation and apoptosis in colorectal cancer stem cells. Since BMP signalling is involved in differentiation and inhibits stem cell self-renewal though inhibition of Wnt signalling, it is expected that cell proliferation within the crypt is also affected. We found again that BMP4 completly abolished proliferation of cells and only addition of noggin, even in the presence of BMP4, maintained proliferation (Fig 3.30). The number of proliferating cells was surprisingly low in the control group and this is possibly due to endogenous BMP ligands that accumulate in culture over 3 days. A similar result was observed by Auclair *et al*. (108) who found that BMP signalling suppresses epithelial cell proliferation and loss of epithelial BMP signalling leads to elongated villi and increased crypt fission, demonstrating the role of BMP signalling in epithelial architecture in the crypt-villus axis. The effect of BMP signalling on crypt morphology was also observed in this study; with BMP4 causing the crypts to shorten in length and lose their polarity over a period of 3 days in culture (Fig 3.31). Noggin was seen to maintain crypt length compared to control and still maintained length and morphology of crypts in the presence of exogenous BMP4. This replicates the results seen with cell proliferation, since loss of proliferation and stem cells will lead to an imbalance in cell renewal which leads to crypt shortening.

Migration, differentiation and compartmentalisation along the crypt-axis is also controlled by TGFβ/Wnt gradients. Barnard *et al*. (192) and Murphy *et al*. (265) have found TGFβRII receptors localised to the differentiated cells of the crypt villi, whilst Koyama *et al*. (193) have also found the receptors at the crypt base. Tang *et al*. (194) have found expression of SMAD4 mostly at the top of the crypts as well as at the crypt base though at lower levels. This result is consistent with the type of pSMAD2,3 labelling we observed along the crypt-axis (Fig 3.32). Although pSMAD2,3 activation was seen all along the

crypt-axis, there was a slighly higher level of activation in the upper region of the crypt. Mishra *et al*. (109) suggest that the presence of TGFβ signalling and absence of Wnt signalling at the top of the crypt is the switch that initiates rapid cell cycle arrest and differentiation. TGFβ signalling has also been shown to be involved in epithelial-to-mesenchymal transition (EMT) (201) (202). Tojo *et al*. (263) found that the alk inhibitor 4/5/7 A83-01 inhibits SMAD signalling and TGFβ induced EMT. We also found in our study that A83-01 inhibited pSMAD2,3 (Fig 3.33) in cultured crypts even in the presence of TGFβ ligand.

TGFβ signalling components are localised in differentiated epithelial cells and have growth suppressive effects. Ishitani *et al.* (266) and Meneghini *et al.* (267) propose that TGFβ signals antagonise Wnt signalling through a route involving the alternative TGFβ effector and MAPKKK and TAK1. They found that in *Caenorhabditis elegans* and mammalian cells, activation of TAK1 stimulates the activity of MAPK NLK, which in turn downregulates TCF. Alternatively, Sasaki *et al.* (268) have shown that TGFβ stimulation inhibits TCF4/β-catenin transactivation of c-Myc via the ability of SMAD3 to physically interact with β-catenin and thereby decouple TCF4/β-catenin complexes (269). The downstream effects of inhibiting TCF/β-catenin complexes are that Wnt target genes such as axin II are downregulated. We found that TGFβ signalling significantly inhibited expression of axin II and this effect was only rescued by addition of the Alk 4/5/7 inhibitor A83-01 (Fig 3.34). The expression of axin II was also inhibited by activin, another activator of the TGFβ signalling pathway (Fig 3.35). Again, A83-01 siginificantly rescued the inhibitory effects of activin stimulation. Activin has a role on cell growth and differentiation mainly through the SMAD-dependent pathways. Kanamaru *et al*. (270) showed that activin A induces growth inhibition on liver cells by downregulating Bcl-xL (anti-apoptotic) expression via SMAD2 or SMAD3. Ho *et al*. (271) also show that activin induces hepatocyte cell growth arrest. We found that both activin and TGFβ ligand inhibited cell proliferation in cultured crypts (Fig 3.40 and 3.38) and A83-01 significantly

rescued this effect by maintaining proliferation. Using an antibody against TGFβ ligand in the cultured crypts, we found that proliferation is maintained, and at the same level as that achieved by A83-01 (Fig 3.39). Potten *et al.* (272) have also showed that TGFβ1 inhibited proliferation in mouse small intestinal crypts. TGFβ can induce antiproliferative gene responses at any point during the cell cycle and inhibits cyclin-dependant kinases as well as downregulating c-Myc. This modulation of cell cycle progression by TGFβ involves SMAD proteins and loss of SMAD 4 has been shown to lead to colorectal cancer (194). As mentioned, TGFβ normally utilises intracellular SMADs to mediate growth suppression, however, Chow *et al*. (273) have found that TGFβ can also activate PI3K to downregulate PTEN for enhancement of cell proliferation that is independent of SMAD proteins and converts TGFβ from a tumour suppressor to tumour promoter.

Constant proliferation of stem cells is important in intestinal tissue to maintain renewal within the crypt to minimise accumulation of mutations. Wnt, BMP and TGFβ pathways are all involved in regulating this renewal and maintaining proliferation and differentiation along the crypt-axis. Since TGFβ is involved in cell differentiation and apoptosis, it is expected that it will have a deletirious effect on stem cells by inhibiting their proliferative capacity and inducing differentiation responsive genes. We found that TGFβ completely abolished $OLFM4^{+}$ stem cells in culture, whilst inhibiting the TGFβ signalling pathway with A83-01 siginificantly increased the number of stem cells compared to control (Fig 3.36). TGFβ also suppressed expression of Lgr5 (Fig 3.37). Potten *et al.* (272) have also shown in mouse that TGFβ1 inhibits proliferation, with the effects particularly pronounced in the lower crypt in the stem cell region. However in embryonic stem cells, TGFβ is involved in their maintanace and characteristics as stem cells. Inhibition of TGFβ/activin/Nodal signaling by SB-431542, a chemical inhibitor of the kinases of type I receptors for TGFβ/activin/Nodal resulted in decreased expression of the markers of undifferentiated states (274). James *et al.* (275) found that phosphorylation and nuclear localization of SMAD2 induced by TGFβ, activin or Nodal signalling was observed in

undifferentiated human embryonic stem cells and decreased upon early differentiation. Watabe *et al*. (276) also found that SB-431542 dramatically decreased the proliferation of mouse embryonic stem cells without decreasing their pluripotency suggesting that activin, Nodal, and/or TGFβ signaling is indispensable for proliferation of embryonic stem cells.

The abolishment of stem cells and inhibited proliferation with TGFβ/activin means that the balance of cell renewal within the colonic crypt is affected. This was seen in our cultured crypts even after 3 days where crypts cultured in TGFβ were dramatically shorter than the control crypts and had lost their morphology and polarity (Fig 3.41). However, the crypts that were cultured in A83-01 increased their lengthcompared to control and inhibited the detrimental effects of TGFβ. As has been demonstrated in this study, inhibition of TGFβ signalling with A83-01 leads to increase of cell proliferation and stem cells which results in crypt renewal and morphology being maintained in culture. This was also demonstrated using real-time time-lapse digital microscopy where crypts cultured in A83-01 increased their length compared to control (Fig 3.42). This increase in length was reflected by the number of mitoses that were observed over the 24 hrs with crypts in A83-01 having a significantly higher mitotic rate compared to the control. The migration rate of cells was also measured, and we observed that migration remained constant in all groups, irrelevant of the growth conditions the crypts were cultured in. This therefore suggests that since migration is constant and proliferation is maintained with TGFβ inhibition, a steady-state crypt renewal is achieved. Abberent proliferation of cells *in vivo* would lead to increased crypt size and crypt fission, therefore a balance between Wnt, BMP and TGFβ signalling is required to regulate the homeostasis and renewal within the colonic crypt.

In this chapter, the role of BMP/TGFβ signalling in human colonic epithelium tissue renewal was demonstrated. BMP/TGFβ signals were found to predominate in the upper region of the crypt and activation of these pathways was deleterious to the maintenance of stem cells and cell proliferation. Inhibition of the BMP/TGFβ pathway was required for human colonic crypt culture for at least 7 days (Fig 3.43).

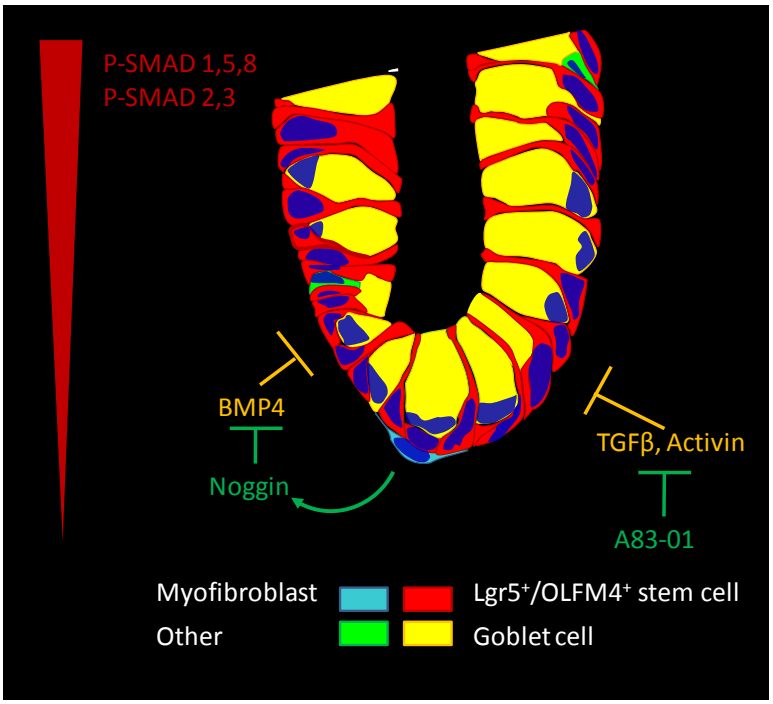


Fig 3.43 Inhibtion of BMP/TGFβ signals maintains intestinal stem-cell driven tissue renewal. *BMP/TGFβ signalling predominates in the upper region of the crypt. Inhibition of BMP/TGFβ signalling maintains stem cells and cell proliferation and expression of Wnt target genes.*

3.3 Intestinal Metaplasia: Development of Barrett's oesophagus crypt culture model

3.3.1 Introduction

Barrett's oesophagus is defined by the presence of columnar epithelium in the lower oesophagus that has replaced the normal squamous cell epithelium as a result of metaplasia and can lead to the development of oesophageal adenocarcinoma. It arises as a repair mechanism to adapt to the harsh intra-oesophageal environment of chronic gastroesophageal reflux disease. The events in the pathogenesis of Barrett's metaplasia are not well understood but signalling pathways such as Wnt, BMP, TGFβ, Hedgehog and NFκB have all been implicated. Abnormal activation of β-catenin is found to be common during metaplastic progression of Barrett's oesophagus and is strongly associated with development of metaplasia (15). Moyes *et al.* (16) have also found that Wnt target genes cyclin D1, Sox9 and c-Myc were upregulated in Barrett's metaplasia and high grade dysplasia compared to normal squamous epithelium. Milano *et al.* (17) have found that the BMP pathway was activated in oesophagitis and Barrett's oesophagus, whilst Zhou *et al.* (226) have shown that acid and bile acid increase the expression of BMP4. TGFβ1 overexpression has been found to be associated with advanced stage of oesophageal adenocarcinoma (239) and induces epithelial-to-mesenchymal transition in the pathogenesis of oesophageal adenocarcinoma (277). Hormi-Carver *et al*. (278) show that Barrett's epithelial cells activate the NFκB pathway after DNA damage allowing them to resist apoptosis which may account for the persistence and malignant predisposition of Barrett's metaplasia.

Research on the cellular and molecular mechanisms involved in the development of Barrett's oesophagus has been difficult due to a lack of human *ex vivo* culture models. Recently however, a Barrett's oesophagus organoid culture model has been developed by Sato *et al*. (20) in which isolated isolated Barrett's crypts formed cystic organoid structures

that resembled colonic organoids. In parallel, the near-native crypt culture model developed during this study in our laboratory, has also paved a way to developing a Barrett's oesophagus crypt culture model in which the single isolated crypts do not form organoids but instead remain in a state of renewal where the morphogenic gradients are maintained. This model will permit the functional interrogation of the status and mechanisms underlying tissue renewal in Barrett's oesophagus, which will aid in the development of strategies to prevent the development of this metaplasia.

3.3.2 Culturing Barrett's epithelium

Isolation of Barrett's oesophagus crypts was carried out in the same manner as that for human colonic crypts. Native tissue was fixed immediately upon removal from the patient undergoing endoscopy or surgery and later microdissected into single crypts. Comparison of Barrett's oesophagus crypts with those of colonic crypts showed great similarity in morphology and the types of cell present (Fig 3.44). Both Barrett's and colonic crypts have a lumen where mucous secreted by the distinctive goblet cells is flushed out. However, Barrett's crypts were found to be about twice the size of colonic crypts.

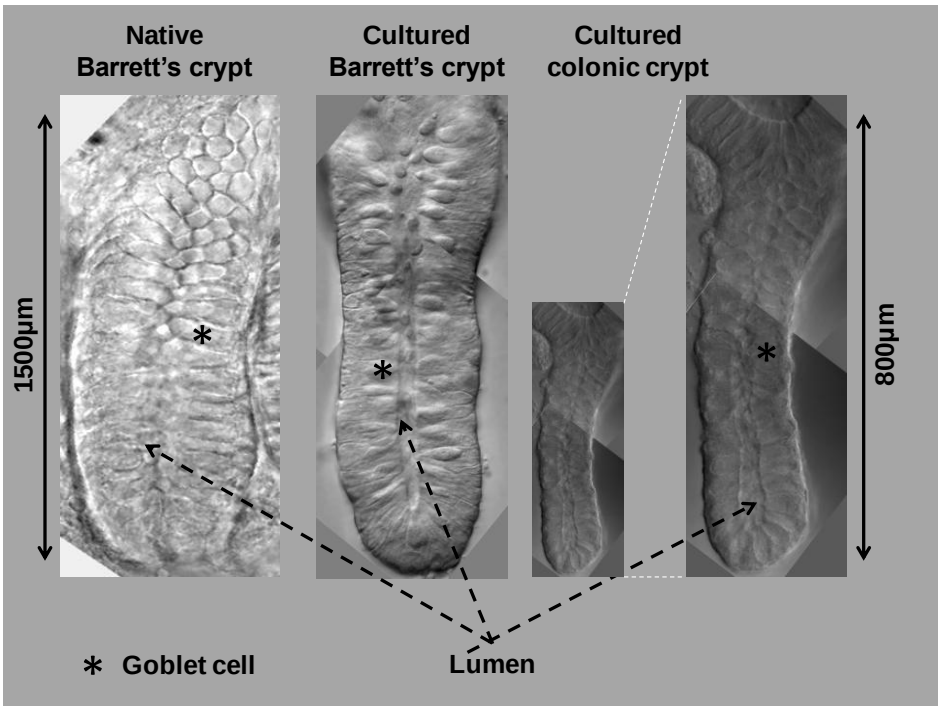


Figure 3.44 Native vs cultured Barrett's oesophagus crypt. *Barrett's oesophagus morphology is very similar to the colonic epithelium, with crypt-like invaginations that contain a lumen and distinctive cells i.e. goblet cells. Barrett's crypts are almost twice the size of colonic crypts and can be isolated from the surrounding squamous tissue in the same manner applied to colonic crypt isolation.*

Once the Barrett's crypts were isolated and placed into culture medium containing growth factors IGF-1, Noggin, R-Spondin1 and Wnt3a, the viability of these crypts was assessed by loading them with Calcein-AM and PI (Propidium Iodide) and then imaging the live crypts on a confocal fluorescent microscope. Calcein-AM is used to demonstrate metabolic esterase activity and therefore viable cells, whilst PI only labels cells that have apoptosed and their membranes have ruptured. As can be seen, cells within the crypt were lit up green with no PI positive cells, whilst at the shedding area at the top of the crypt there are a few PI positive cells (Fig 3.45).

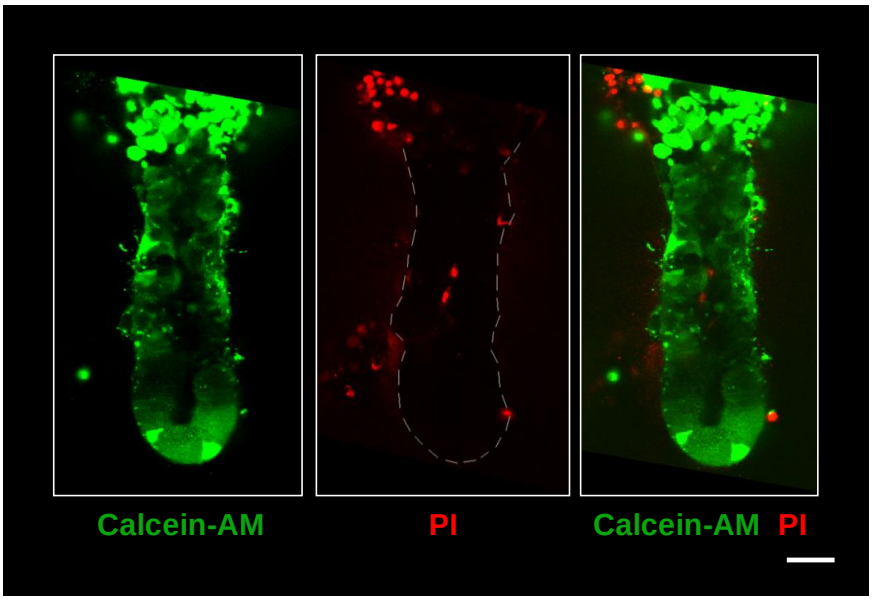


Figure 3.45 Viability of Barrett's oesophagus crypts. *Barrett's crypts were cultured for 24 hrs then loaded with Calcein-AM and PI in the media then imaged live with a confocal fluorescent microscope. Cells that took up Calcein-AM have lit up green demonstrating viability by metabolic esterase activity. PI is excluded from the cells in the body of the crypt, whilst at the top of the crypt, cells that have been shed are positive for PI.*

Observation of mitotic events using real-time time-lapse microscopy provides another method of determining the viability of cells within the isolated Barrett's crypts (see appendix: movie 3). By observing the crypts over a period of 24 hrs from day 1-2, proliferation of cells along the crypt axis can be seen and their rate of mitosis calculated. Fig 3.46 shows snapshots of a mitotic event which takes about an hour to complete, with a rate of about 0.8 mitoses per hour at the base of the crypt. There is a decrease in mitotic events at the top of the crypt although this is not statistically significant possibly due to low numbers of crypts.

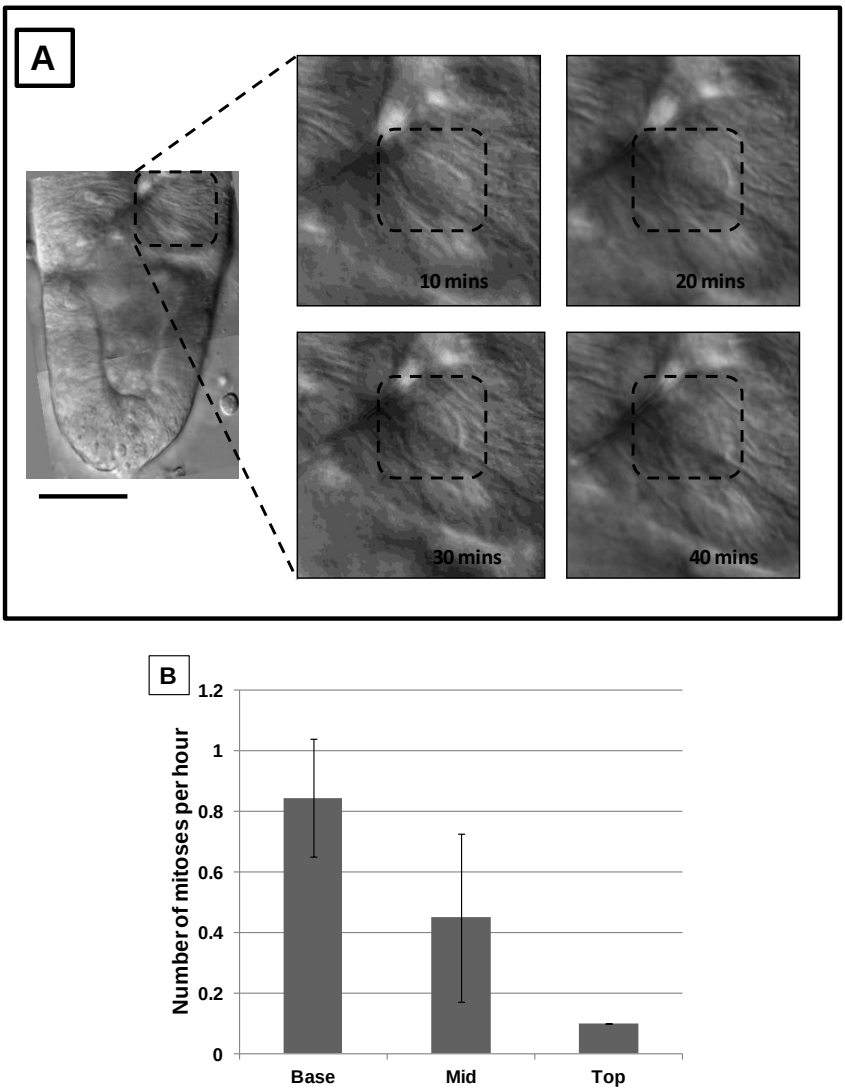


Figure 3.46 Mitotic events in Barrett's oesophagus crypts. *A) Barrett's crypts were cultured for 24 hrs then placed on a time-lapse microscope for 24 hrs with images of the crypt cells being taken every 5 mins. B) Mitotic events can be observed at a rate of 0.8 mitoses per hour with more proliferation occurring at the base of the crypt and very minimal at the top. Culture media: IGF-1 (50ng/ml), Noggin (100ng/ml), R-Spondin 1 (500ng/ml), Wnt3a (100ng/ml). (N=4 crypts from 1 patient).*

3.3.3 Signalling in Barrett's epithelium

Having observed mitotic events in the isolated cultured Barrett's crypts, the proliferative status of cells in cultured crypts was compared to the native microdissected crypts. Both native and cultured crypts were processed by immunocytochemistry and labelled for the

proliferation maker Ki67. Fig 3.47 shows that both the cultured and native crypts have higher proliferation at the crypt base although no statistical gradient along the crypt axis is observed.

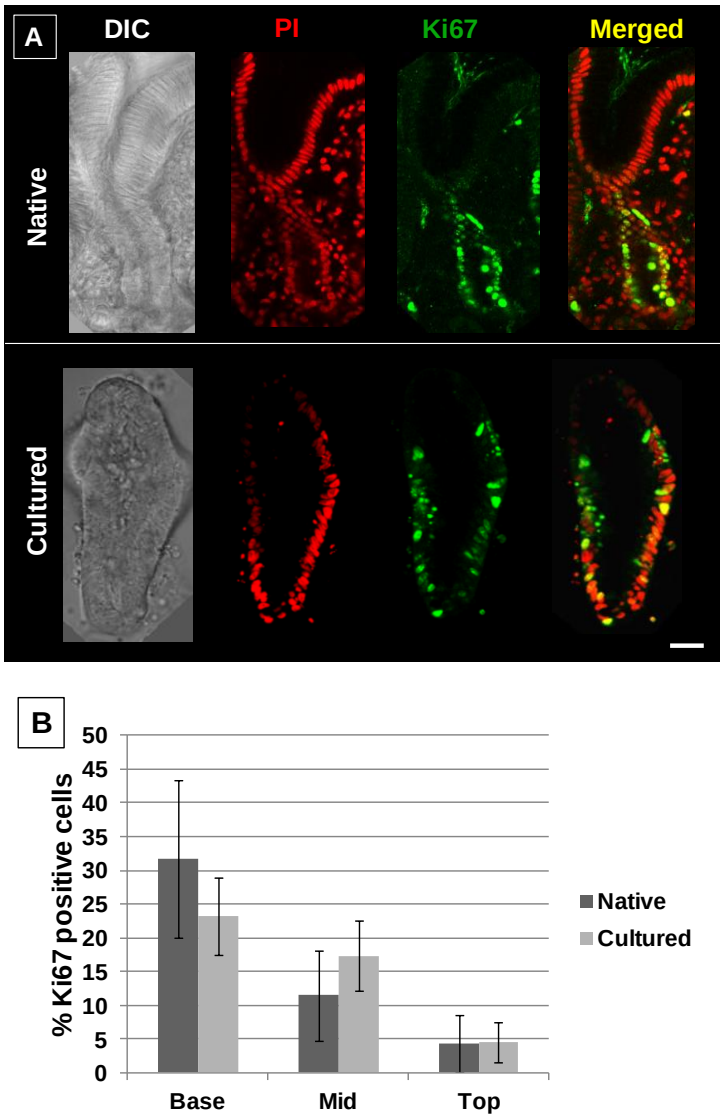


Figure 3.47 Proliferation in native and cultured Barrett's oesophagus crypts. *A) Both native and cultured Barrett's crypts were processed by immunocytochemistry and labelled for Ki67. Scale bar represents 50µm. Culture media: IGF-1 (50ng/ml), Noggin (100ng/ml), R-Spondin 1 (500ng/ml), Wnt3a (100ng/ml). B) Analysis for percentage of* $Ki67^{+}$ *cells in native and cultured Barrett's crypts. (N=6 crypts from 2 patients).*

The Wnt signalling pathway is highly important in maintaining proliferation in colonic crypts and has been implicated in neoplastic progression of Barrett's oesophagus. To

determine the status of Wnt signalling within our human native and cultured Barrett's crypts, immunocytochemistry was carried out on these crypts and then labelled for β-catenin. Fig 3.48 shows that Wnt signalling is indeed active in Barrett's crypts and there are distinct nuclear β-catenin cells at the base of the crypt (arrows). A gradient can be seen along the crypts axis with higher expression of nuclear β catenin at the base of the crypt and significantly lower in the top region of the crypt. Again it can be seen that the cultured crypts maintain their gradients that are observed in native crypts. This demonstrates that this is a good Barrett's crypt culture model that is reflective of the *in vivo* status of human Barrett's oesophagus and is ameanable to functional experiments that cannot be carried out on tissue sections or organoid models.

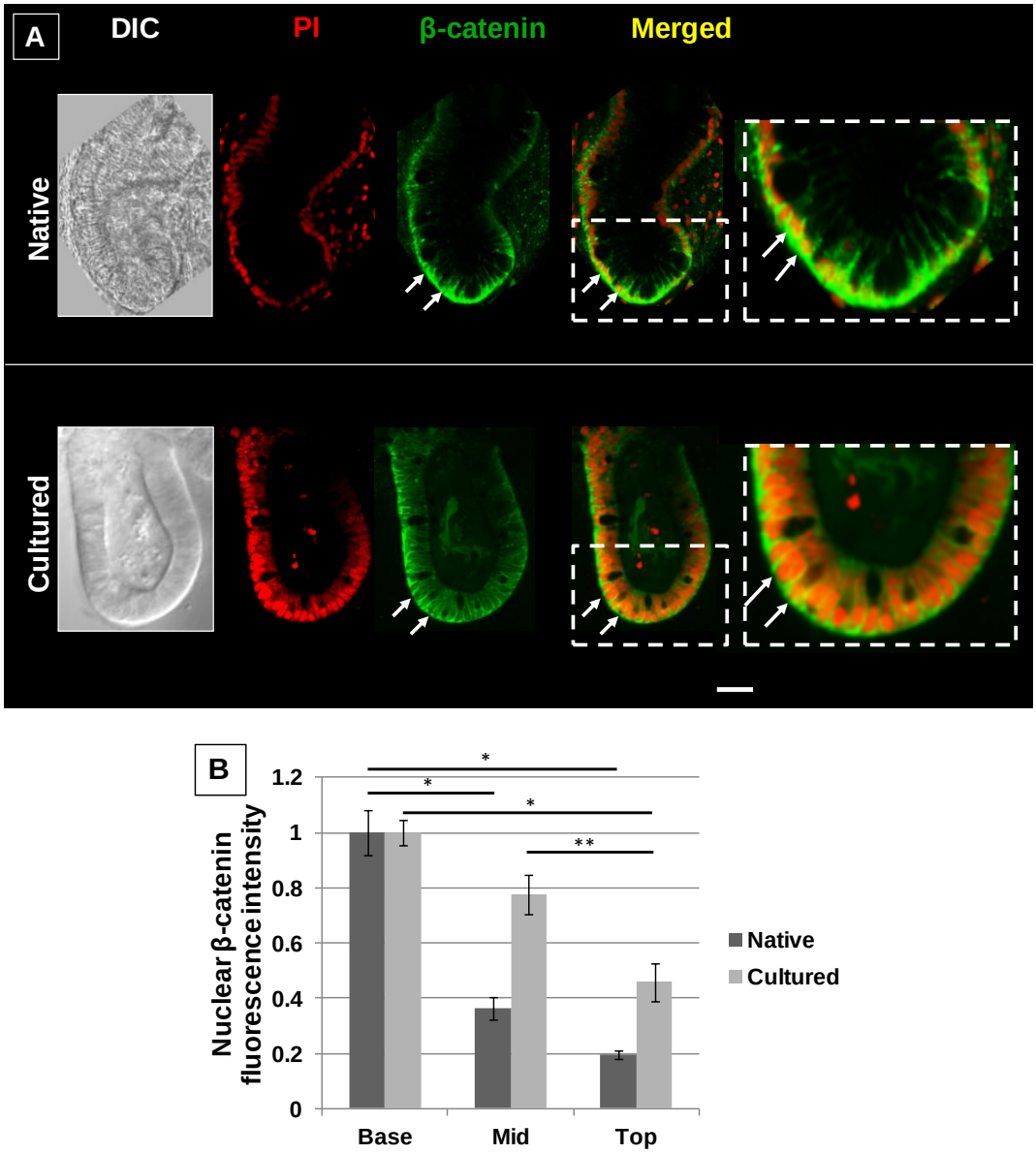


Figure 3.48 Wnt signalling status in Barrett's oesophagus crypts. *A) Native and cultured Barrett's crypts were processed by immunocytochemistry and labelled for β-catenin. Arrows indicate an example of nucler β-catenin in cells. Scale bar represents 50μm. Culture media: IGF-1 (50ng/ml), Noggin (100ng/ml), R-Spondin 1 (500ng/ml), Wnt3a (100ng/ml). B) Analysis for nuclear β-catenin in native and cultured crypts. Higher expression of β-catenin can be seen at the base of the crypt in both native and cutured crypts. (*p=0.0, **p=0.003, N=4 crypts from 2 patients).*

Moyes *et al*. (16) have found that the Wnt target genes cyclin D1, Sox9 and c-Myc were upregulated in Barrett's metaplasia and high grade dysplasia compared to normal

squamous epithelium. The status of Wnt target c-Myc was determined in native microdissected crypts using immunocytochemistry. Fig 3.49 shows that c-Myc is present in Barrett's crypts but no significant gradient along the crypt axis could be observed. However, this could be due to only one patient being analysed which would not reflect the overall status of c-Myc in Barrett's crypts.

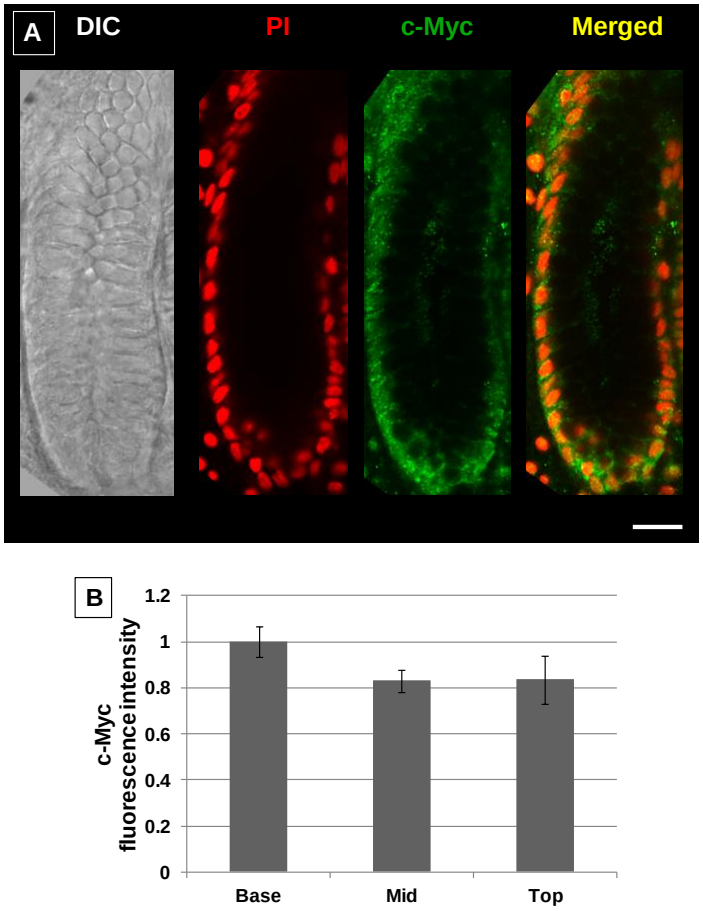


Figure 3.49 c-Myc status in Barrett's oesophagus crypts. *A) Native Barrett's oesophagus crypts were fixed immediately upon removal from the patient and microdissected into single crypts. Crypts were then processed by immunocytochemistry and labelled for c-Myc. Scale bar represents 50μm. B) Analysis for nuclear c-Myc in native crypts. No gradient can be seen along the crypt axis. (N=3 crypts from 1 patient).*

The transcription factor NFκB (Nuclear Factor κB) is a key player in inflammatory response and regulates processes such as cell proliferation and cell survival. Incorrect regulation of NFκB has been linked to cancer and inflammatory diseases. The signalling

status of NFκB in native crypts was determined using immunocytochemitry. Fig 3.50 shows that there is nuclear NFκB present in native Barrett's crypts (arrows) and some in the surrounding squamous tissue. This suggests that the NFκB pathway is activated in the inflammed tissue due to the acid and bile acid reflux. Since Barrett's oesophagus develops as a result of chronic gastroesophageal reflux, irritation of the squamous epithelium leads to the accumulation of inflammatory cytokines in the epithelium. One of the cytokines implicated in inflammatory and malignant processes is Tumour Necrosis Factor-α (TNF-α). TNF-α signalling occurs through several intracellular pathways including NFκB. Tselepis *et al*. (230) have shown elevated levels of TNF-α in Barrett's metaplastic epithelium compared to normal squamous epithelium and that TNF-α expression persisted and intensified during the progression of Barrett's oesophagus to adenocarcinoma. This increased expression of TNF-α along the metaplasia-dysplasia-adenocarcinoma sequence suggests a relatively early role for it in the formation of the adenocarcinoma.

Having found that NFκB signalling is active in native Barrett's crypts, TNF-α was used to stimulate cultured crypts and determine its effects on NFκB. Fig 3.50 shows that TNF-α stimulated NFκB p65 to translocate from the cytoplasm of the cells into the nucleus where antiapoptotic genes can be induced, making the cells more persitant and malignant contributing to metaplasia.

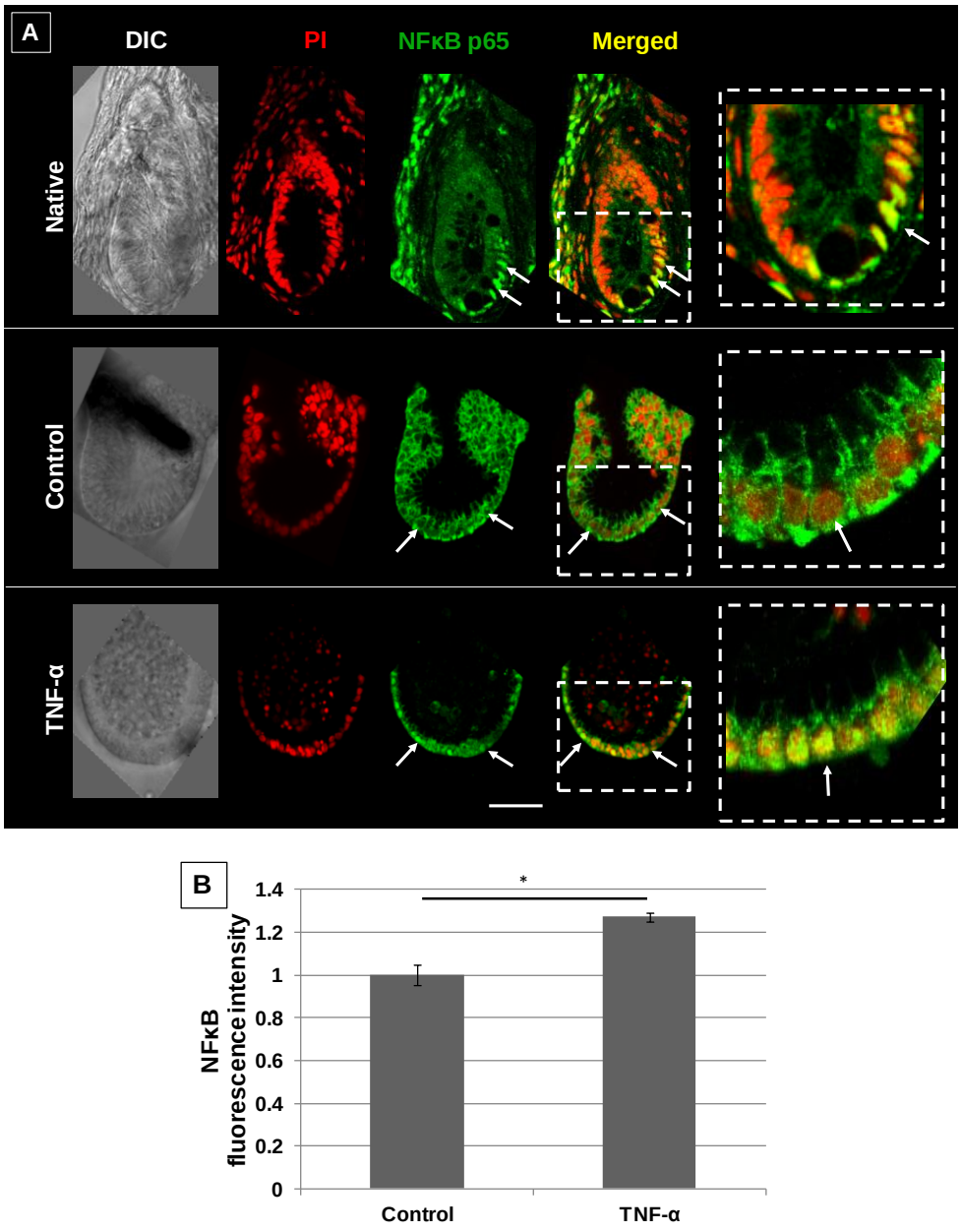


Figure 3.50 NFκB signalling in Barrett's oesophagus crypts. *A) Native Barrett's oesophagus crypts were fixed immediately upon removal from the patient and microdissected into single crypts. Isolated Barrett's crypts were stimulated with TNF-α and fixed on day 2. Control media: IGF-1 (50ng/ml), Noggin (100ng/ml), R-Spondin 1 (500ng/ml), Wnt3a (100ng/ml). Both native and cultured crypts were then processed by immunocytochemistry and labelled for (NFκB) p65. Arrows indicate example of nuclear p65 in cells. Control crypts show p65 in the cytoplasm of the cell which then translocates to the nucleus upon stimulation with TNF-α. Scale bar represents 50μm. B) Analysis for*

*nuclear NFκB (p65) in control and stimulated crypts. (*p=0.037 N=3 crypts from 1 patient).*

No robust markers of stem cells for Barrett's oesophagus have yet been identified. However, since Barrett's oesophagus is a type of intestinal metaplasia it is possible that intestinal stem cell markers such as Lgr5 and OLFM4 could be used to identify stem cells within a Barrett's crypt. Becker *et al.* (237) have shown some staining of $Lgr5^+$ cells at the base of Barrett's crypts and intensity of staining increased with high dysplasia compared to non-dysplastic Barrett's tissue. To determine if our cultured Barrett's crypts labelled for any intestinal stem cell markers, immunocytochemistry was performed on cultured crypts and labelled for OLFM4. Fig 3.51 shows that the intestinal stem cell marker OLFM4 is highly present in Barrett's crypts and seems to be maintained in (day 2) cultured crypts. As seen in colonic crypts, there is a higher percentage of $OLFM4^+$ stem cells at the base of the crypt. There also seems to be a high number of $OLFM4^+$ stem cells in the Barrett's crypt and this could be due to the inflammatory nature of the tissue.

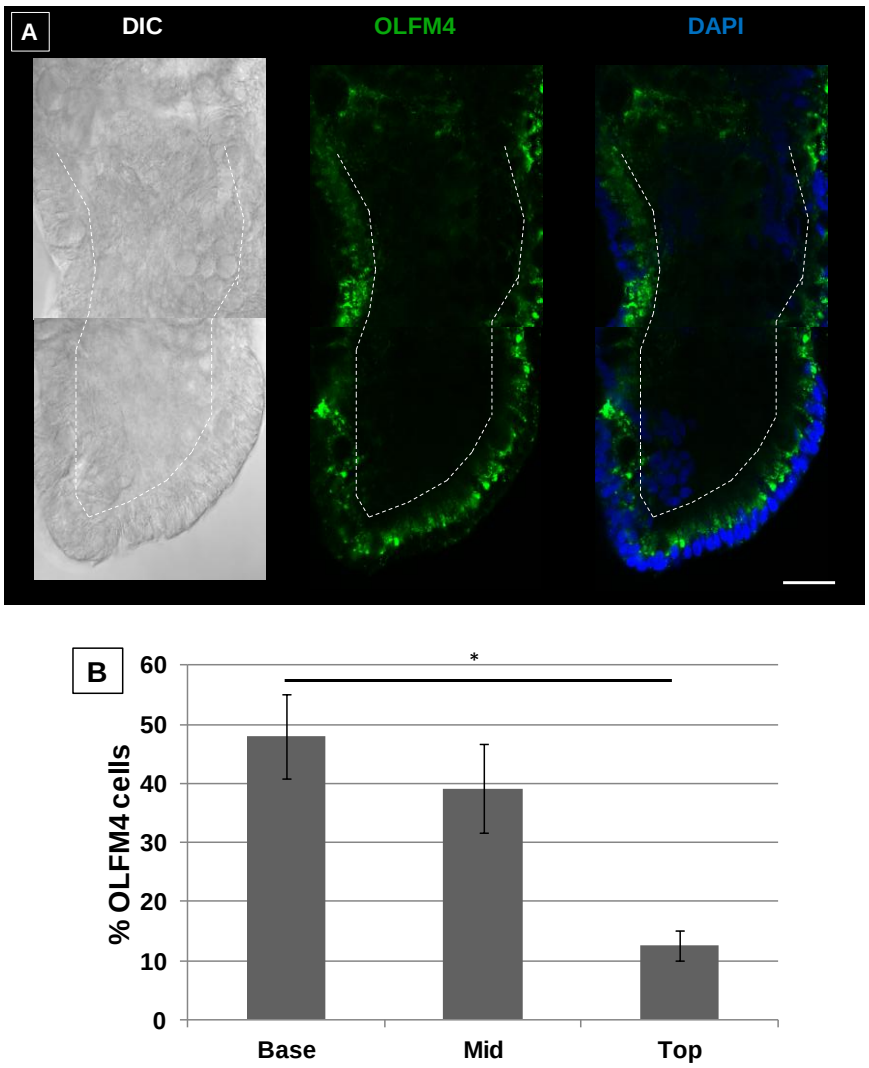


Figure 3.51 Stem cell status in Barrett's oesophagus crypts. *A) Cultured Barrett's oesophagus crypts were fixed on day2, processed by immunocytochemistry and labelled for OLFM4. Scale bar represents 50µm. B) Analysis of percentage of OLFM4$^+$ cells in Barrett's crypts. Higher percentage of OLFM4$^+$ cells can be seen at the base of the crypt compared to the top. (*p=0.043, N=3 crypts from 1 patient).*

3.3.4 Discussion

In this chapter, the potential of a new Barrett's oesophagus culture model was demonstrated. Currently most studies are carried out on cell lines and tissue sections either from surgically manipulated mouse models or human sections from oesophageal adenocarcinomas, Barrett's oesophagus with varying degrees of dysplasia and normal

squamous epithelium. Cell lines can be used to understand the molecular basis of carcinogenesis and data such as viability, apoptosis and tracking of certain signalling molecules and proteins can be determined. This models drawback however, is the lack of conditions and interactions the cells would be subjected to in their natural environment. Surgically manipulated animal models are also used mainly in the development of therapeutics, for example where treatment with COX-2 inhibitors showed a reduced risk of oesophageal adenocarcinoma (242). Recently, a new model to study Barrett's oesophagus was developed by Sato *et al.* (20) using a technique they developed for culturing intestinal organoids. Due to the similarity of the crypt-like invaginations in Barrett's, culturing of these crypts led to cystic organoid structures that formed buds resembling small crypts. These organoids could also be cultured for more than 3 months, which was unachievable up until then.

In parallel, the near-native crypt culture model developed in our laboratory has paved the way for developing a human Barrett's crypt culture model that could survive in culture and be amenable to functional experiments. This model is different to that developed by Sato *et al*. (20) since the single crypts remain intact without forming organoids and still maintain the morphological and signalling gradients that maintain hierarchy of cells along the crypt axis. We used native microdissected crypts as our control for determining the native status of signalling pathways and their gradients and compared to the cultured Barrett's crypts to determine if these gradients were maintained.

Isolation of Barrett's crypts revealed that they were about twice the size of colonic crypts (Fig 3.44) and distinctive cells such as goblet cells were easily seen, clearly demonstrating the conversion of the squamous epithelium to a glandular columnar epithelium that is distinctive of Barrett's. To demonstrate that the cells with the cultured Barrett's crypt were still viable after a few days in culture, loading crypts with Calcein-AM and PI revealed that cells within the body of the crypt were viable and only cells that have been shed at the top of the crypt were PI positive (Fig 3.45). Time-lapse microscopy was also used to show

that the cells within the cultured crypts were still proliferating and maintained gradient along the crypt axis, with higher number of mitoses occurring at the base of the crypt (Fig 3.46). The proliferative status of cultured crypts was also demonstrated by labelling for Ki67 and comparing the percentage of proliferating cells in cultured crypts to the native microdissected crypts (Fig 3.47). As with the mitotic counts, a gradient of higher proliferation was observed at the base of the crypt. This shows that not only are the Barrett's crypts viable in culture, but they also maintain their proliferative gradients.

Another gradient that was seen to be maintained in cultured Barett's crypts was β-catenin labelling, the hallmark of Wnt signalling. Abnormal activation of β-catenin has been found to be common during neoplatic progression of Barrett's oesophagus (15). Moyes *et al.* (16) have found that there is increased nuclear β-catenin in Barrett's compared to suqamous tissue. Gonzalez *et al.* (223) however, have shown that mutations in β-catenin, axin and APC are rarely detected in Barrett's oesophagus and as demonstrated by Clement *et al.* (224), it is *APC* and *SFRP1* silencing by promoter hypermethylation as well as *Wnt2* upregulation that is involved in neoplastic progression. Since Wnt signalling seems to be involved in Barrett's metaplasia and is highly important in maintaining proliferation in colonic crypts, it is no surprise that Wnt3a and R-Spondin 1 were required to be present in the culture media for both our cultured and crypts and the Barrett's organoids developed by Sato *et al.* (20). The Wnt target gene involved in proliferation, c-Myc, was expressed in the native Barrett's crypts (Fig 3.49), but unfortunately, no cultured comparison was carried out. Moyes *et al.* (16) have shown that expression of c-Myc was increased in Barrett's metaplasia compared to normal squamous tissue, whilst Schmidt *et al.* (225) have also found a linear correlation of c-Myc over-expression along the metaplasia-dysplasia-adenocarcinoma sequence.

Barrett's oesophagus develops as a result of chronic gastroesophageal reflux which leads to irritation of the squamous epithelium and causes inflammatory cytokines to accumulate in the epithelium. One of the cytokines implicated in inflammatory and malignant

processes is TNF-α. Tselepis *et al.* (230) have shown elevated levels of TNF-α in Barrett's metaplastic epithelium compared to normal squamous epithelium and that TNF-α expression persisted and intensified during the progression of Barrett's oesophagus to adenocarcinoma. This increased expression of TNF-α along the metaplasia-dysplasia-adenocarcinoma sequence suggests a relatively early role for it in the formation of the adenocarcinoma. TNF-α induces signalling though pathways such as NFκB. NFκB is a protein complex that acts as a transcription factor and plays a key role in regulating the immune response to infection. However, dysregulation of NFκB has also been linked to cancer, inflammatory and autoimmune diseases. We determined the signalling status of NFκB using immunocytochemistry and found that there is nuclear NFκB p65 present in the native Barrett's crypt and some in the surrounding squamous tissue (Fig 3.50). This suggests that the NFκB pathway may be activated in the inflamed tissue due to chronic acid and bile acid reflux. TNF-α was then used on our cultured crypts to determine if the NFκB pathway could be stimulated. We found that in the control crypts NFκB p65 was expressed mostly in the cell cytoplasm, but upon activation with TNF-α, NFκB p65 was translocated to the nucleus (Fig 3.50). This translocation of NFκB would be expected to activate anti-apoptotic genes such as Bcl-2 and Bcl-xL (278) making the cells more persistant and malignant.

No robust markers for stem cells in Barrett's oesopgagus have yet been identified. Due to the similarity of Barrett's crypts to intestinal crypts, Becker *et al.* (237) examined the pattern of Lgr5 expression in Barrett's crypts and found a few $Lgr5^{+}$ cells at the base of the crypt. They later found variable staining of Lgr5, ranging from predominatly low intensity in non dysplastic Barrett's oesophagus to high intensity in dysplasia. No Lgr5 expression was seen in normal squamous tissue but high Lgr5 expression in adenocarcinoma was associated with worse survival (238). Increased expression of Lgr5 in Barrett's oesophagus was also seen by Von Rahden *et al*. (239) and Vega *et al*. (240). Two other stem cell markers DCAMKL-1 and Msi-1 were also upregulated in Barrett's oesophagus

compared to normal squamous tissue (240). We determined whether the stem cell marker OLFM4 was present in our cultured Barrett's crypts and found that indeed there was OLFM4 staining along the crypt axis similar to that observed in colonic crypts. There were a higher number of $OLFM4^+$ stem cells at the base of the Barrett's crypt which decreased dramatically in the top region of the crypt (Fig 3.51). Surprisingly there seemed to be a high percentage of $OLFM4^+$ stem cells within the crypt, but this could be due to the inflammatory nature of the tissue. Data by another colleague in the laboratory has shown that colonic crypts from inflammatory bowel disease patients have a much higher percentage of $OLFM4^+$ stem cells compared to normal patients. This high percentage of stem cells, some of which could aquire mutations, leave the patient with a much higher risk of developing adenocarcinoma, as colonic crypts from cancer patients were also seen to have a very high percentage of $OLFM4^+$ stem cells.

In this chapter we have demonstrated the development of an *ex vivo* Barrett's oesophagus crypt culture model that is amenable to functional bioimaging approaches. The potential role of Wnt signalling in the maintanance of Barrett's crypt tissue renewal was also shown. Identification of Barrett's oesophagus stem cells and the use of the *ex vivo* culture model will help the development and translation of novel strategies for prevention of Barrett's oesophagus and oesophageal adenoscarcinoma.

Chapter 4 General Discussion

4.1 Signalling tissue renewal in the human colonic epithelium

Intestinal tissue renewal is fundamental to life-long health. The processes by which the intestinal epithelium renews itself has been well described in the mouse, but the molecular and cellular mechanisms that govern tissue renewal in the human colonic epithelium are less well understood partly because of a lack of human *ex vivo* culture models. The intestinal epithelium is one of the most dynamic tissues in the body with 10 billion cells shed from the gut epithelium which are replaced by intestinal stem cell progeny. The hierarchy of tissue renewal is thought to minimise the accumulation of molecular damage by positioning the long-lived stem cells at the crypt base away from bacterial toxins, metabolites, dietary antigens and mutagens. Therefore the molecular mechanisms that regulate the processes of tissue renewal are of great interest since they are disrupted in conditions such as inflammatory bowel disease and colorectal cancer.

The intestinal epithelium is an ideal tissue to study adult stem cell processes such as self-renewal and multipotent differentiation. The regulation of these processes is intricate and interconnected and understanding the interplay of the different components and signalling pathways is very important. Clinical tissue samples and studies of model systems ranging from cancer cell lines to genetically modified mice have made a dramatic contribution to the understanding of the biology of intestinal tissue renewal in health and disease. However, to gain a better understanding of the molecular mechanisms involved in human colonic epithelium tissue renewal, *ex vivo* tissue models that are amenable to bioimaging and functional genomic approaches are required. Recently, intestinal organoid culture models have been developed that are composed predominantly of immature stem/progenitor cells that can be induced to differentiate by withdrawal of Wnt stimulation (20) (220). Complementary to these organoid culture models, our laboratory has developed a near-native human colonic crypt culture model that can be used to investigate the regulatory mechanisms involved in tissue renewal. The culture conditions used by

Sato *et al.* (20) promoted re-modelling of colonic crypt morphology that was followed by multiple budding events characteristic of the intestinal organoids. Replacing EGF with IGF-1 however, supported renewal of the crypt cell population by maintaining hierarchy of crypt cell proliferation, migration, differentiation and shedding, whilst the crypt length remained relatively constant. The optimisation of the human colonic crypt culture conditions suggested that activation of Wnt pathway and suppression of BMP and TGFβ pathways was required for the homeostasis of tissue renewal. The status and functional role of these signalling pathways in the regulation of human colonic crypt renewal and intestinal stem cell biology was subsequently interrogated.

Intestinal stem cells play a central role in tissue renewal and a strategy to label these cells in the human colonic epithelium is imperative. In the mouse, several theories as to their location have been proposed, but identifying them in the human colonic epithelium has proven to be more challenging. The stem cell zone model originally proposed by Cheng and Leblond (36) demonstrated the existence of slender cells wedged between Paneth cells in the mouse small intestine that divided once every day. These slender cells were referred to as crypt base columnar (CBC) cells and lineage tracing using 3H-thymidine exposure revealed the stemness nature of the CBC cells. Potten *et al*. (279) proposed the +4 model when they found that radiation-sensitive label-retaining cells (LRCs) resided directly above the differentiated Paneth cell compartment ranging from position +2 to +7, but on average at position +4. However, these LRCs were shown to be actively proliferating every 24 hrs and this label-retention was due to asymmetric segregation of old and new DNA strands during subsequent cell divisions (40).

The intestinal stem cell marker Lgr5, was found to be a receptor for the Wnt agonist R-Spondin by forming a Wnt receptor complex and activated by Wnt signals (259). Isolation and culture of $Lgr5^{+}$ stem cells has been shown to generate complex three dimensional organoid structures in small intestine and colon (67) (68). Recently Yui *et al*. (68) demonstrated long term engraftment after transplantation of organoids derived from a

single Lgr5^{+} colon stem cell after extensive *in vitro* expansion. Gene expression and proteome profiling of Lgr5^{+} cells revealed an Lgr5 'stem cell signature' with these genes contributing to stemness (69). Van der Flier *et al*. (69) showed that genetic ablation of one of these genes, Ascl2, resulted in rapid in stem cell death, whilst overexpression resulted in expansion of the stem cell compartment. Van der flier *et al*. also showed that the Lgr5^{+} cells were also highly enriched in OLFM4, and proposes it as a robust marker of Lgr5^{+} stem cells. In this study we used confocal imaging of whole mount intact human colonic crypts to visualise double labelling of OLFM4 protein and either Lgr5 protein or Lgr5-mRNA. A number of Lgr5^{+}/OLFM4^{+} slender cells at the crypt base were identified, that were reminiscent of the Lgr5-GFP positive cells in the mouse colon (34). In the small small intestine, Lgr5^{+} stem cells are found between Paneth cells whilst in the human colon we found that these slender cells were interspersed between goblet-like cells and comprise about 30% of the cell population at the base of the crypt (Fig.4.1). Movie 1 (Appendix) demonstrates clearly how the slender Lgr5/OLFM4^{+} stem cells are tightly wedged between goblet cells and the 3D reconstruction shows the mosaic nature of the stem cells with other differentiated cell lineages.

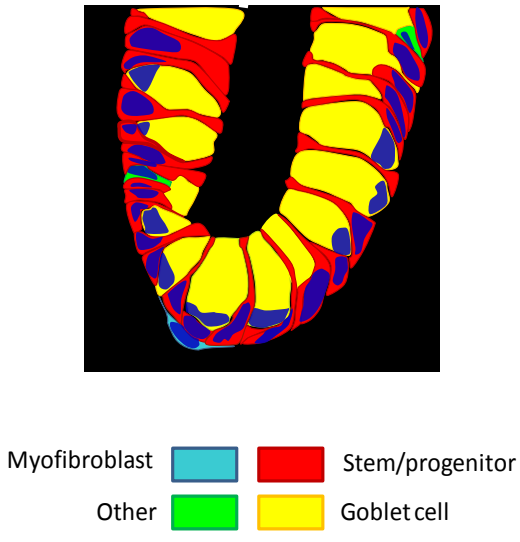


Figure 4.1 Schematic diagram of slender CBC stem cells wedged between goblet cells.

The importance of the Wnt signalling pathway in maintaining homeostasis of the intestinal epithelium was demonstrated by Kuhnert *et al.* (2) who showed that adenoviral expression of the Wnt antagonist Dkk1 in adult mice resulted in inhibited proliferation in the small intestine and colon with progressive loss of crypts, villi and glandular structure. Korinek *et al*. (252) also demonstrated that disruption of TCF4 led to loss of stem cell proliferative compartments in the crypts, whilst Muncan *et al.* (141) showed that deletion of the Wnt target gene c-Myc led to loss of intestinal crypts. These studies demonstrate that Wnt and its downstream targets are important in maintaining proliferation of stem cells. We found that Wnt signalling predominates at the crypt base, as demonstrated by β-catenin, axin II and c-Myc labelling, and that inhibition of the pathway causes loss of proliferation, stem cells and expression of Wnt target genes.

Pericryptal myofibroblasts, which reside immediately beneath the basal crypt epithelial cells, maintain the stem cell niche by secreting Wnt ligands. In the mouse small intestine, Paneth cells have also been found to secrete Wnt3 (34). Gregorieff *et al*. (142) showed that in mice, Wnt ligands such as Wnt3, Wnt9b and Wnt5a predominated at the base of the crypt along with the LRP5/6 coreceptors whilst work by another colleague in our laboratory has shown that mRNA for Wnt ligands *Wnt1, Wnt2, Wnt2b, Wnt3, Wnt3a, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7b* and *Wnt11* can be found in isolated human colonic crypts. Farin *et al*. (25) have also shown that although deletion of Paneth cell derived Wnt3 in the mouse intestinal epithelium showed no effect *in vivo*, it was required for growth and sustainability in organoid *in vitro* culture. Co-culturing of mesenchymal derived Wnt2b ligand restored the growth of organoids, suggesting that there is a compensatory mechanism in the mouse small intestinal stem cell niche that safeguards against stem cell dysfunction. However, the mouse colon does not express Wnt ligands (142) which presumably reflects a difference between the dependence of the mouse and human colonic crypts on non-epithelial derived Wnt ligands.

In an opposing effect to Wnt signalling, the BMP signalling pathway is thought to be involved in inhibition of stem cell self-renewal and promotion of cell differentiation. He *et al.* (8) demonstrated that BMP signalling suppresses Wnt signalling, which allows for a balanced control of stem cell self-renewal. However, inhibition of BMP signalling by noggin results in the formation of ectopic crypts with an expansion of stem cell and progenitor cellular populations (7). Therefore, crosstalk between the two signalling pathways is important in maintaining the stem cell niche and balanced renewal within the crypt (Fig 4.2). Several BMP components are found to be highly expressed in the top region of the the colonic crypts (74) with Hardwick *et al*. (179) and Haramis *et al*. (7) showing that BMP2 and BMP4 to be strongly expressed in the intervillus mesenchyme near the villus tips, with a decreasing expression gradient towards the crypt base. The BMP receptor BMPR1 and phosphorylated SMADs are also found along the villus (8). In this study we show that generation of pSMAD1,5,8, a downstream mediator of BMP signalling, predominates in the upper region of the crypt. BMP antagonists such as noggin and gremlin have been found to be expressed in the pericryptal myofibroblasts (8) (74) and maintain the correct BMP/Wnt gradients along the crypt axis by inhibiting BMP signalling at the crypt base. We show that BMP inhibition with noggin led to increased β-catenin and axin II expression with downstream effects of maintaining stem cells and cell proliferation. It is thought that stem cell self-renewal is activated by transient expression of noggin which overrides the BMP signal and releases β-catenin inhibition by PTEN (8).

Along with BMP signals, it is thought that TGFβ signals predominate in the upper region of the crypt where they are thought to influence crypt cell positioning, differentiation and apoptosis, thus maintaining the normal size, shape and function of the polarised gut epithelium. The linear migration, differentiation and compartmentalisation along the mouse intestinal crypt-axis has been shown to be controlled by TGFβ and Wnt gradients, with TGFβ controlling cell polarisation proteins and Wnt controlling the expression of EphB sorting receptors (109). Mishra *et al*. (109) show that the presence of TGFβ signals

and the absence of Wnt signals at the villus compartment results in rapid cell cycle arrest and differentiation with SMAD4 and TCF4 being the dominant switch between the proliferative progenitor and the differentiated epithelial cell. In colorectal cancer this switch may be permanently reversed with TGFβ signalling being inactivated and TCF4 being constitutively active by mutations in the Wnt cascade. We found that activation of the TGFβ pathway inhibited cell proliferation and stem cell self-renewal by interacting and inhibiting Wnt signalling components such as axin II. Interaction between SMAD7 (an inhibitory SMAD of TGFβ signalling) and β-catenin was demonstrated by Edlund *et al.* (199) who found that TGFβ cells stably transfected with inducible SMAD7 resulted in increased β-catenin, LEF1 and c-Myc expression. They propose that a complex with SMAD7, β-catenin and LEF1 forms in response to TGFβ signalling with the complex then driving c-Myc and LEF1 production.

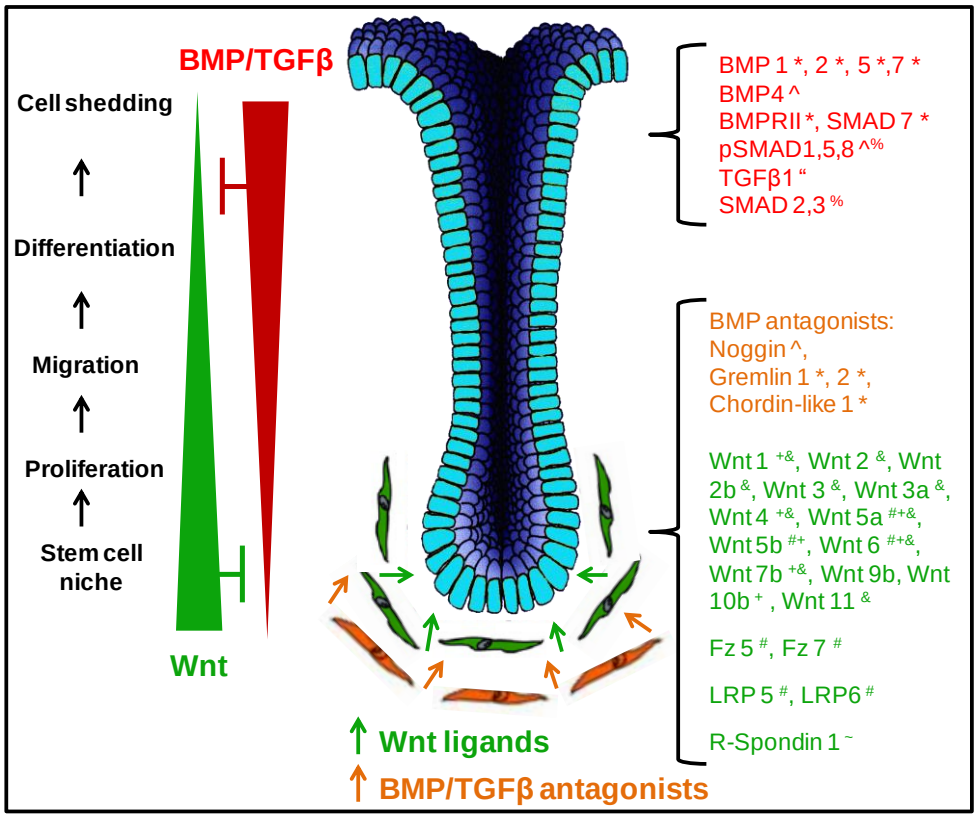


Figure 4.2 Reciprocal Wnt and BMP/TGFβ signalling gradients maintain colonic crypt tissue renewal. **Gene microarray analysis of BMP ligands and antagonists, Kosinski et al.* (74). *^ Expression of BMP pathway components and antagonists in mouse small intestine, He et al.* (8). *“TGFβ ligand mRNA expression in rat intestinal epithelium,* (192). *% Expression of BMP/TGFβ activation in native human colonic crypts, Williams (data unpublished). # mRNA Wnt ligand and receptor expression in mouse intestinal crypts, Gregorieff et al.* (142). *+ mRNA Wnt ligand expression in human intestinal crypts, Holcombe et al.* (127). *& mRNA Wnt ligand expression in human isolated intestinal crypts, Williams (data unpublished). ~ R-Spondin proteins are ligands for Lgr4/5/6 receptors, de Lau* (5).

The gradients arising from the interaction between Wnt, BMP and TGFβ signalling pathways ensures that stem-cell driven tissue renewal is maintained in a balanced manner. An imbalance in the gradients could lead to either crypt hyperproliferation and increased crypt fission or stem cell death and loss of proliferation resulting in crypt degeneration.

Investigation of the modulators that establish these morphogenic gradients along the crypt-axis and their influence on the efficiency of stem cell-driven tissue renewal in health and disease will provide insights into disease risk and prevention.

4.2 Colorectal cancer risk and ageing

Colorectal cancer is the third most common cancer in the UK, and the Wnt, BMP and TGFβ signalling pathways have all been implicated in colon carcinogenesis (122) (177) (183). Inactivating mutations in BMP and TGFβ signalling pathways and constitutively activating mutations in the Wnt pathway paves the way to the development of adenocarcinomas.

About 90% of all colorectal cancers will have an activating mutation of the canonical Wnt signalling pathway, ultimately leading to the stabilisation and accumulation of β-catenin in the nucleus of the cell. These mutations lead to early premalignant lesions in the intestine, such as aberrant crypt foci and small polyps (122). Mutations of APC were first identified in patients with familial adenomatous polyposis (FAP). These patients develop hundreds of polyps in the colon after inactivation of the remaining wild-type allele. Although FAP is quite rare, mutations of APC account for up to 85% of all sporadic colorectal cancers. Hypermethylation of the wild-type APC allele is also found in some sporadic colorectal cancers which may be an alternative mechanism for APC gene inactivation (126).

Patients with juvenile polyposis, a rare autosomal dominant hamartomatous polyposis syndrome have an increased risk for the development of colorectal cancer. Patients with this syndrome have mutations in BMPRI and SMAD4, which suggests a role for the BMP pathway in the initiation of colorectal neoplasia. Hardwick *et al.* (179) found that BMP2 inhibits colonic epithelial cell growth *in vitro*, promoting apoptosis and differentiation and inhibiting proliferation and that BMP2 expression is lost in the microadenomas of familial adenomatous polyposis patients suggesting that BMP2 acts as a tumour suppressor. Loh *et al*. (180) have also found BMP3 to be growth suppressive and its expression is frequently

lost through promoter methylation, again suggesting a tumour suppressor role for BMPs in colorectal cancers. However, BMP4 expression has been shown to be up-regulated in the transition from primary colorectal adenomas to adenocarcinomas (181). Deng *et al*. (181) demonstrated that overexpression of BMP4 can protect colon cancer cells from apoptotic death under stress environment and drive these cancer cells to a more migratory and invasive phenotype through induction of uPA activity. This suggests that BMP4 promotes invasive behaviour of colon cancer cells. By examining pSMAD1,5,8 activation, Kodach *et al*. (177) found that loss of BMP signalling occurs during the transition from late adenoma to early carcinoma suggesting that BMP signalling is involved in tumour progression rather than as an initiator of carcinogenesis.

The TGFβ signalling pathway has a role in both tumour suppression as well as tumour progression. TGFβ1 switches from an inhibitor of tumour cell growth to a stimulator of growth and invasion during human colon carcinoma progression (183). Trobridge *et al*. (206) have demonstrated that a combination of inactivation of the TGFβ signalling pathway and expression of oncogenic Kras leads to the formation of invasive intestinal neoplasms through a β-catenin independent pathway and that these adenocarcinomas have the capacity to metastasize. Munos *et al*. (207) also found that the loss of TGFβRII in intestinal epithelial cells promotes the invasion and malignant transformation of tumours initiated by APC mutation suggesting that Wnt signalling deregulation and TGFβ signalling inactivation cooperate to drive the initiation and progression, respectively, of intestinal cancers *in vivo*.

The highest risk factor to the development of adenocarcinoma is the ageing epithelium (280). Preliminary data from our laboratory suggests that there is a perturbed Wnt signalling gradient along the crypt-axis as well as an expansion of the stem cell niche with increased age. Since the hierarchy of tissue renewal is compromised in the ageing colonic epithelium, it may exacerbate the risk of colorectal cancer. It would therefore be interesting to investigate more fully the status of human colonic tissue renewal and the

influence of compromised Wnt and BMP/TGFβ gradients with respect to age and disease risk (Fig 4.3).

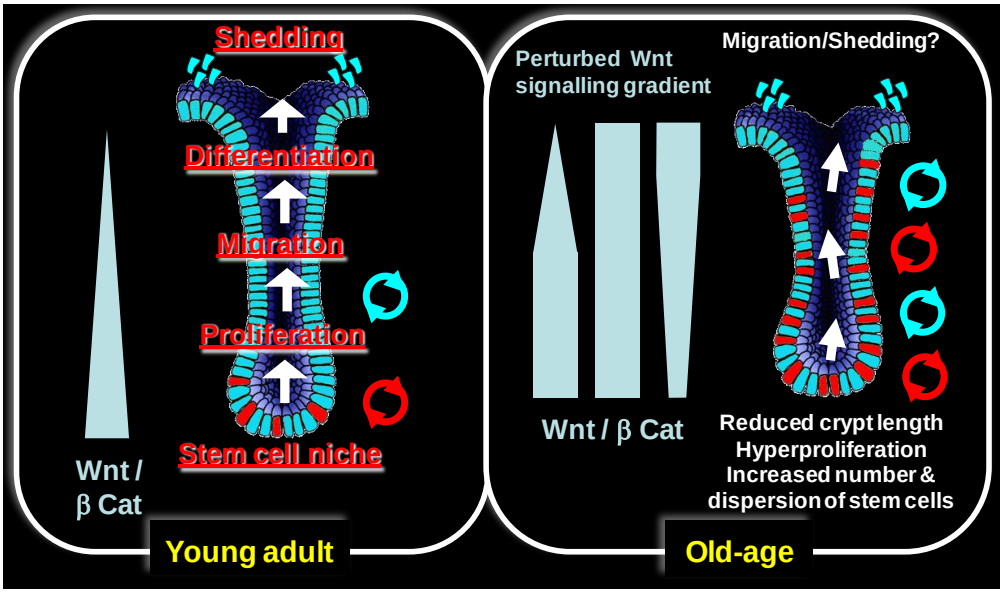


Figure 4.3 Compromised tissue renewal in the ageing human colonic epithelium. *Williams et al. (unpublished).*

4.3 Development of Barrett's oesophagus culture model

The molecular mechanisms that drive Barrett's oesophagus metaplasia and maintain the renewal of the glandular structures termed crypts had proven to be difficult to investigate. Research on mouse models and cell lines has suggested that Barrett's oesophagus arises as a result of gastroeosophageal reflux, where the squamous tissue is replaced by glandular columnar epithelium that is more resistant to acid and bile acids. Investigation in human Barrett's oesophagus crypts has been hampered by a lack of an *ex vivo* culture model. The last aim of this thesis therefore was to develop an *ex vivo* Barrett's oesophagus culture model in which the functional role of signalling pathways involved in Barrett's crypt renewal could be investigated.

A model to study Barrett's oesophagus was very recently developed by Sato *et al.* (20) using a technique they developed for culturing intestinal organoids. Due to the similarity of the crypt-like invaginations in Barrett's oesophagus, culturing of these crypts led to cystic organoid structures that formed buds resembling small crypts and could also be cultured for more than 3 months. In parallel, and during the course of this study, we also developed an *ex vivo* Barrett's oesophagus crypt culture model that was ameanable to functional bioimaging techniques. Using the techniques developed for the near-native colonic crypt culture model developed in our laboratory, single Barrett's oesophagus crypts were isolated from the surrounding squamous tissue and placed into culture conditions developed for the colonic crypts. The isolated Barrett's crypts maintained their morphology in culture, were viable and maintained their proliferation gradient in the same manner as seen in the native microdissected Barrett's crypts.

This study demonstrated the importance of Wnt signalling in stem cell-driven tissue renewal in the human colonic crypts, and since Barrett's oesophagus crypts are similar in structure and morphology, Wnt signalling may also be important in the maintanance of Barrett's crypts. We show that Wnt signals are active in Barrett's oesophagus crypts in a similar fashion to colonic crypts, with β-catenin predominating at the crypt base. Bian *et al*. (15) show that there is abnormal activation of β-catenin during neoplastic progression of Barrett's oesophagus. Clement *et al*. (224) found that *APC* and *SFRP1* silencing by promoter hypermethylation may be responsible for activation of the Wnt pathway in the development of oesophageal adenocarcinoma in Barrett's oesophagus. Moyes *et al.* (16) also found that Wnt target genes cyclin D1, Sox9 and c-Myc all had increased expressed in Barrett's oesophagus compared to normal squamous epithelium.

In human colonic crypts we demonstrate that as well as Wnt signals being required for tissue renewal by promoting proliferation, the BMP and TGFβ signalling pathways may also play a reciprocal role by inducing cell differentiation and apoptosis. During this study, we did not have time to investigate the role of BMP and TGFβ signalling on

Barrett's crypt survival and renewal, but the literature suggests that BMP signalling is involved in the metaplasia of Barrett's oesophagus from normal squamous epithelium. Zhou *et al*. (226) show that acid and bile acid increase the expression of BMP4 and promoted expression of ID2 and CDX2. Milano *et al.* (17) found in both human and rat tissue that the BMP pathway was activated in oesophagitis and Barrett's oesophagus. Incubation of squamous cells with BMP4 showed a shift in cytokerartin expression was consistent with columnar epithelium. Wang *et al*. (18) have recently shown that epithelial Hedgehog ligand expression may contribute to the initiation of Barrett's oesophagus through induction of stromal BMP4 which triggers reprogramming of squamous epithelium in favour of a columnar phenotype. TGFβ signalling may also play a part in Barrett's oesophagus related adenocarcinoma. Von Rahden *et al*. (228) found that TGFβ1 overexpression is associated with advanced stage of oesophageal edonocarcinoma and has a negative impact on survival. SMAD4 mRNA has been found to be progressively reduced in the mataplasia-dysplasia-adenocarcinoma sequence along with SMAD4 promoter methylation in majority of Barrett's adenocarcinomas (229). Mendelson *et al*. (19) also observed loss of SMAD4 and TGFβRII in Barrett's oesophagus and adenocarcinoma tissues. Sato *et al*. (20) also show that as well as Wnt signals being required for Barrett's organoid survival, the BMP antagonist noggin and Alk 4/5/7 inhibitor A83-01 and the p38 MAP kinase inhibitor SB202190 are also essential.

Stem cell markers of Barrett's oesophagus have yet to be identified, but due to the similarity of Barrett's oesophagus crypts to intestinal crypts, and studies showing that Lgr5 is upregulated in Barrett's tissue compared to squamous (238) (240), we demonstrated that the intestinal stem cell marker OLFM4 could be used to identify stem cells within Barrett's tissue. We found that OLFM4 labelling was consistent with that found in colonic crypts, and although the percentage of $OLFM4^+$ cells was much higher than that found in normal colonic epithelium, it is quite consistent with the percentage of $OLFM4^+$ cells found in IBD patients: work by another colleague in the laboratory has shown that in active IBD

there are about 40% of OLFM4^{+} stem cells at the base of the crypt, which is similar to that found in the Barrett's crypts. We have also found that colorectal cancer patients have a much higher percentage of OLFM4^{+} stem cells (about 70%), (William's lab, data unpublished) so it is no surprise that Barrett's oesophagus which is an inflammatory disease predisposes patients to developing adenocarcinoma. Identification of Barrett's oesophagus stem cells and the use of the *ex vivo* culture model will help the development and translation of novel strategies for prevention of Barrett's oesophagus and oesophageal adenocarcinoma.

4.4 Future Work

4.4.1 Renewal of the ageing human colonic epithelium in health and disease

The highest risk factor to the development of adenocarcinoma is the ageing epithelium (280). Xiao *et al*. (281) have shown that ageing enhances proliferation, but attenuates apoptosis in the mouse intestinal epithelium whilst Martin *et al.* (282) show that stem cells in the ageing mouse have a reduced ability to regenerate following injury. An increase in clonogens (stem/progenitor cells) and a greater degree of apoptosis at the crypt-base following irradiation of the ageing mouse intestinal epithelium has also been reported (283). In drosophila, the ageing intestinal epithelium is also characterised by an increase in stem cell proliferation that is associated with mis-differentiation (284).

It has been suggested that the Wnt signalling pathway is augmented in the ageing mouse intestine. In the Klotho mouse model of accelerated ageing, Liu *et al.* (285) show that young Klotho mice had a decrease in stem cell number and an increase in progenitor cell senescence, and that continuous Wnt exposure triggered accelerated cellular senescence. In human biopsy tissue samples, a Wnt-dependent and age-related phenotype in the human colonic epithelium was raised by the recent demonstration of age-related CpG island methylation of Wnt pathway inhibitors (286).

Given that age is a major risk factor for colorectal cancer (287), there is a surprising lack of data regarding the status of, and cellular signals for, tissue renewal in the normal ageing human colonic epithelium. It would therefore be interesting to characterise the functional status of the Wnt signalling pathway and stem cell-driven tissue renewal in the ageing and diseased human colonic epithelium.

Any differences in the morphology and crypt length between young (<40 years of age) and old patients (>70 years of age) could be investigated by measuring crypt length in native microdissected crypts. Crypt cell proliferation can be assessed using Ki67 immunolabelling, whilst Lgr5/OLFM4^{+} stem cells status can be determined by double immunolabelling of OLFM4 protein with Lgr5 protein or *Lgr5* mRNA. This will demonstrate if there is an expansion of the stem cell niche with age. To determine any differences in the status of the Wnt pathway, native microdissected crypts from young and old patients can be immunolabelled for β-catenin and Wnt target genes, axin II and c-Myc. The human colonic crypt culture system can then be used to investigate the differential sensitivity of crypts derived from young and old patients to either exogenous and/or endogenous Wnt stimulation and the sensitivity to inhibition of Wnt signals with the Wnt secretion inhibitor IWP2. These experiments could demonstrate an ageing phenotype in the human colonic epithelium that is driven by increased Wnt signals and increase our understanding of human ageing and age-related conditions such as colorectal cancer.

Intestinal stem cells have been suggested to be the cells of origin of colorectal cancer (220). A possible scheme relating the age-related expansion of intestinal stem cells along the human colonic crypt-axis to an increased risk of colorectal cancer is illustrated in Fig. 4.4. Although the details are not yet clear, it is conceivable that inefficient tissue renewal and migration of intestinal stem cells away from their safe harbour at the base of colonic crypts renders them more vulnerable to neoplastic transformation.

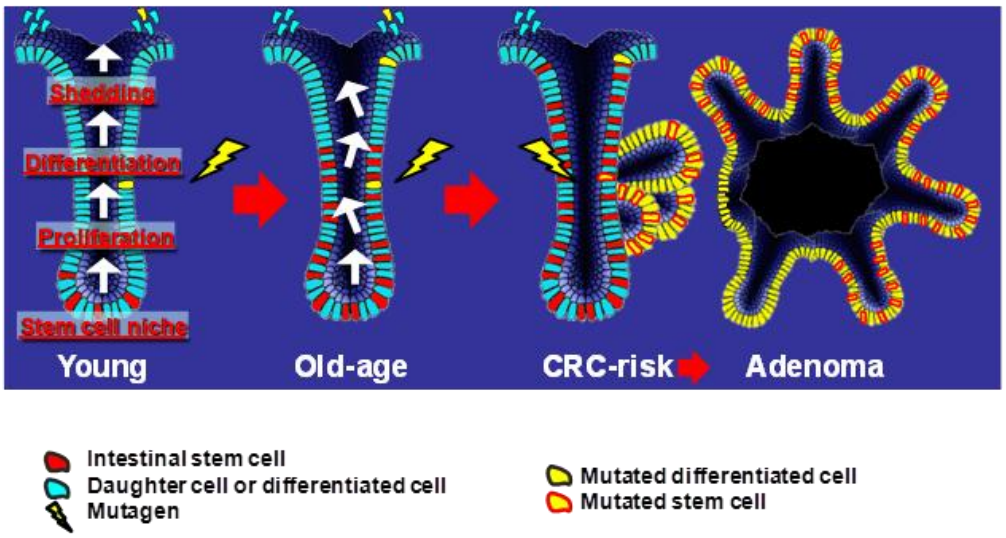


Figure 4.4 Age-related expansion of intestinal stem cells along the human colonic crypt-axis and inefficient tissue renewal and migration of intestinal stem cells away from their safe harbour at the base of colonic crypts renders them more vulnerable to neoplastic transformation.

4.4.2 Inflammatory mediators for the survival and expansion of Barrett's oesophagus

Barrett's oesophagus is thought to be provoked and sustained by inflammation associated with gastro-oesophageal reflux disease. The major protagonists include bile acids, the acidic pH of the refluxate and prostaglandin E_2 (PGE_2), which is produced in response to damage to the oesophageal epithelium. Short- term exposure of Barrett's tissue to acid has been shown to increased cell proliferation and survival and activated MAPK (ERK and p38) signalling and COX2 expression (288) (289), and Souza *et al.* (290) suggest that the consequences to acid exposure may be mediated at least in part by PGE_2. Bile salts have also been shown to stimulate cell proliferation via MAPK dependent pathways (291) and upregulate COX2 (292) in models of Barrett's oesophagus. As bile salts can also stimulate the signalling effects of antiapoptotic pathways such as NFκB it is likely that continuous bile damage enhances dysregulated cell proliferation (293). Alterations in the Wnt signalling pathway during neoplastic progression of Barrett's oesophagus have also been demonstrated (224). In the colon, Buchanan *et al.* (294) have suggested that PGE_2 stimulation transactivates the Wnt pathway and this may be true for Barrett's oesophagus.

These studies suggest that inflammatory mediators such as bile acids, acidic pH and PGE_2 may modulate cellular signalling pathways (Wnt, MAPK, NFκB) to sustain a columnar epithelial cell type and promote expansion of Barrett's oesophagus by cell proliferation and cell survival. Therefore the functional effects of these inflammatory mediators on Wnt, MAPK and NFκB signalling pathways on the regulation of human Barrett's crypt renewal , survival and intestinal stem cell biology can be interrogated using the developed *ex vivo* culture model for Barrett's oesophagus.

The expression and localisation of prostaglandin receptors (EP1-EP4) could be determined by immunolabelling of native and cultured Barrett's crypts. EP receptor subtype-specific agonists and antagonists can be used to identify which second messenger signalling pathways and specific target genes are activated by those receptors. PGE_2 transactivation of Wnt, phosphoIKKβ/NFκB and EGFR/MAPK pathways could be tested in a similar

manner. The functional consequences of PGE_2 signalling can be investigated by assessing stem cell number, cell proliferation using Ki67 labelling and BrDU incorporation, cell migration by real time digital time lapse microscopy and cell survival/apoptosis by calcein/propidium iodide labelling of live/dead cells in conjunction with immunolabelling of activated caspase 3. The functional consequences of acid and bile acid exposure on Barrett's crypt survival and tissue renewal can be assessed in the same manner.

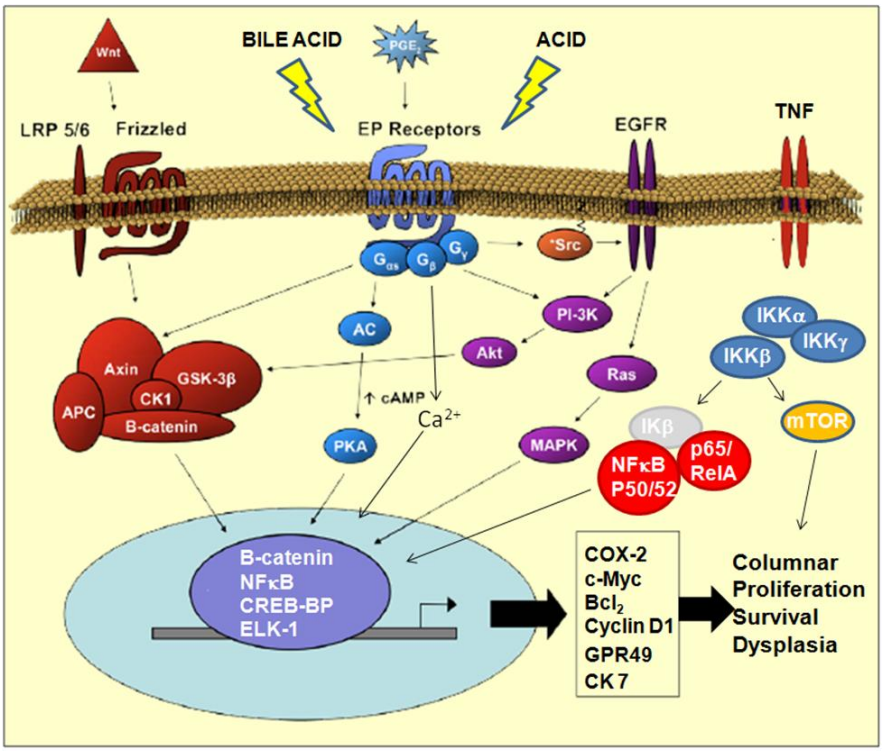


Figure 4.5 Inflammatory mediators (bile acids, acidic pH, PGE_2) modulate cellular signalling pathways (Wnt, MAPK, NFκB) to sustain a columnar epithelial cell type and promote expansion of Barrett's oesophagus by cell proliferation and cell survival.

Appendix

1) Movie 1: 3D reconstruction of confocal image stack of Lgr5/OLFM4/E-cadherin immunolabelling.
2) Movie 2: Timelapse movie of colonic crypt proliferation and migration (x20 objective).
3) Movie 3: Timelapse movie of Barrett's oesophagus crypt (x20 objective).

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