Wnt Signals and Imaging Tissue Renewal in the Human Colonic Epithelium

A thesis submitted for the degree of Masters by Research

by

Loren Bigwood

School of Biological Sciences
University of East Anglia

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Contents

Abstract

Background The human intestinal epithelium is one of the most rapidly renewing tissues in the mammalian body, with 10 billion cells being shed from its surface every day. Renewal of the intestinal epithelium takes place at the base of flask-like epithelial invaginations known as crypts. Within these crypts reside a stem cell population that continuously repopulates the epithelium with all of its specialised cell lineages. Many signalling pathways are involved in the regulation of these activities, the predominant one being the canonical Wnt signalling pathway. The Wnt signalling pathway is known to be dysregulated in many cancers and has been posed as a potential therapeutic target in such conditions.

Objective To investigate the status of canonical Wnt signalling in human colonic crypts and assess the regulation of crypt cell proliferation and migration in the context of tissue renewal.

Design In the first instance**,** the status of the canonical Wnt signalling pathway in relation to the proliferation hierarchy was characterised in native human colonic crypts. The functional link between canonical Wnt signals and regulation of human colonic crypt cell renewal was investigated using near-native, cultured human colonic crypts. Live intact human colonic crypts were isolated from mucosal tissue samples and placed into 3D culture in which conditions were optimised for steady-state tissue renewal. Crypts were observed either in control conditions, stimulated with Wnt3a or denied Wnt exposure due to the presence of its antagonist Dkk1. From these observations, it was possible to determine the effect of Wnt signalling on crypt cell kinetics.

Results Cell proliferation and canonical Wnt signalling pathway activity predominated at the base of native human colonic crypts. Colonic crypt morphology was preserved in culture, demonstrated by the presence of goblet cells, enteroendocrine cells and cell polarity. Proliferation in the crypt-base was significantly higher in crypts stimulated with Wnt3a compared to control conditions, although the rate of cell migration between the two groups was not significantly different. The absence of Wnt ligand or the presence of the Wnt inhibitor Dkk1 caused crypts to shrink in length compared to Wnt3a and control groups without affected crypt cell migration.

Conclusions Canonical Wnt signalling is required for steady-state tissue renewal in the human colonic epithelium. Proliferation and migration within the crypt are regulated independently of each other, thus appropriate regulation of cell Cproliferation is constantly required to maintain the crypt cell population.

Abbreviations

APC	Adenomatous polyposis coli
BMP	Bone morphogenic protein
BrdU	5'Bromodeoxyuridine
CBC	Crypt base columnar
CRC	Colorectal cancer
DIC	Differential interference contrast
Dkk	Dickkopf
Fz	Frizzled
GSK3β	Glycogen synthase kinase 3β
IBD	Inflammatory bowel disease
IGF	Insulin-like growth factor
ISC	Intestinal stem cell
PBS	Phosphate buffered saline
ROI	Region of interest
SFRP	Secreted frizzled related protein
TGFβ	Transforming growth factor β

Chapter 1 Introduction

1.1 General Introduction

The most common cause of death worldwide is cancer. Around 16,000 deaths per year are as a result of bowel cancer, making it the UK's third most common cause of cancer mortality (Bowel cancer UK). These statistics have prompted extensive research into the causes of cancer and the possible targets for treatment.

The human colonic epithelium has a cellular turnover rate of approximately 10 billion cells per day, making it one of the most rapidly self-renewing tissues in the adult mammalian body. The continuous cycle of proliferation, migration, differentiation and shedding of cells from the epithelium is under tight regulation by a range of intracellular signals. The most prominent regulatory pathways within the human colonic epithelium is the β-catenin dependent canonical Wnt signalling pathway (Korinek et al., 1998). In fact deregulated homeostasis of the Wnt signalling pathway is linked to malignancies in many self-renewing adult tissues, as well as inflammatory conditions of the bowel. There is currently a good understanding of the influence of Wnt signalling perturbation and malignant transformation of self-renewing epithelial tissues, although a great deal of this understanding comes from murine models.

The colonic epithelium is organised into millions of flask-like invaginations known as crypts, each crypt represents the functional unit of the tissue. These crypts are the site of cell loss and subsequent replenishment as the hosts of a small population of long-lived stem cells at the base of each crypt, conferring the ability to repopulate the entire epithelium every 5-7 days. The base of the crypt is also the most Wnt-rich region, providing stimulation for proliferation of intestinal stem cells. Recent years

have seen a great deal of interest in identification of the elusive intestinal stem cell. However, Barker et al. (2007) identified the marker gene *Lgr5* in the mouse intestinal stem cells, an apparent Wnt target gene found to be down regulated when under the influence of Wnt pathway inhibition (Barker et al., 2007). Loss of Wnt signals have grave consequences for the epithelium. Transgenic mice expressing the secreted Wnt inhibitor Dkk1 demonstrated a loss of proliferative activity and subsequently the presence of intestinal crypts (Pinto et al., 2003).

Our 3D model for near-native culture of human colonic crypts has allowed us to interrogate the effects of the Wnt signalling pathway on crypt cell proliferation, differentiation and migration and to manipulate its ability to function by use of the Wnt inhibitor Dkk1. Identification of the Wnt signalling status in this model under both agonistic and antagonistic conditions has demonstrated the essentiality for homeostatic regulation of Wnt signals in order to ensure physiological well being in the mammalian colon.

Epithelial cancers are often found to be Wnt-driven. Cells under the control of hyper-activated Wnt signalling have been found to proliferate uncontrollably and without any apoptotic activity, thus causing the appearance of tumours. Antagonising this aberrant Wnt signalling in cultured colon cancer cells inhibits further growth of the cells and promotes differentiation (Tetsu and McCormick, 1999; Van de Wetering et al.,2002). Such observations pose great opportunities for cancer therapeutics in terms of targeted inhibition of the dysregulated Wnt pathway (Barker and Clevers, 2007).

1.2 Physiology of the Human Colon

1.2.1 Colon Function

The human colon (large intestine) functions fundamentally to absorb water and electrolytes from a flow of chyme, or food residues found to be indigestible by the small intestine (Kaji et al., 2011). This absorption allows the formation of faeces which is later expelled from the body via the rectum.

The intestine receives approximately 9L of water per day. Of this 9L, around 2L is derived from dietary intake and the remaining 7L from the secretion of digestive juices by the gastrointestinal tract in order to aid the break down of food. Around 84% of this 9L of fluid is absorbed in the small intestine, leaving between 1.5 and 2L to move into the large intestine for absorption (Kunzelmann and Mall, 2002). Only approximately 100ml of fluid is expelled per day in the faeces (Murek et al., 2009).

The homeostatic absorptive and excretory processes in the colon must be constantly regulated in order to ensure the maintenance of water and electrolyte balance. These activities occur across a barrier of epithelial cells connected by tight junctional proteins located in the apical membrane of the cell. The apical and basolateral membranes of these epithelial cells, as well as the crypts of Lieberkuhn express multiple proteins for the transport of ions in the form of channels, carriers and pumps (Kunzelmann and Mall 2002) responsible for the establishment and maintenance of chemical and electrical gradients between the gut lumen and its surrounding environment (Figure 1.1). It is by these gradients that the movement of water along its osmotic gradient is determined.

Either innate or acquired disturbances in the equilibrium between the bidirectional movement of water and ions across the epithelial barrier can manifest in one of two potentially fatal states. For example, diarrhoea due to bacterial infection by the enterotoxin *Vibrio cholera*, leads to dehydration due to stimulation of fluid secretion with the stool (Murek et al. 2009). During an episode of secretory diarrhoea, the secretion of NaCl and KCl surpasses the rate of their absorption, thus leading to a shift in the osmotic gradient (Kunzelmann and Mall 2002). Constipation occurs frequently in cystic fibrosis patients, later leading to bowel obstruction as the mutated chloride channel CFTR present in this phenotype interferes with chloride secretion, causing the osmotic gradient to shift in the opposite direction, in turn causing dehydration of the stool (Murek et al. 2009).

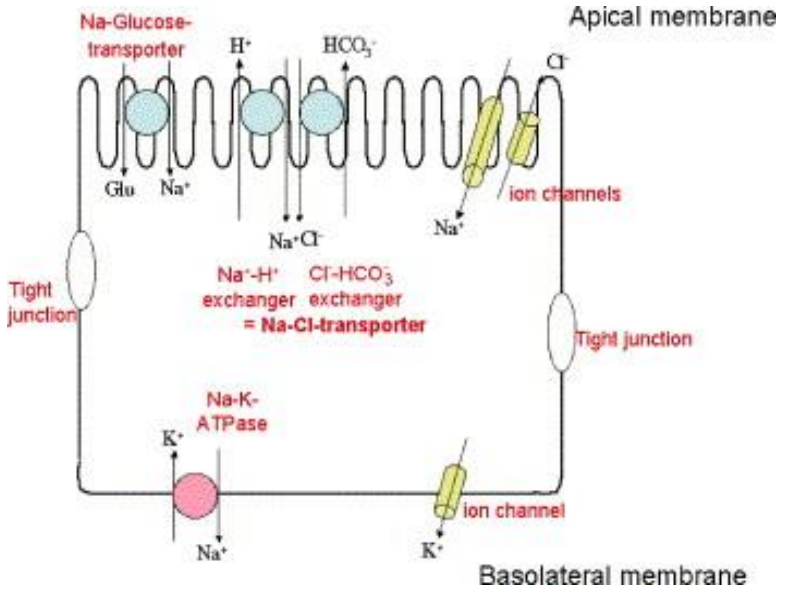

Figure 1.1 Mechanisms of electrolyte transport across the enterocyte. *Schematic of enterocyte cell demonstrating methods of ion transport across the apical and basolateral membranes (Hinsberger and Sandhu 2004).*

The colon is densely populated with bacteria. The majority of these are anaerobes such as bacteroides, eubacteria and streptocci, with bacteroides making up over 30% of the population. The presence of these bacteria allows production of the short chain fatty acids (SCFA) acetate, propionate and butyrate following the hydrolysis of carbohydrates from the diet and bacterial fermentation. Non-strarch polysaccharides are known to be the major component of dietary fiber. These complex carbohydrates are largely indigestible until their arrival at the large intestine.

1.2.2 Colon structure

The human intestine is a tube-like, muscular organ which consists of two physiologically distinct parts, the small intestine and the large intestine, or colon. The colon can be further divided into four sections; the ascending colon, the transverse colon, the descending colon and the sigmoid colon (Vander's Human Physiology, 2005) (Figure 1.2).

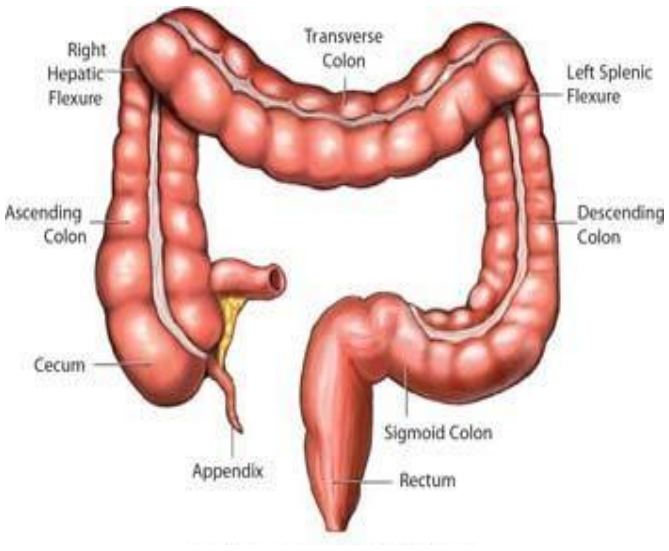

Figure 1.2 Gross Anatomy of the Human Colon. *The colon ascending from the caecum and terminating at the rectum (http://www.crcftlauderdale.com).*

The colon initiates at the top of the caecum, from where it ascends and turns to the left, at which point the transverse colon begins, extending horizontally from the right hand to the left hand side. This is the section of the colon with the greatest length, approximately 40-50cm. The transverse colon turns and forms the descending colon, which descends down the left hand side to form the S-shaped sigmoid colon, the lower end of which terminates at the rectum (http://www.helium.com).

The muscular wall of the colon consists of multiple layers (Figure 1.3), a characteristic maintained throughout the whole of the gastrointestinal tract. These layers are, moving in from the outermost layers: serosa; longditudinal muscle; circular muscle; submucosa, muscularis mucosae and finally the mucosal layer lining luminal surface of the colon (PACE and Williams, 1969).

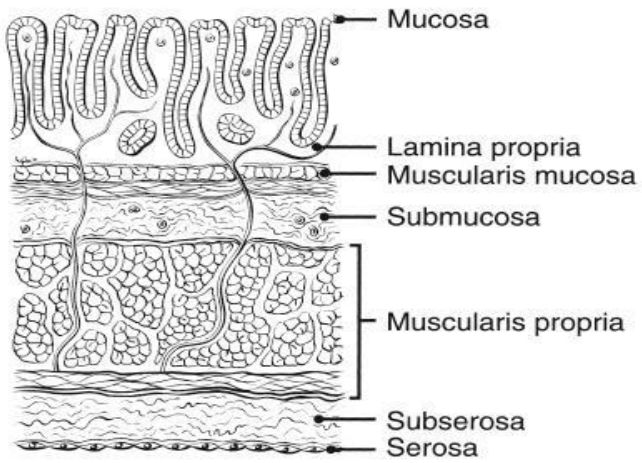

Figure 1.3 Layers of the Intestinal Wall. *Schematic of a cross sectional segment of the colonic wall, showing the four distinct layers, the serosa, muscularis, submocosa and mucosa (http://beneficialbacteria.net).*

The serosa is a single sheet of serous membrane surrounding the colon. It is a connective tissue layer used to anchor the colon to the abdominal wall, ensuring that it remains in position within the abdominal cavity. Directly below the serosa are two distinct muscular layers which together form the muscularis propria. The first of these two layers is a layer of londitudionally aligned muscle fibres arranged in three bands known as the taenia coli, the second layer is one of circular smooth muscle. These muscle layers mobilise in synchrony to aid peristalsis in the gut, as the circular muscle contracts, causing a narrowing of the lumen, thus preventing any back flow of luminal contents, which is instead propelled in a forward motion by the contraction of the longditudinal muscle (Pace and Williams, 1969).

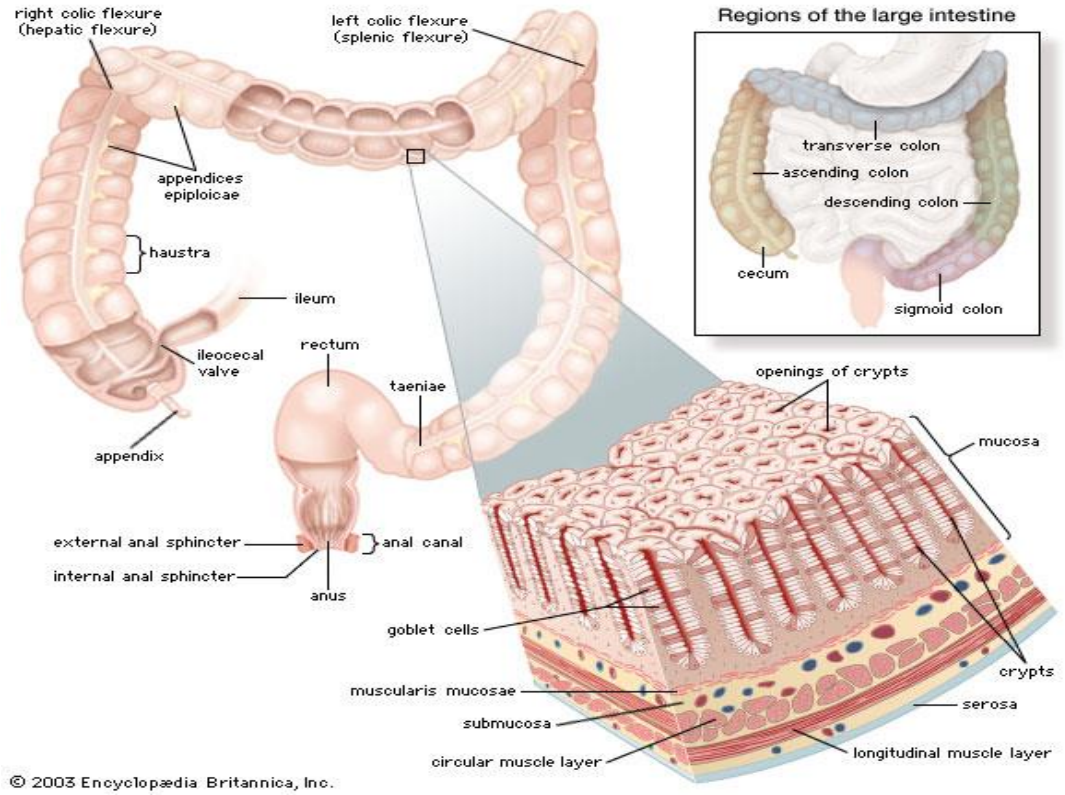

Figure 1.4 Structural anatomy of the colon. *(www.anatomytopics.wordpress.com).*

Between the muscularis propria and the mucosa lies the submucosa, a layer of connective tissue. The submucosa, via its connective tissue properties, attaches the mucosa to the underlying muscle layers of the colon wall and is also densely populated with blood vessels, lymphatic vessels and nerve cells, all of which supply the above mucosal layer. The innermost layer of the colon wall and that lining the surface of the colon lumen is the mucosa. Three layers comprise the mucosa; the surface epithelium, the lamina propia and the muscularis mucosae. The innermost layer of these three is the simple columnar epithelium, a smooth surface lacking the projecting villi found in the small intestine, Figure 1.5 shows a comparison between the small and large intestinal wall structure. The lamina propia, also known as the basement membrane is a layer of connective tissue situated between the surface epithelium and the underlying muscularis mucosae. The muscularis mucosae is the outermost layer of the mucosa, furthest from the luminal side and in direct contact with the submucosa, composed entirely of smooth muscle (Vander's Human Physiology, 2005).

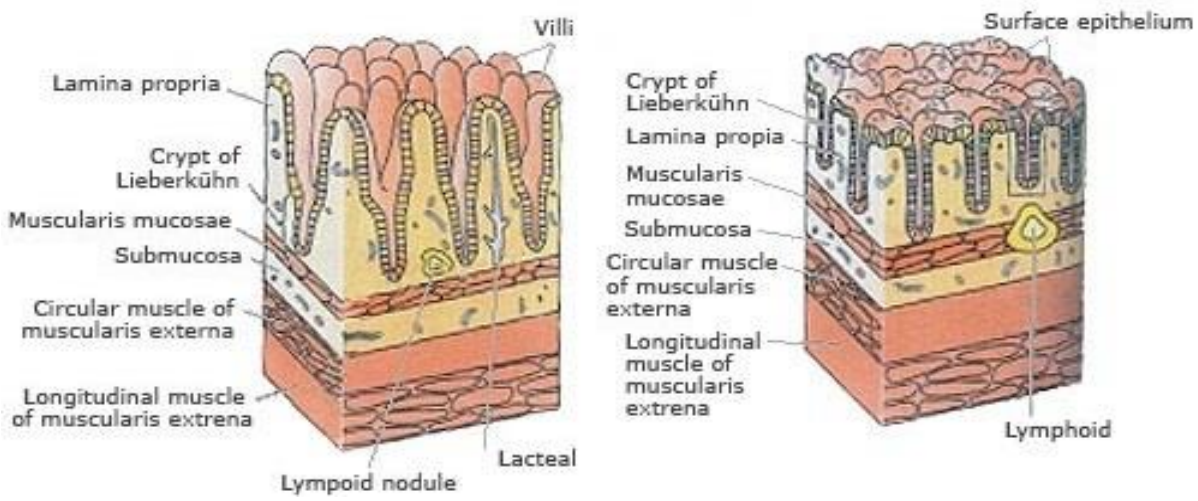

Figure 1.5 Cross Sectional Views of the Structural Components of the Intestinal Wall. *In the small intestine, finger-like projections known as villi extend from the top of the crypts, from the mucosal surface. In the large intestine however, such structures are absent, leaving a smooth, flat epithelial layer (http://beneficialbacteria.net).*

Figures 1.3 – 1.5 demonstrate the invaginated nature of the intestinal epithelium. These invaginations, the crypts of Lieberkun, extend down from the surface epithelium throughout the mucosal layer and are found in both the small and large intestine. There are approximately 10 million crypts found throughout the colon, each of which are able propagate every type of epithelial cell from a small number (4-6) of monoclonal stem cells located at the crypts base (Clevers H., 2006).

1.2.3 Specialised Cell Types of the Colonic Epithelium

The nature of the cell lineages found in the intestinal epithelium is largely conserved between the large and small bowel. The three key epithelial lineages are: enterocytes, goblet cells and enteroendocrine cells (Pinto and Clevers, 2005). In the small intestine, the base of the crypt is also occupied by Paneth cells, which are not found within the normal human colon (Medema and Vermeulen, 2011). Enterocytes or columnar cells are the absorptive cells of the intestine and are characterised by the presence of a brush border on their apical surface (van der Flier and Clevers, 2009) composed of large numbers of microvilli, providing an increased surface area for the absorption of water, glucose and ions such as sodium (Figure 1.1.) from the intestinal lumen.

Goblet cells are far more abundant in the colon than in the small intestine. These goblet cells are responsible for the production and secretion of a sticky mucus layer covering the luminal wall (Pinto and Clevers, 2005). This mucus has an essential protective function within the colon, it provides lubrication in order to facilitate the expulsion of luminal contents, in turn preventing damage to the smooth lining of the colon. The proportion of goblet cells increases approximately four fold from the small intestine to the descending colon (van der Flier and Clevers, 2009).

Enteroendocrine cells account for around 1% of the total population of epithelial cells throughout the intestine and are, like the goblet cells, a secretory lineage. Enteroendocrine cells are found scattered throughout the mucosa, where they synthesize and secrete specialised hormones such as gastrin and serotonin responsible for secretion and motility respectively (Sternini et al., 2008). These cells are classified into approximately fifteen subtypes based upon the specific peptide they secrete (van der Flier and Clevers, 2009).

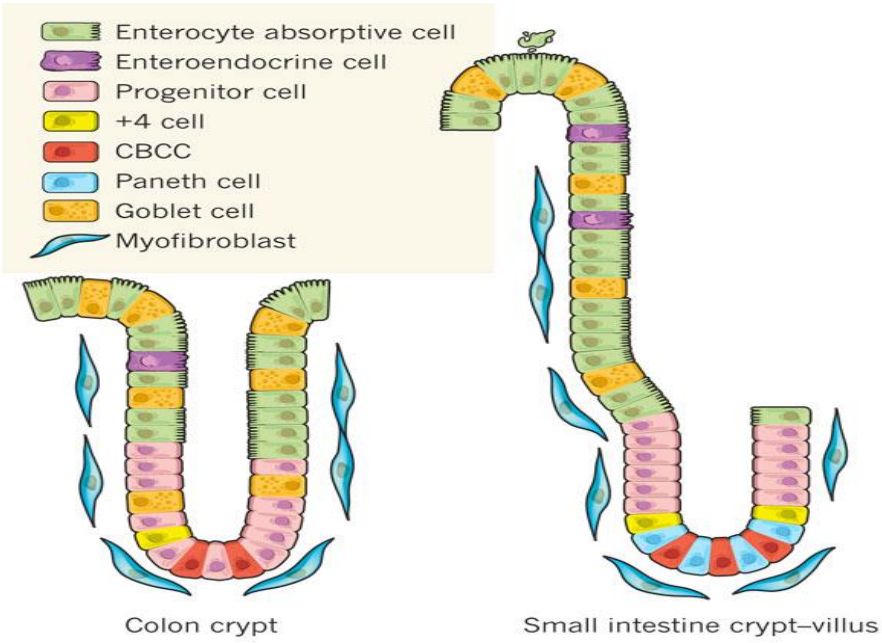

Figure 1.6 Specialised cell types of the intestinal epithelium. *Paneth cells at the base of small intestinal crypts are absent in the colonic crypts (Medema and Vermulen, 2011).*

1.3 Tissue renewal in the intestinal epithelium

1.3.1 The intestinal stem cell

The defining characteristics of stem cells are their abilities to self-renew and to give rise to progeny that can differentiate into all specialised lineages. It is the microenvironment in which the stem cell resides, its 'niche' that determines its characteristics. Adult stem cells have now been identified in the hematopoietic system, the epithelial system, neural system and epithelial system (Xie and Li, 2007). The stem cell niche can be cellular, non-cellular or the cancer stem cell niche. Cellular and non-cellular components interact with each other to convey stemness properties and to

regulate symmetric and asymmetric division. The cancer stem cell niche contains stem cells with acquired mutations. Within this niche, tumour initiating, multi-potent stem cells can reside un-regulated.

The intestinal stem cell niche contains adjacent epithelial cells, subepithelial myofibroblasts, enteric neurons, endothelial cells, intraepithelial lymphocytes and the basement membrane, all of which regulate stem cell fate (Chia and Kosinski, 2011). Wnt ligands are known to be of key importance in stem cell maintenance. The source of Wnt ligands in the stem cell niche are the subepithelial myofibroblasts (Brittan and Wright, 2002).

In vitro cultured intestinal stem cells have been found by Sato et al. (2009) to require additional signals to grow, signals which may not originate from the epithelium. With the addition of factors such as R-Spondin 1, EGF and Noggin, Lgr5+ cells are able to grow into organoids. In addition to this, Sato et al. (2011) found that co-culture of Lgr5+ stem cells with Paneth cells led to increased survival and number of organoids. Paneth cells express EGF, TGFα, Wnt3 and the Notch ligand D114, all of which are essential signals for stem cell maintenance in culture. Lgr5+ stem cells were lost *in vivo* on genetic removal of Paneth cells. The growth of these Lgr5+ stem cells and promotion of organoid formation was supported by cKit+ goblet cells expressing D111, D114 and EGF in the colon (Rothenberg et al., 2012).

The stem cell niche maintains a constant number of stem cells. Occurance of genetic mutations can disrupt the homeostatic control of stem cell division. Stem cells produced by symmetrical division are retained in the niche or become desensitised to regulatory signals and cause crypt expansion leading to neoplastic transformation. Signalling pathways responsible for the maintenance of crypt homeostasis include bone

morphogenic protein (BMP), transforming growth factor β (TGFβ), Notch and Hedgehog (Hh). TGFβ and BMP signalling function to inhibit intestinal stem cell activation and promote cell differentiation (He et al., 2004). The key regulator of cellular proliferation in the intestinal epithelium is the Wnt signalling pathway. The effects of Wnt signalling inhibition by the Wnt inhibitor Dkk1 has been found to result in loss of proliferation and subsequently, loss of crypts (Pinto et al., 2003).

1.3.2 Migration

Following stem cell proliferation at the base of the intestinal crypt, the resulting daughter cells migrate along the crypt axis, undergoing differentiation as they go, until they reach the surface. T-cell factor (TCF) is a transcription factor activated by the binding of β-catenin (Booth et al., 2002). It is the interaction of B subclass ephrins with EphB receptors that is responsible for the control of stem cell positioning within the intestinal stem cell niche (Holmberg et al., 2006). Ephrin proteins and their Eph receptors are membrane bound proteins. The Ephrin/Eph molecules are known Wnt target genes and function under the control of the β-catenin/ TCF transcription interaction (Scoville et al., 2008). Batlle et al. (Batlle et al., 2002) provide supportive evidence for the proposal that positioning of cells along the small intestinal crypt axis is controlled by regulated expression of EphB and ephrinB genes. The regulator of this gene expression is β-catenin/TCF signalling.

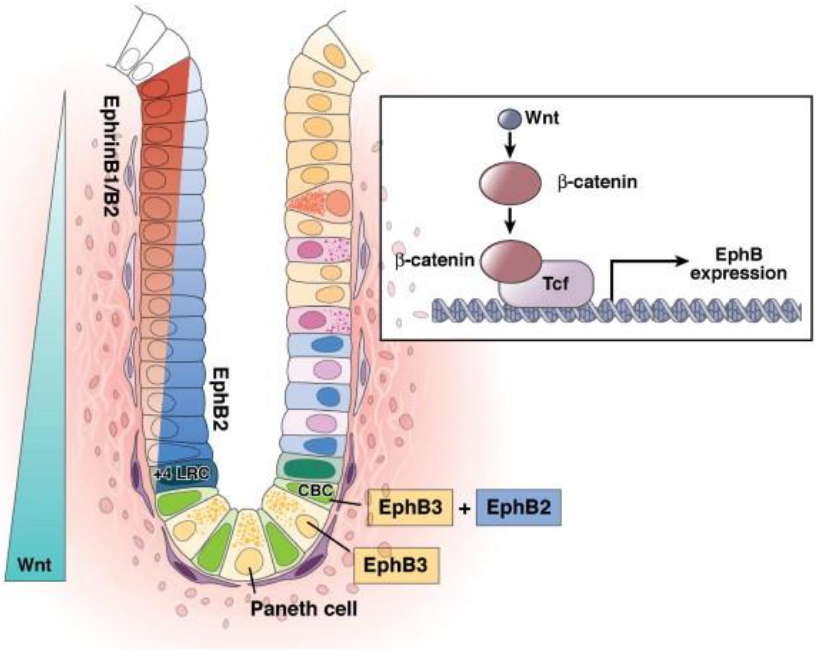

Figure 1.7 Control of crypt cell migration is by EphB receptors and Ephrin-B ligands. *Cells at the crypt-villus junction express high levels of Ephrin-B1/B2 ligands. EphB2 has an opposing gradient and instead is stronger at the crypt base, in the same way that the Wnt signalling gradient decreases further along the crypt axis. The migratory behaviour of crypt cells is determined by Ephrin-B ligands and EphB receptors (Scoville et al., 2008).*

The location of a cell within the crypt determines the state of that cell as it will be influenced by the molecules secreted in that region. Paneth cells do not express any Ephrin- B ligands, only EphB3. Therefore, Paneth cells migrate down towards the base of the cell and that is where they remain. This is different to CBCs and +4 LRCs which are both forced to migrate upwards toward the crypt-villus junction due to their expression of EphB2 and EphB3 (Scoville et al., 2008). These gradients function by repulsion between the EphB/Ephrin-B interaction. According to investigations by Batlle et al. (2002), CRC progression is greatly influenced by EphB expression. Loss of EphB expression at the adenoma-carcinoma transition in human

CRC correlates strongly with the degree of malignancy. In the colon and rectum of the APC(Min/+) mouse, tumorigenesis is accelerated by a decrease in EphB expression, leading to aggressive adenocarcinomas.

1.3.3 Cell Shedding

In order for crypt homeostasis to be maintained, the number of actively dividing cells must match the number of cells shed from the epithelium. Apoptotic cells signal to their neighbouring cells which subsequently contract to form an actomyosin ring that squeezes the apoptotic cell from the epithelium into the surrounding lumen (Eisenhoffer et al., 2012). Sphingosine-1-phosphate (S1P) is the signal secreted by the dying cell, which in turn triggers Rho-mediated contraction to squeeze out the dying cell. Extrusion of the apoptotic cell can be inhibited if sphingosine kinase activity is inhibited or if S1P is denied access to its receptor. Cells adjacent to a cell undergoing apoptosis display an S1P(2) receptor to which S1P can bind in order to initiate extrusion of the apoptotic cell (Gu et al., 2011).

Eisenhoffer et al. (2012) stimulated cellular overcrowding by culturing MDCK cells on a silicone membrane stretched to 28% of its original length and then released from the stretch. Pre-release level of cells were restored within 6 hours after crowding. Homeostatic controls acted to eliminate additional numbers of cells. Perpetual shedding of cells from the intestinal epithelium by a 'programmed cell death' mechanism, reduces the risk of colorectal cancer by preventing damaged or mutated cells continuing to proliferate and populate the epithelium.

Findings from Potten et al. (1997), demonstrated additional spontaneous apoptosis of cells in the stem cell region further down the crypt, where 10% of the cells were

apoptotic. Potten et al. (1997) suggested a regulatory mechanism in order to control the number of stem cells in the adult epithelium, in turn maintaining crypt size as appropriate. This spontaneous mitosis is a far more regular occurrence in the small intestine compared to the colon, in addition these apoptosing cells are found scattered throughout the colonic crypt rather than being restricted to the proliferative region as in the small intestine. This in itself could offer an explanation as to why the incidence of cancer is higher in the colon than in the small intestine. A greater ability to remove any mutated stem cells from the small intestine could conceivably decrease the likelihood of malignant transformation (Potten et al., 1997).

1.4 Intestinal Wnt signalling

1.4.1 The Wnt Signalling Pathway

30 years ago marked the discovery of the mouse proto-oncogene Int-1 (Nusse and Varmus, 1982). The Int-1 gene was later renamed Wnt1, an almagamation of Int-1 and the homologous fly *wingless* gene *wg* (Rijsewijk et al., 1987), known to be responsible for segment polarity in the developing fly larvae (Nüsslein-Volhard and Wieschaus, 1980).

Wnt signalling plays a fundamental role in ensuring physiological harmony in both the developing embryo and the adult mammalian body. Appropriate regulation of cell proliferation, differentiation and migration, as well as fate decisions in the developing embryo rely on tight regulation of this signalling cascade (MacDonald et al., 2009; Nusse et al., 1991 and 1992; Angers and Moon, 2009; Logan and Nusse, 2004). 19 known Wnt genes reside in most mammalian genomes, including that of the

human (Nusse, 2010), although single-cell organisms lack the presence of any Wnt genes (Petersen and Reddien, 2009). Wnts are secreted cysteine-rich glycoproteins each of around 350-residues (MacDonald et al., 2009). Intestinal sub-epithelial myofibroblasts (ISEMFs), found adjacent to crypt epithelial cells are thought to be a source of Wnt ligands (Bienz and Clevers, 2000; Madison et al., 2005), indicated by Powell et al. (1999), who found in their culture system that these ISEMFs have the ability to stimulate epithelial cell proliferation.

Both a canonical and several noncanonical Wnt signalling pathways exist within the body (Figure 1.8), noncanonical pathways being defined as those which function independently of β-catenin. In contrast, the canonical Wnt signalling pathway, also known as the Wnt/β-catenin pathway is β-catenin-dependent and results in the stabilisation and nuclear translocation of the β-catenin protein which in turns facilitates the transcription of target genes. Here we will concentrate on the canonical Wnt signalling pathway.

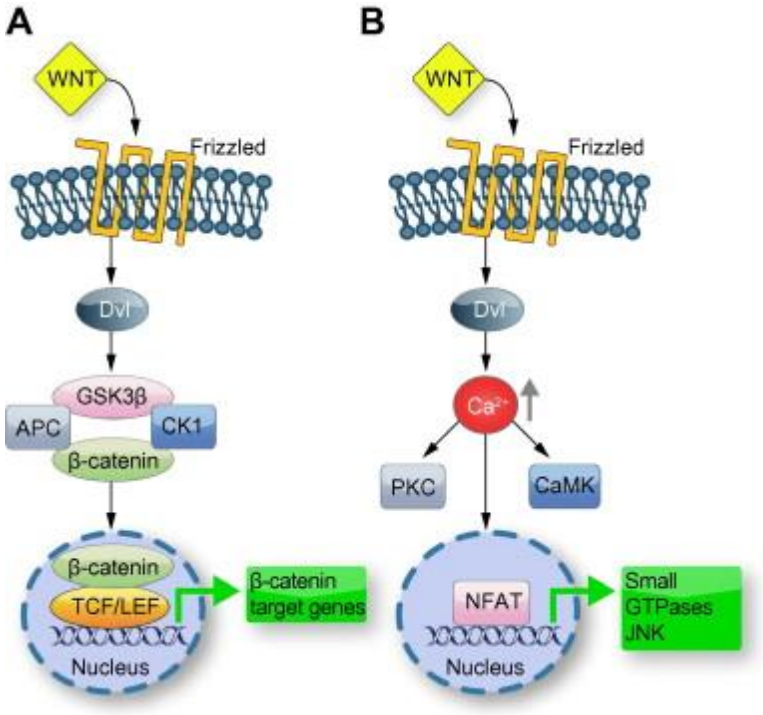

Figure 1.8 Canonical and non-cannonical Wnt signalling. *A) Canonical Wnt signalling. Wnt binds to Fz receptors, activating Dvl, thus stabilising β-catenin and allowing its nuclear translocation, where it interacts with TCF/LEF transcription factors, activating Wnt target genes. B) Non-canonical Wnt signalling. Wnt isoforms Wnt 4, 5a and 11 bind Fz and activate Dvl, but instead of β-catenin involvement is small GTPases and JNK. Dvl activation leads to increased intracellular calcium levels, activating proteins such as PKA, CaMK and NFAT. These proteins then act as transcription factors in the nucleus. (Strazzabosco and Fabris, 2012).*

The canonical Wnt signalling pathway has been well characterised to date (Figure 1.9). Stimulation of the pathway occurs as a secreted Wnt ligand binds to its cell surface receptor, frizzled (Fz), a seven pass transmembrane receptor and the low-density lipoprotein receptor-related co-receptor protein 5 or 6 (LRP5 or LRP6), both single span transmembrane proteins (Tamai et al., 2000;Wehrli et al., 2000). This Fz receptor hosts an N-terminal cysteine rich domain (CRD) which acts as the binding

site for Wnt ligands (He et al., 2004). After translation, palmitate and/or palmitoleic acid is added to a cysteine residue and/or serine residue, therefore making crypts hydrophobic (Takada et al., 2006; Willert et al., 2003). In order for secreted Wnt proteins to be fully active, acylation must occur. The Wnt lipid group is thought to engage directly with Fz-CRD, although the need for acylation is not entirely clear (Bazan and Sauvage, 2006). The acyltransferase Porcupine has been indicated as necessary for the palmitoylation of Wnt ligands by Wnt-secreting cells (Hausmann and Basler, 2006). The acylation of Wnts may be required to aid their morphogenic activity, by anchoring them to cell membranes (Mulligan et al., 2012).

Activation of the Wnt signalling pathway by Wnt ligands is selectively antagonised during homeostasis, ensuring that cells are not under continuous instruction to proliferate. Specific antagonists of the canonical Wnt signalling pathway include secreted frizzled-related proteins (sFRPs), Dickkopf (Dkk) and Wnt inhibitory factor (WIF-1) (Kawano and Kypta, 2003; Hsieh et al., 1999; Niehrs, 2006). sFRPs bind directly to Wnt ligands, preventing interaction with Fz. Dkk1 works together with Kremen to block access of Wnt ligands to the LRP5/6 co-receptors (He et al., 2004).

At the basal level, when the Wnt ligand is not bound to its membrane receptor, β-catenin is bound to a multiprotein degradation complex, known as the destruction complex. This complex contains Axin as a scaffold protein, the APC tumour suppressor gene protein, casein kinase 1 (CK1) and glycogen synthase kinase 3β (GSK 3β), the β-catenin binding domains (Schneikert and Behrens, 2007). β-catenin is targeted for phosphorylation at its amino-terminal Serine S45 residue by CK1 (Amit et al., 2002; Liu et al., 2002). GSK3β is then recruited to phosphorylate additional serine

and threonine residues (Rubinfeld et al., 1996). β-catenin is subsequently ubiquitinated by E3 ubiquitin ligase, containing TrCP. Following ubiquitination, β-catenin is degraded by proteolysis in the cytosolic proteasome. Under these conditions, Wnt target genes would not be transcribed.

The aim of canonical Wnt signalling activation is to stabilise β-catenin (Logan and Nusse, 2004). On binding of the Wnt ligand to its cell surface receptors, Axin is recruited to and docked at the plasma membrane, resulting in the degradation of this scaffold protein and subsequent dissociation of the destruction complex, from which stable β-catenin is discharged and allowed to accumulate in the cytoplasm. This non-phosphorylated β-catenin translocates to the nucleus where it interacts with T cell factor/ lymphoid enhancer factor (TCF/LEF) transcription factors (Hurlstone and Clevers, 2002), displacing the co-repressor Groucho. This bipartite complex then functions to transcribe Wnt target genes such as the oncogene *c-myc*. The Wnt signalling cascade and thus transcription of Wnt target genes ceases on removal of the stimulating Wnt ligand.

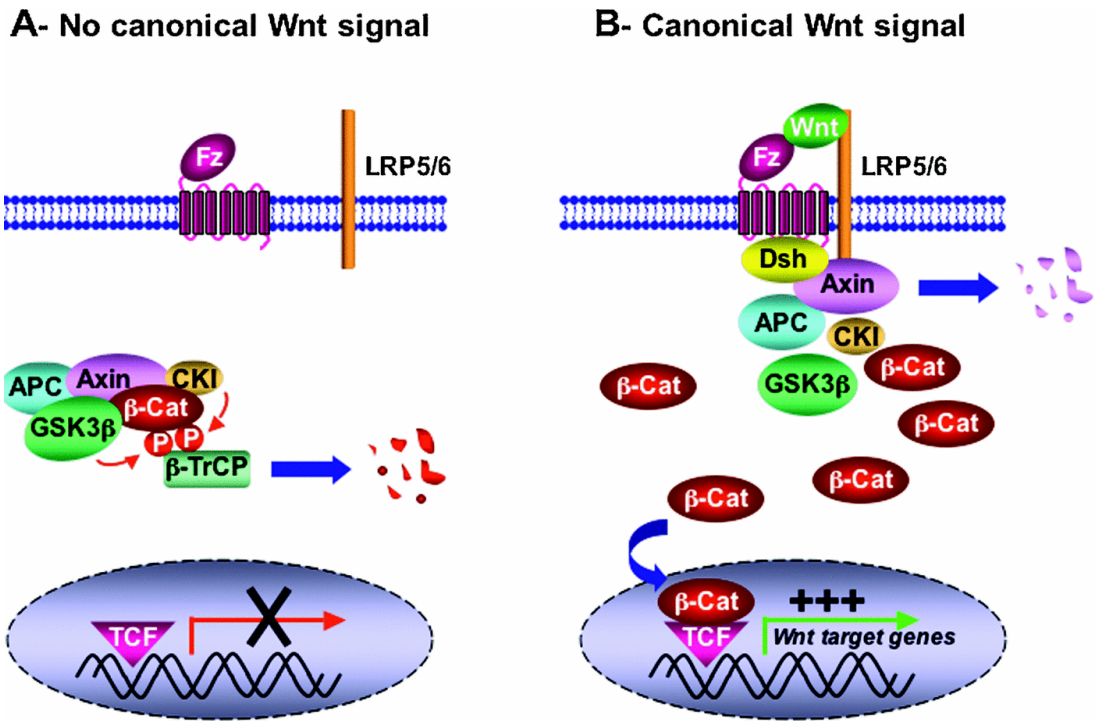

Figure 1.9 Canonical Wnt signalling. *A) Basal state un-stimulated Wnt cascade. B) Wnt cascade stimulated by the binding of a Wnt ligand to the Fz and LRP5/6 receptors, leading to membranal Axin recruitment, destruction complex dissolution and discharge of stabilised β-catenin to the cytoplasm, which then translocates to the nucleus. In the nucleus, β-catenin binds to TCF/LEF transcription factors and Wnt target genes are transcribed (He, 2003).*

In vivo mouse studies have demonstrated that apropriate Wnt signalling activation is essential in the maintenance of normal intestinal epithelial architecture and that precursor-cell proliferation requires constant stimulation of the Wnt pathway. This constant stimulation is demonstrated by the accumulation of nuclear β-catenin at the base of normal crypts, the hallmark for active Wnt signalling (Clevers, 2006). Kuhnert et al. (2004) and Pinto et al. (2003) transgenically expressed the secreted Wnt inhibitor Dkk1, which led to loss of intestinal crypts, as well as significantly reducing epithelial proliferation (Pinto et al., 2003). This loss of intestinal crypts is also seen in mice lacking TCF4 (Korinek et al., 1998) or β-catenin (Ireland et al., 2004). In

addition to this finding, Ireland et al. (2004) also noted that β-catenin deletion increased apoptosis and depleted goblet cell numbers (Ireland et al., 2004). Intestinal hyperplasia occurs as the result of activating mutations in β-catenin simultaneously to inactivation of *APC* (Reya and Clevers, 2005).

Using *in situ* hybridisation, Gregorieff et al. (2005) investigated the expression patterns of all Wnts, Frizzleds, LRPs, Wnt antagonists and TCFs in the mouse small intestine and colon. They tested 19 Wnt genes and found that 7 Wnts were readily detected in the intestine, although *Wnt2b* and *Wnt3* were absent in the colon. *Wnt3* and *Wnt9b* were found only at the very base of small intestinal crypts, in the same region as Paneth cells. *Wnt9b* was detected in epithelial progenitor cells above the Paneth cell compartment but colonic *Wnt9b* was found throughout the local epithelium. *Wnt6* was expressed throughout the small intestinal and colonic crypts and was strongly expressed in adenomas. *Wnt5a* was found in abundance in villus tips and less so towards the crypt-villus junction. In the colon *Wnt5a* and *Wnt5b* were found only in the mesenchyme beneath the surface epithelium. *Wnt4* was uniformly expressed along the villus mesenchyme and was restricted to the mesenchyme beneath the surface epithelium of the colon. *Wnt2b* was strongly expressed in the mesenchymal layer of the villi but only in the endothelial or smooth muscle cells in the colon (Gregorieff et al., 2005).

Frizzled receptors 5 and 7 were found to be expressed in epithelial cells at crypt bases, *Fz4* was restricted to the differentiated epithelial villi cells. *Fz6* was expressed uniformally throughout the whole intestinal epithelium. Co-receptors *LRP5* and *6* were expressed in the proliferative epithelial crypt cells.

sFRPs are a family of secreted factors and the functional counterparts of the Fz receptors. sFRPs antagonise Wnt signalling by competing for Wnt binding. Fzs and sFRPs share an equivalent Wnt-interacting cysteine-rich domain (Gregorieff et al., 2005). Gregorieff et al. (2005) also found abundant levels of *sFRP1* in the mesenchymal cells immediately adjacent to crypts. *sFRP5* expression was found in cells located immediately above the Paneth cell compartment (Gregorieff et al., 2005).

WIF antagonises Wnt signalling by binding directly to Wnt ligands, this together with *Dkk2*, an LRP binding Wnt antagonist, was only detected in adenomas (Gregorieff et al., 2005). *Dkk3* was weakly expressed in the villus mesencyme and up-regulated in adenomas, *Dkk1* and *Dkk4* were undetected in the intestine.

When investigating TCF expression, Gregorieff et al (2005) 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. *TCF3* was expressed in the proliferative compartment of the colon only and *LEF* was detected in intestinal polyps but absent in normal epithelium.

The Wnt signalling pathway can also be activated by a family of secreted proteins known as the R-Spondin family, all vertebrates contain four R-Spondin family members, each of which potentiates the canonical Wnt signalling pathway (Kim et al., 2008), thus R-Spondins are Wnt pathway agonists. Each of the four proteins are able to bind Lgr4, 5, and 6 (Lau et al., 2011). Lgr4 ablation had no effect on signalling by Wnt3a, but abolishes the R-Spondin 1-mediated signal enhancement in canonical signalling initiated by Wnt3a. Re-expression of LGR4, 5 or 6 rescues this effect (Lau et al., 2011). Using transgenic mice, induced to ectopically express R-Spondin 1,

Kimm et al. (2005) found hyperproliferation in the small and large intestine leading to a 25% increase in the colon of these mice. Increased levels of nuclear β-catenin was also noted in these hyperproliferating cells (Kim et al., 2005). Binding of R-Spondin 1 to membranes of cells expressing Lgr4 or 5 led to the simultaneous internalisation of R-Spondins and LGR's. The supportive effect of R-Spondins on Wnt singnalling were lost on LGR5 depletion (Carmon et al., 2005).

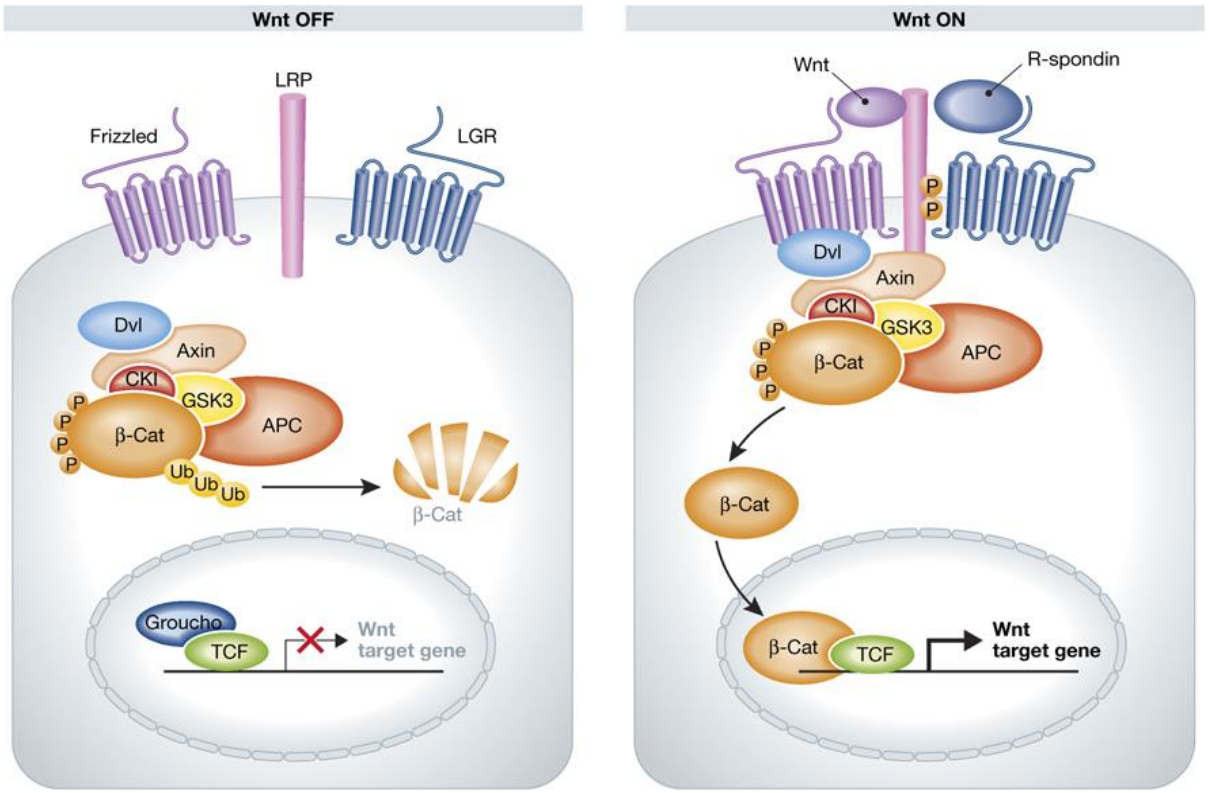

Figure 1.10 R-Spondin-LGR interaction at the cell surface. *LGR homologues interact with Frizzled/LRP5/6 receptors and bind with R-Spondins to enhance Wnt signalling. (Schuijers and Clevers, 2012).*

1.4.2 Wnt signalling and colorectal cancer

The term colorectal cancer (CRC) refers to cancer of the colon (72%) and that of the rectum (28%). Approximately 95% of CRC cases are the result of sporadic somatic mutations, up to 80% of which contain an APC mutation. The remaining 5% are the result of inherited germline mutations, most commonly Hereditary Nonpolyposis Colorectal Cancer (HNPCC) and Familial Adenomatous Polyposis (FAP) (Centelles, 2012). Around 90% of all CRC contain a Wnt-pathway activation mutation. This in turn leads to the stabilisation and accumulation of β-catenin within the cell nucleus. Wnt signalling is well established as a driving force in tumorigenesis.

Vast numbers of genetic studies on human colorectal neoplasms have been carried out for many years due to the relative ease in obtaining tissue samples at each stage of tumorigenesis, from small polyps to advanced adenocarcinomas. FAP is currently the most widely known understood example of a Wnt-pathway mutation that leads to production of tumours. It was genetic analysis of thesse FAP patients which first identified the tumour suppressor APC (Groden et al., 1991; Kinzler and Vogelstein, 1996). FAP is autosomal dominant in its transmission, therefore one inherited defective *APC* allele is required to exhibit the mutated phenotype (Kinzler et al., 1991; Nishisho et al., 1991). This genotype results in the FAP phenotype in which hundreds of polyps or adenomas are developed in young adults. Although these polyps are benign, the remaining wild type *APC* allele is often inactivated at a later stage, causing malignant transformation of some of the polyps to progress into adenocarcinomas. FAP is a relatively rare condition, although many sporadic CRC do

lose both *APC* alleles, resulting in β-catenin stabilisation and accumulation (Kinzler and Vogelstein, 1996).

APC is a large protein of 312kDa. Within this protein, four 15aa repeats bind β-catenin and seven 20aa repeats are involved in its binding and downregulation and AXIN1/AXIN2 binding of APC in the destruction complex. The β-catenin inhibitory domain (CID) has been located following the second 20aa repeat, conferring to APC, the ability to regulate β-catenin signalling (Kohler et al., 2009). Mutations in APC cause truncation at the protein's C-terminus, deleting five or more of the seven 20aa repeats. At least three of these APC repeat sequences are required for down-regulation of β-catenin (Rubinfeld et al., 1997). Further studies have shown that highly conserved NES sequences exist at the N-terminus of APC as well as within the 20aa repeat region. β-catenin was found to accumulate in the nucleus in the absence of NES sequences (Rosin-Arbesfeld et al., 2000; Henderson, 2000; Neufeld et al., 2000).

APC mutation is also thought to prevent it carrying out its role as a nuclear-based regulator of β-catenin (Fabbro and Henderson., 2003). Within the nucleus APC promotes the export β-catenin and therefore deactivation of TCF-mediated transcription. Mutated APC is unable to effectively carry out this function (Henderson, 2000).

β-catenin is encoded by the gene *CTNNB1*. When β-catenin is mutated, the CK1/GSK3β phosphorylation sites at its N-terminus are disrupted (Ilyas et al., 1997; Morin et al., 1997). Mutated β-catenin is therefore rendered unrecognisable by the β-TrCP, resulting in its stabilisation, subsequently β-catenin is able to enter the nucleus and constitutively activate transcription via TCFs. Inappropriate complex formation between TCF4 and β-catenin causes activation of the Wnt target gene c-myc (He et

al., 1998). c-myc is a proto-oncogene and an important regulator involved in growth control and cell cycle progression. When over- expressed, c-myc causes the cell-cycle to become deregulated, and prevents induction of apoptosis (Breckenridge and Shore, 2000). This over-expression of c-myc can be detected in colorectal carcinomas at both early and late stages (Smith et al., 1993; Imaseki et al., 1989; Sikora et al., 1987).

Hypothesis

Renewal of the human colonic epithelium is maintained by a canonical Wnt signalling gradient along the colonic crypt axis

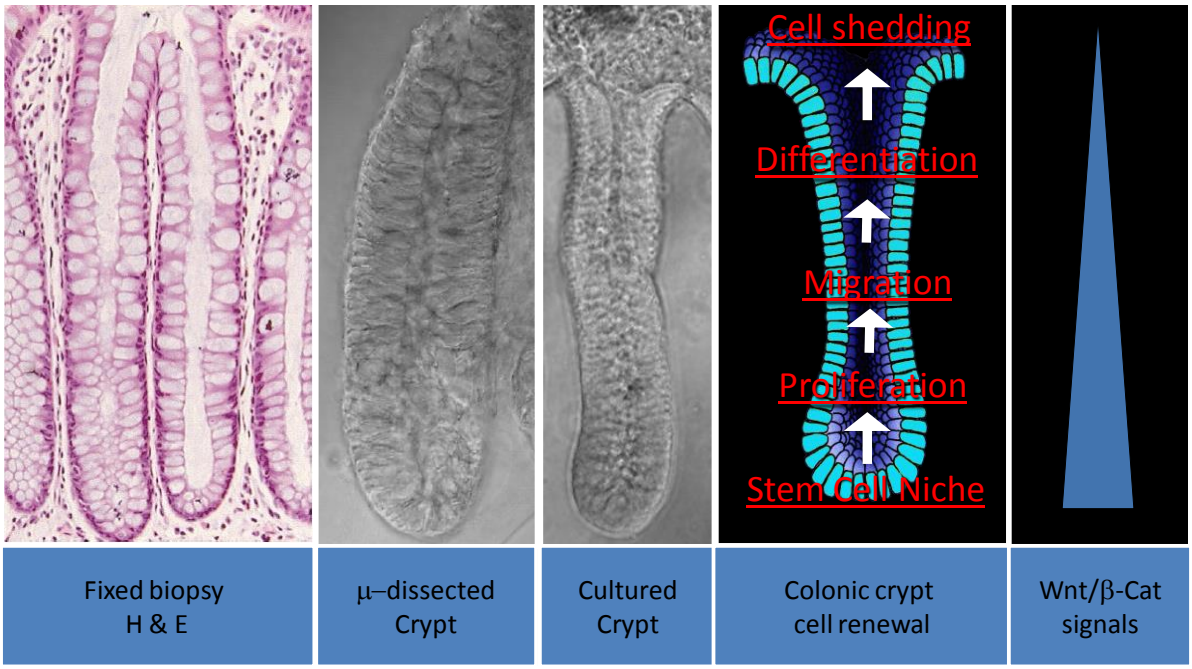

Figure 1.11: Hypothesis - Renewal of the human colonic epithelium is maintained by a canonical Wnt signalling gradient along the colonic crypt-axis. *The left-hand panel depicts a H&E section of a fixed colorectal biopy tissue sample. The adjacent panel depicts an intact native colonic crypt that has been microdissected from a fixed biopsy sample. Intact colonic crypts can be liberated from 'live' biopsy samples and placed into culture. These systems will be used to investigate the functional link between the tissue renewal hierarchy and the Wnt signalling pathway along the human colonic-crypt axis.*

Aims

- Determine the Wnt signalling status in the native colonic epithelium.
- Investigate the consequences of Wnt activation and inhibition on cultured human colonic crypt morphology, proliferation and migration.

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Chapter 2 Materials and Methods

2.1 Human Colonic Crypt Culture

2.1.1 Isolation and culture of human colonic crypts

Epithelial tissue samples were obtained from the sigmoid colon of patients undergoing either colonoscopy or bowel resection for cancer. Biopsies were taken from macroscopically normal areas. Samples were obtained with the approval of the Norwich District Ethics Committee, Norfolk and Norwich University Hospital. Isolation of human colonic crypts was carried out using a similar method to that described previously (Reynolds et al, 2007). Biopsies 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 1mM EDTA (Diaminoethanetetra-acetic acid disodium salt) and 1mM DTT (Dithiothreitol) for 1 hour at room temperature (reagents from Fisher Scientific, Loughborough, UK). Vigorous shaking liberated single crypts which were seeded onto non-fluorescent, grade 0, glass coverslips (Sigma) and embedded in matrigel (growth factor reduced, phenol free: BD Bioscience, Oxford, UK). Coverslips were placed into 12-well plates (Sigma, Gillingham, UK) prior to isolation. The matrigel was polymerized for 10 minutes at 37°C in a humidified 95% air, 5% CO_2 incubator. Crypts were covered with 500µl of medium (Advanced DMEM/F12: Invitrogen, Paisley, UK) supplemented with penicillin (5000 units/ml) / streptomycin (5mg/ml) (Invitrogen, Paisley, UK), L-Glutamine (2mM; Invitrogen, Paisley, UK), N2 (a serum-free growth supplement)

(Invitrogen, Paisley, UK), B27 (supportive serum substitute for cell culture) (Invitrogen, Paisley, UK) and 1mM N-acetylcysteine (Sigma, Gillingham, UK). Different experimental groups were created by the addition of various combinations of growth factors to the colonic crypt culture medium, including: human recombinant insulin-like growth factor 1 (IGF-1), 50 ng/ml; human recombinant noggin, 100 ng/ml; human recombinant R-spondin-1, 500 ng/ml; human recombinant Wnt 3A (100 ng/ml). Crypts for use in immunocytochemistry experiments were fixed for 1 hour in 4% paraformaldehyde (Sigma, Gillingham, UK) after 3 days in culture, and those required for longer term experiments were kept in culture for up to 1 week. The culture media was replaced every two days.

2.2 Immunocytochemistry

2.2.1 Native Human Colonic Crypts

1-5mm biopsies taken from the colonic epithelium of patients undergoing either a colonoscopy or surgical resection were fixed immediately on removal from the patient, in 4% paraformaldehyde (Sigma, Gillingham, UK) for 1.5 hours at 4°C. Biopsies were left in PBS overnight and then microdissected into single crypts and placed in 0.5% Triton-X (Roche Applied Sciences, Sussex, UK) for 1 hour. Crypts were then placed into Matrigel (BD Bioscience, Oxford, UK) and seeded onto non-fluorescent glass coverslips (Sigma, Gillingham, UK) in a 12 well plate (Sigma, Gillingham, UK). After polymerization of the Matrigel in the incubator, crypts were post-fixed and then washed in PBS. Crypts were made permeable with Triton-X-100 and after a final PBS wash, non-specific binding sights were blocked with blocking serum composed of 10%

goat (Abcam, Cambridge, UK) or donkey serum (Sigma, Gillingham, UK) and 1% bovine serum albumin (Sigma, Gillingham, UK) for 2 hours. The protein of interest was labelled by incubating crypts overnight with a specific antibody. Primary antibodies (Table 1) were diluted 1:100 in PBS. Secondary antibodies (Molecular Probes) (Table 2), were added for 2 hours, 1:200 dilution in PBS, followed by a PBS wash before mounting on poly-L-lysine coated slides (BDH) in vectashield mounting media (Vector labs, Petaborough, UK) with DAPI or PI stain.

2.2.2 Near-native Cultured Human Colonic Crypts

On completion of live experiments, the cultured human colonic crypts were fixed in 4% paraformaldehyde for 1 hour at 4°C and washed in PBS before processing for immuno labelling similar to that described above for native human colonic crypt. Fixed cultured crypts were permeabolised with 0.5% Triton-X for 30 minutes followed by three PBS washes. For BrdU epitope retrieval, crypts were denatured in 1M HCl for 10 minutes. After a final PBS wash, non-specific binding sites were blocked with blocking serum composed of 10% goat or donkey serum and 1% bovine serum albumin for 2 hours. Crypts were incubated with the primary antibody overnight at a 1:100 dilution in PBS and secondary antibodies added for 2 hours at a 1:200 dilution in PBS, before mounting on poly-L-lysine coated sides in vectashield mounting media with DAPI or PI stain. Non-specific labelling of secondary antibodies was determined by omitting the primary antibody or substituting it with the appropriate immunoglobulin.

Primary Antibody	*Species Origin*	*Supplier*
Anti BrdU	Rat polyclonal	Abcam
Anti Chromagranin A	Rabbit polyclonal	Abcam
Anti Ki67	Mouse monoclonal	Dako
Anti Mucin 2	Rabbit polyclonal	Santa Cruz
Anti Active β-Catenin	Mouse monoclonal	Millipore

Table 1 Primary antibodies used

Secondary Antibodies	
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 Secondary antibodies used

2.3 Crypt Morphology

2.3.1 Length Measurements

Multiple experimental groups of crypts from a single sample were maintained in culture as described previously (Section 2.1.1). Crypts from each group were imaged every 24 hours using a Zeiss Axiovert epifluorescence microscope housed in a humidified chamber maintained at 95% air, 5% CO_2 and at 37°C. Bright field images were taken using a x100 magnification. These experiments continued for as long as the crypts lived in culture, usually up to one week. One-way ANOVA and Tukey's post-hoc analysis were used to determine statistical differences between each experimental group. The groups used were: control (Advanced F12/DMEM culture medium supplemented with noggin, IGF-1 and R-spondin-1 as described in Section 2.1.1), Wnt3a (100ng/ml: R&D Systems, Abingdon, UK), DKK1 (800ng/ml: R&D Systems, Abingdon, UK) and a combination of Wnt3a and DKK1.

2.4 Crypt Cell Proliferation

2.4.1 BrdU Incorporation

At day 2 in culture, human colonic crypts were incubated with 10μM BrdU added to the culture media at 5% CO_2 and 37°C for 24 hours. After the incubation period crypts were fixed and underwent immunocytochemistry (see section 2.2).

2.4.2 Real-time Digital Time-lapse Microscopy

Human colonic crypts cultured on glass coverslips (Sigma, Gillingham, UK) in 12 well plates (Sigma, Gillingham, UK) were placed within the climate-controlled chamber of a Zeiss inverted motorised time-lapse system, maintained at 5% CO_2, 37°C. The locations of selected crypts from both Wnt 3A and control groups were programmed using Axiovision 4 software and DIC images taken every 5 minutes at x200 total magnification, over a 24 hour period. This was repeated for as long as the crypts survived in culture, with the culture media being replaced every 2 days. It was then possible to manually count the number of mitoses occurring in the base, or bottom third of each crypt over a given period. Differences in the number of mitoses occurring between the two groups were calculated and statistical differences found using one-way ANOVA and Tukey's post-hoc analysis.

2.5 Crypt Cell Migration

Crypts were placed in the Zeiss inverted time-lapse system and again imaged at 5 minute intervals over a 24 hour period. Using Axiovision 4 software, the migration of three selected cells per crypt moving from the mid region (the middle third) of the crypt up towards the surface of the crypt were followed. The rate of migration of each of the three cells was determined, and differences between the experimental groups, i.e. control and Wnt 3A, were assessed

2.6 Visualisation and Semi-quantitative Analysis

Colonic 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 total magnification. Semi-quantitative 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. For each crypt, 10 representative cells from each region of the crypt 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 proliferatioin experiments, the number of BrdU positive cells were manually counted for each region of the crypt: base, middle and top 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.

Chapter 3 Results

Introduction

The colonic epithelium is one of the most dynamically renewing tissue in the human body, with a daily turnover rate of 10 billion cells. Each cell that is shed from the surface epithelium must be replaced in order to repopulate the tissue. This replenishment is achieved by the tightly regulated proliferation activity of the local stem cell population located at the base of the colonic crypt. Following mitosis, the stem cell progeny migrate along the crypt-axis toward the surface epithelium, upon arrival at which they will undergo apoptosis and after being shed into the intestinal lumen. During their journey along the crypt-axis each cell will differentiate into one of three key lineages: enteroendocrine cells, enterocytes or goblet cells.

Wnt signalling has long been acknowledged as a key mediator in cell proliferation, differentiation and migration and is recognised as active within cells that demonstrate a nuclear accumulation of the protein β-catenin. Beinz showed that removal of the surface Wnt ligand results in the exportation of β-catenin from the nucleus and its subsequent degradation (Bienz, 2002). Murine studies such as those by Kongkanuntn and colleagues have demonstrated that even very the earliest neoplastic changes are reliably indicated by an increased level of nuclear β-catenin expression (Kongkanuntn et al., 2009).

In this chapter we investigate the effects of canonical Wnt signalling on crypt cell

proliferation and migration and maintenance of colonic crypt morphology in an *ex-vivo* human tissue culture system.

3.1 Morphological assessment of crypts in culture

Samples of colonic epithelium were obtained from macroscopically normal regions of the sigmoid colon of patients undergoing either a colonoscopy or surgical resection. From each patient, at least one biopsy was immediately fixed in 4% paraformaldehyde, while the others were maintained in hepes-buffered saline. The fixed samples were later microdissected into single crypts (Figure 3.1 A). The live portion of the sample underwent isolation as previously described by Reynolds et al. (2007) and crypts were placed in culture (Figure 3.1 B).

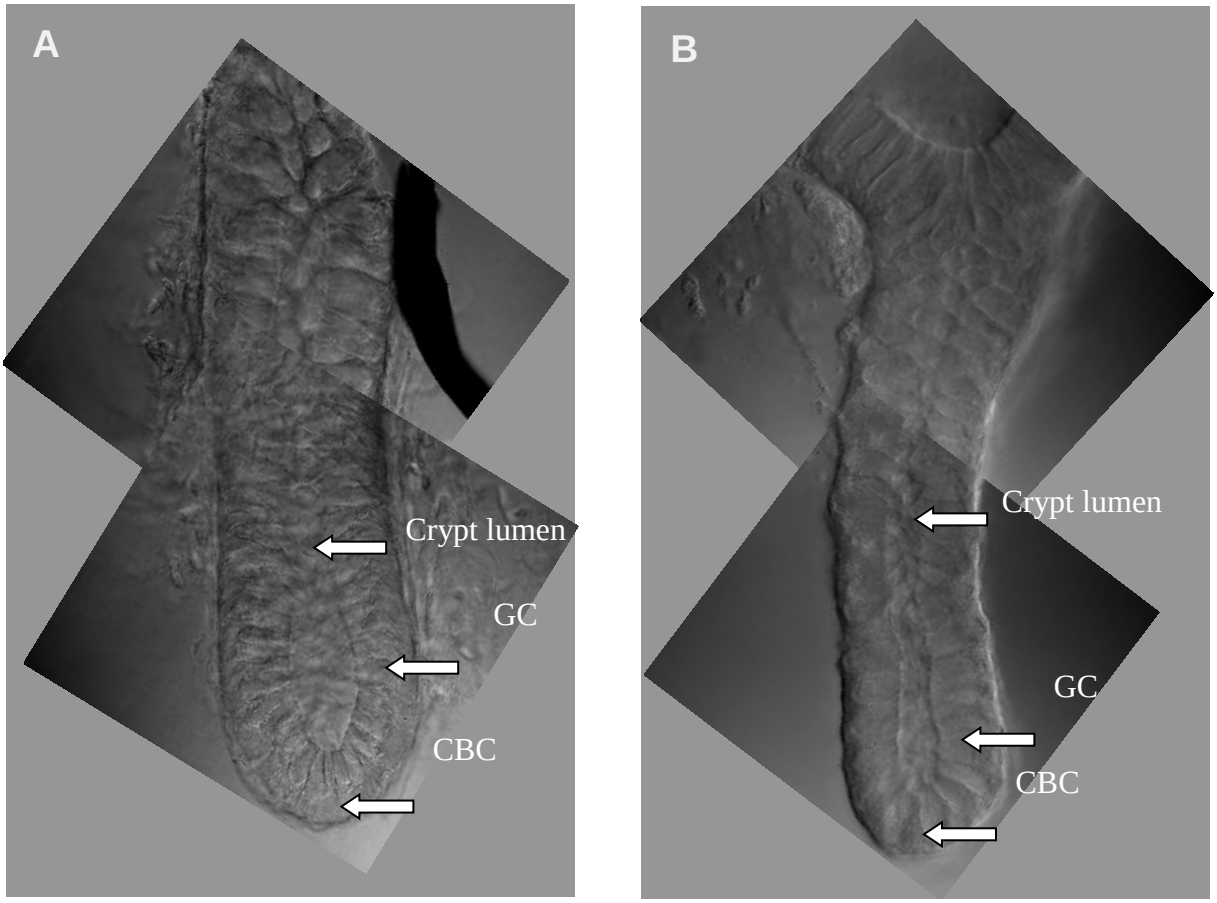


Figure 3.1. Morphological assessment of human colonic crypts ex-vivo. *DIC images of human colonic crypts. A) x200 magnification of a microdissected crypt from a fixed biopsy sample, B) x200 magnification image of an isolated crypt liberated from a live biopsy, demonstrating the maintenance of the crypt lumen, crypt base columnar cells (CBC) and goblet cells (GC), as would be found in the native tissue.*

Cultured human colonic crypts maintain their flask-like structure, with a narrow needle-like lumen, as well as their polarised nature whilst in culture, usually for up to seven days during which time it is possible to clearly visualise individual cells (Figure 3.2).

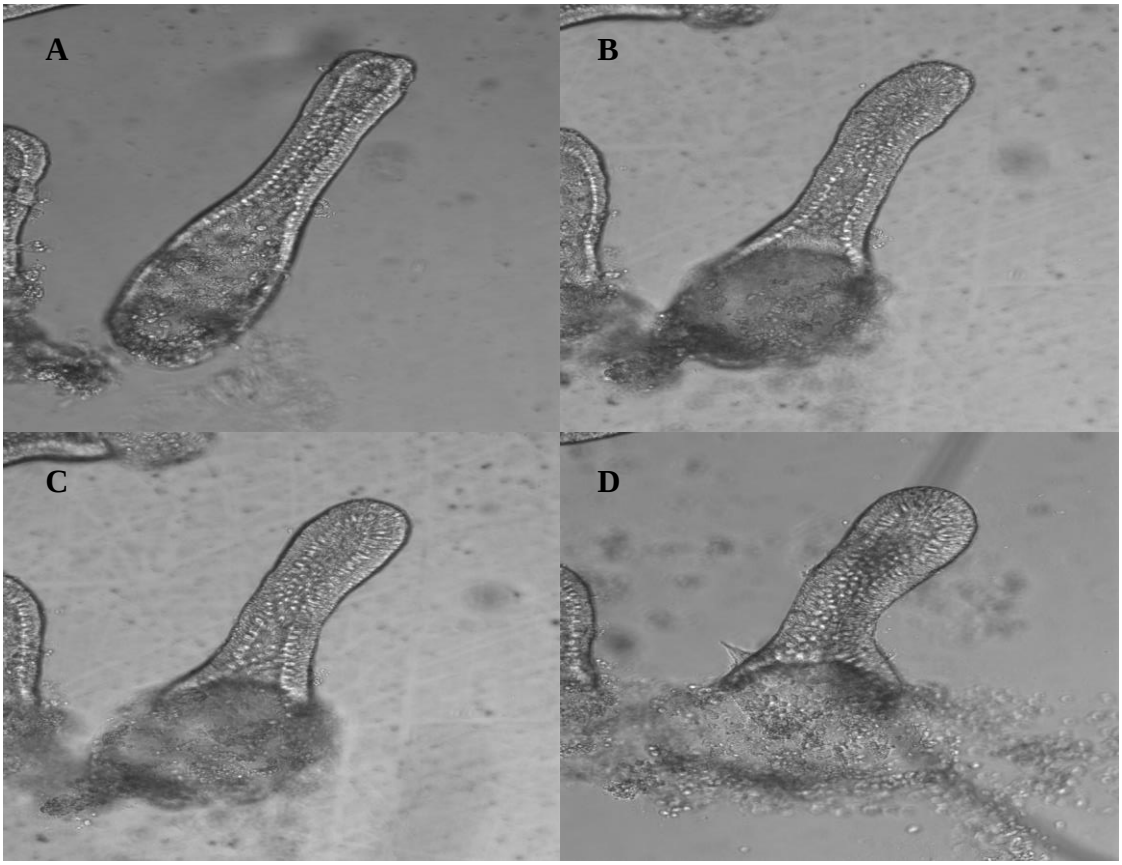


Figure 3.2. Maintenance of crypt morphology in culture for four days. *DIC images at x200 magnification of colonic crypts in culture on days 0 (day of isolation), 1, 2 and 3, A), B), C) and D) respectively. Flask-like shape of the crypt is maintained and individual cells remain visible throughout, demonstrating polarity.*

By fixing crypts at various stages in the culture period and processing them by immunocytochemistry as previously described in chapter 2, it was possible to verify the preservation of key crypt cell lineages during their time in culture using fluorescence microscopy (Figure 3.3).

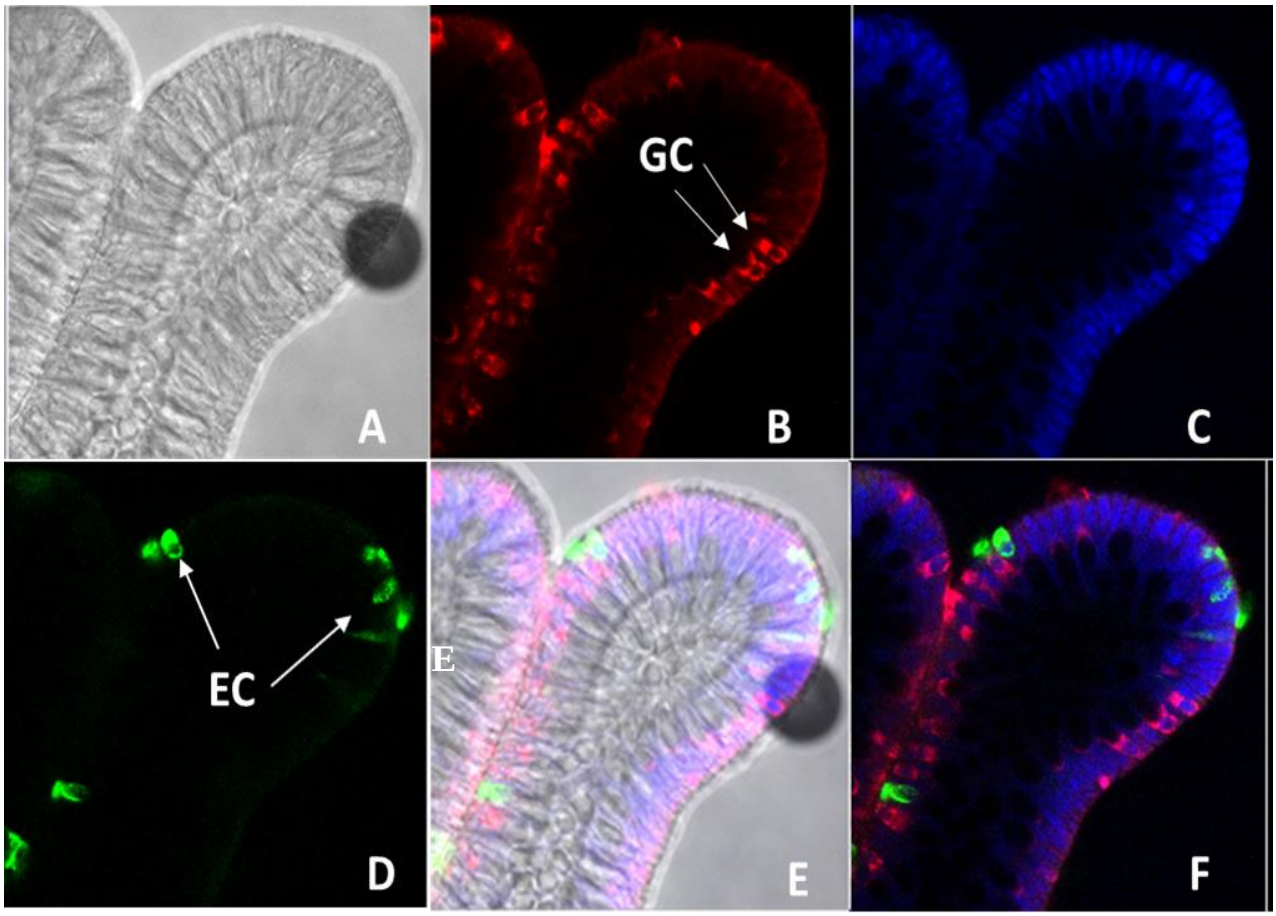


Figure 3.3 Goblet cells, enteroendocrine cells and cell polarity are maintained in culture. *A) DIC image of cultured human colonic crypt fixed and processed for immunocytochemistry, B) MUC2 labelling of goblet cells preserved in the crypt base, C) DAPI labelling of cell nuclei in a cultured human colinic crypt cultured for three days,, D) chromogranin A labelling of enteroendocrine cells in the crypt base below the goblet cells, E) DIC merged image of MUC2 and Chromogranin A labelling, F) merged fluorescent image of MUC2, chromogranin A and DAPI labelling.*

3.2. Wnt-driven maintenance of crypt cell renewal

Crypts microdissected from fixed biopsies underwent immunocytochemistry and were labelled for β-catenin (Figure 3.4A) in order to investigate the Wnt signalling status of the human colonic crypt *in-vivo*. Analysis indicated that the highest levels of nuclear β-catenin were at the base of the crypt, progressively decreasing along the crypt-axis towards the top of the crypt (Figure 3.4B).

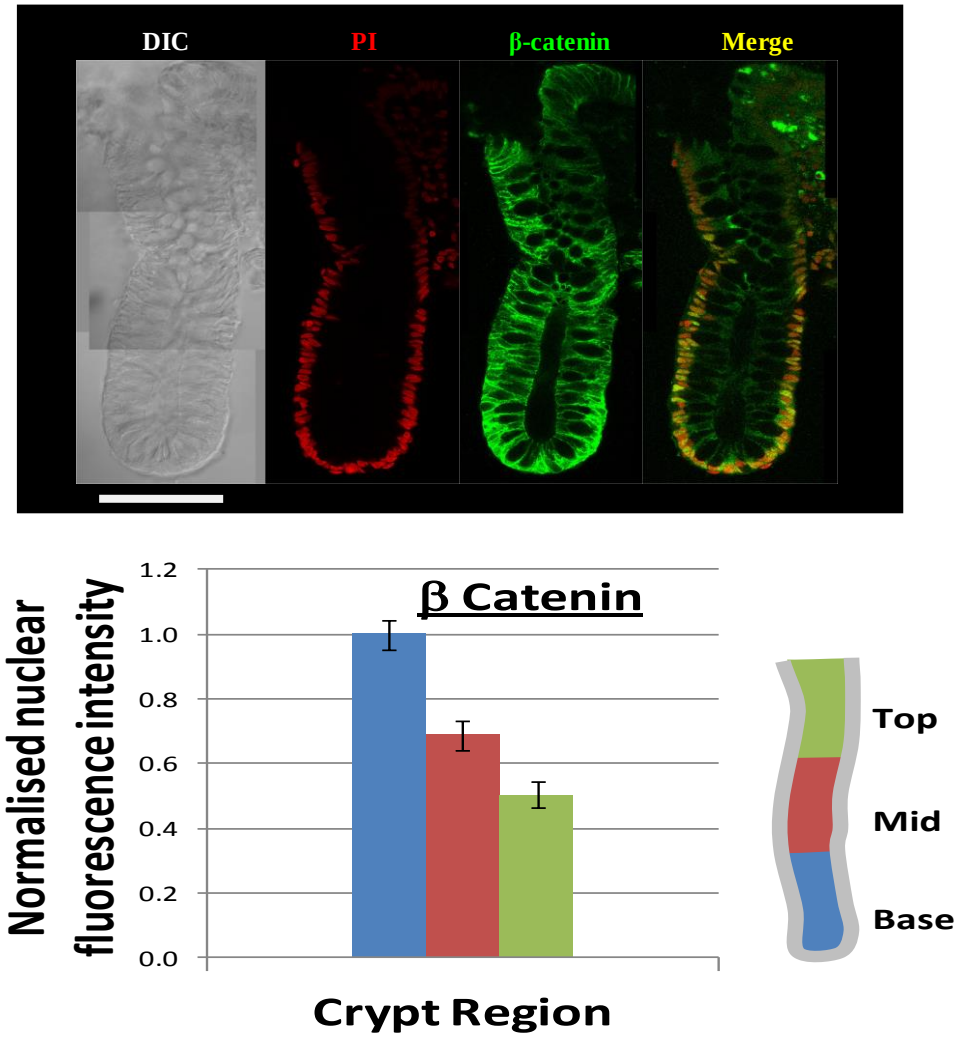


Figure 3.4 Nuclear β-catenin as an indicator of active Wnt signalling*. A) Immunolabelling for β-catenin demonstrates native Wnt signalling status in the colonic crypt. The β-catenin gradient decreases along the crypt-axis. Scale bar represents 100µm. B)Semi-quantitative analysis of nuclear β-catenin along the crypt axis.*

The requirement for Wnt signalling in ensuring the delicate balance between cell proliferation, differentiation, migration and the eventual shedding from the surface of the crypt has been extensively studied *in-vivo* in mouse models. Pinto et al. (2003) published the first definitive finding that Wnt ligands are directly required to drive the proliferation of the intestinal epithelium and the differentiation of its secretory cell lineage. This finding was brought about by means of a transgenic mouse in which the ectopic expression of the Wnt inhibitor Dkk1 was induced. In these mice there was a significant reduction in epithelial proliferation, presence of secretory cell lineages and nuclear β-catenin levels (Pinto et al., 2003).

In order to investigate the requirement for canonical Wnt signals to maintain human colonic crypt cell renewal, we cultured human colonic crypts in media that promoted or inhibited Wnt pathway activation (Figure 3.5). The control media included IGF-1 (50 ng/ml), Noggin (100 ng/ml) and RPSO-1 (500 ng/ml). Canonical Wnt pathway was activated by inclusion of Wnt 3A (100 ng/ml), and inhibited by the presence of DKK-1 (800 ng/ml). Daily images were taken of crypts in each of the experimental groups and length measurements taken. Crypt length was maintained in the Wnt3A group, however, crypts in the remaining groups all decreased in length. The presence of Dkk1, a known inhibitor of Wnt signalling, led to disruption of crypt morphology. Inspection of time-lapse video microscopy revealed that without Wnt pathway activators, crypt cell migration and cell shedding from the crypt surface epithelium was accompanied by migration of the crypt base towards the crypt opening and concomitant shortening of the crypt length. This observation suggested that the rate at which cells were shed from the surface of the crypt exceeded the rate at which stem cells at the crypt-base were able to divide and repopulate the crypts, thus causing the crypts to decrease in length.

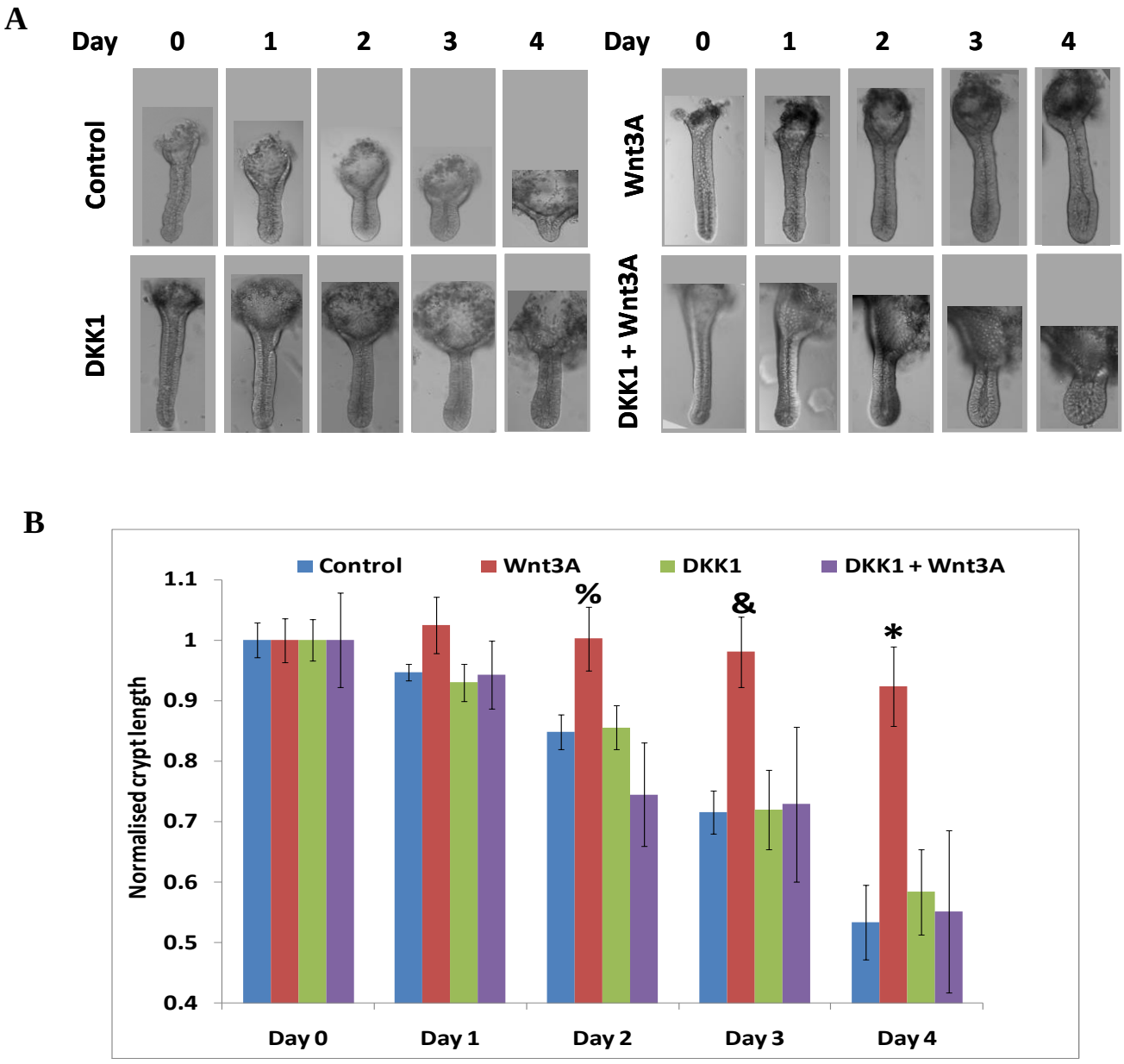
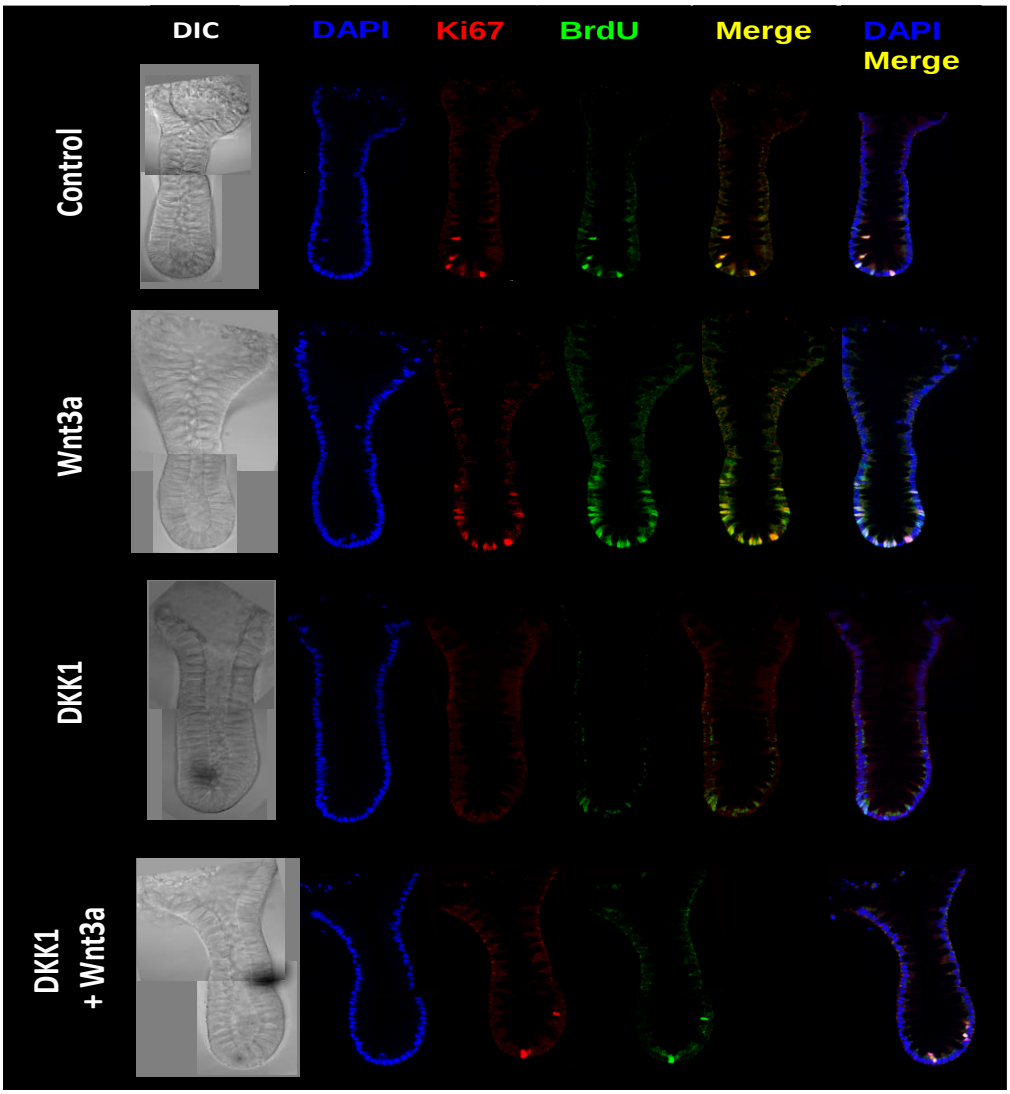


Figure 3.5 Canonical Wnt pathway activation is required to maintain human colonic crypt homeostasis ex vivo. *A) Colonic crypts cultured in either control media* [IGF-1 (50 ng/ml), Noggin (100 ng/ml), RPSO-1 (500 ng/ml)] alone or supplemented with, *Wnt3a and/ or Dkk1. Crypt in each experimental group were imaged daily for 5 days and their lengths measured from these images. B) Crypts in the presence of Wnt3a maintained their length. On days 2, 3 and 4. p= 0.02, p=0.002 and p=0.017 respectively, the difference in length between the Wnt3a treated crypts and those in the other experimental groups was significant, p=<0.05. Significant differences are represented by %, & and *. Number of crypts, n ≥ 4. Number of subjects N ≥ 2.*

In order to investigate the effect of Wnt signalling on cellular proliferation at the crypt base, BrdU incorporation was carried out on cultured crypts. BrdU is a thymidine analogue which can be incorporated into the newly synthesised DNA of cells undergoing replication or in the S phase of the cell cycle, thus acting as a marker of cell proliferation when visualised by immmunolabelling. Isolated crypts were cultured for two days in either control media, Wnt3a, Dkk1 or Wnt3a + Dkk1, after which their media was replaced with fresh media containing BrdU for an incubation period of 24 hours. These crypts were then fixed and processed by immunocytochemistry (Figure 3.6 A) and the number of BrdU positive cells counted manually on the equatorial plane of each crypt. From this data it was possible to calculate the percentage of the total number of cells that were BrdU positive (Figure 3.6 B). Ki67, a second marker of proliferation was used as a double label, as it is a protein which is known to be present throughout the entire active period of the cell cycle. Presence of Wnt3a maintained the proliferative status of the crypt, whilst proliferation was inhibited by Dkk1, even in the presence of Wnt3a.

A


DIC
DAPI
Ki67
BrdU
Merge
DAPI
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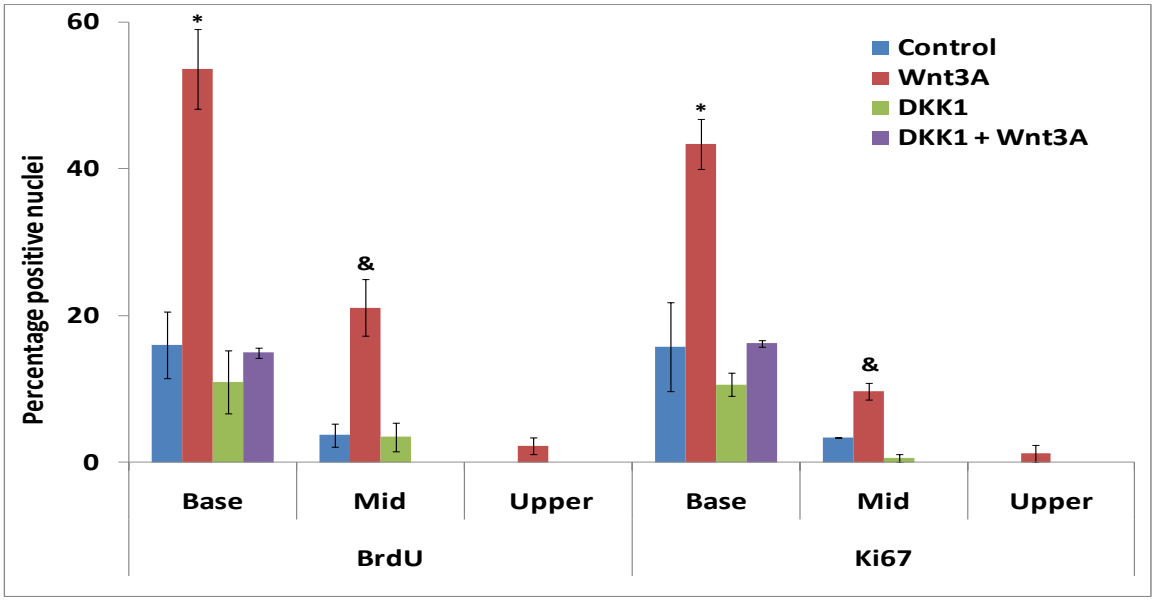
Control
Wnt3a
DKK1
DKK1
+ Wnt3a

B


Percentage positive nuclei
60
40
20
0
*
&
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&
Control
Wnt3A
DKK1
DKK1 + Wnt3A
Base
Mid
Upper
Base
Mid
Upper
BrdU
Ki67

Figure 3.6 Proliferation is maintained by Wnt3a and inhibited by Dkk1. *A) Crypts maintained in culture for 2 days in 4 experimental groups in the presence of Wnt3a ligand or Dkk1. BrdU and Ki67 were added to the media on day 2 to identify proliferating cells. Proliferation was maintained in the base of crypts when Wnt3a was present in the media, such activity was inhibited by Dkk1. Both Ki67 and BrdU labelling illustrate a proliferation gradient along the crypt-axis, with proliferation being primarily seen in the crypt base and diminishing further up the crypt. B) Percentage of BrdU and Ki67 positive nuclei in the presence of Wnt3a and/or Dkk1. Significant differences between: con base vs Wnt base (BrdU) p=0.0 (*), con mid vs Wnt mid (BrdU) p=0.006 (&), con base vs Wnt base (Ki67) p=0.0 (*), con mid vs Wnt mid (Ki67) p=0.009 (&). Number of crypts n ≥ 4, number of subjects N ≥ 2.*

3.3 Real-time imaging of crypt cell renewal in the human colonic epithelium

In order to demonstrate the effect of Wnt3A on crypt cell kinetics, isolated crypts were imaged using real-time time-lapse microscopy 24 hours post-isolation. Crypts were cultured either in control media (IGF, Noggin, R-spondin-1) or Wnt media (IGF, Noggin, Wnt3A and R-Spondin-1). 24 hours post -isolation (day 1), crypts in culture under both sets of conditions were transferred to the time-lapse microscope where they remained for the next 24 hours. During this period a x200 magnification DIC image was taken of each crypt every 5 minutes. From the resulting movies, it was then possible to count the number of mitoses occurring per crypt -base in order to calculate the average number of mitoses occurring per hour. These results were then plotted (Figure 3.7) and a direct comparison made between the two groups. There is a significant difference in the number of mitoses occurring between the groups, with a significantly greater number of mitoses observed in the crypt bases that had been stimulated with Wnt3a compared to those remaining in the control media.

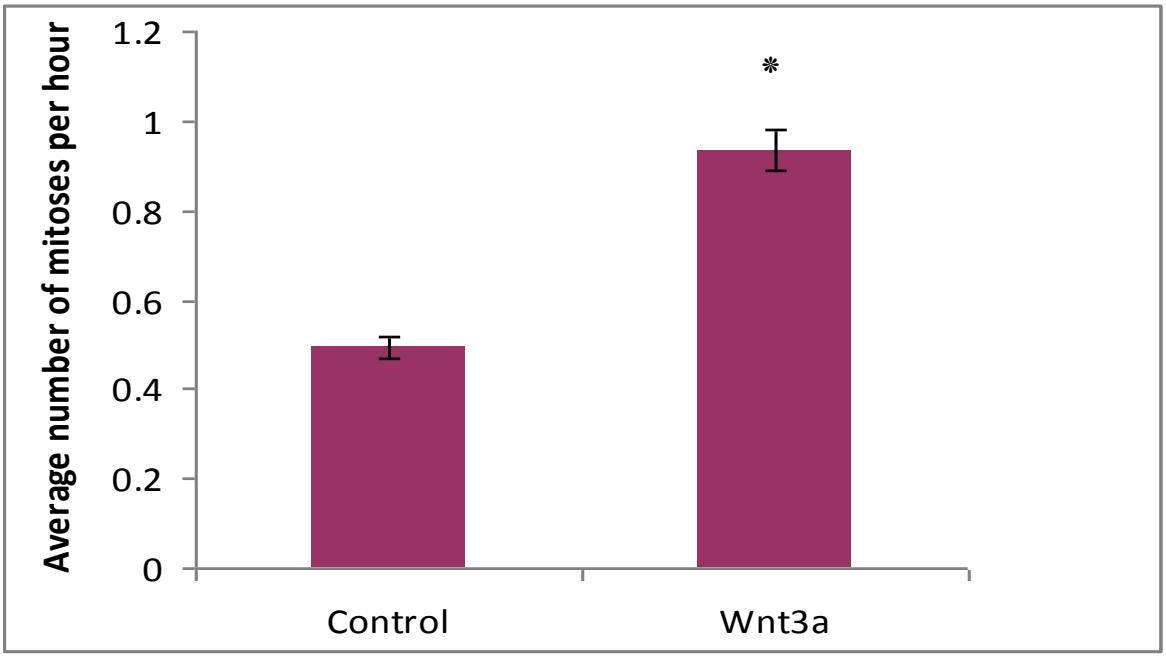

Figure 3.7 Wnt3a promotes crypt cell division. *Number of mitoses is significantly higher in crypts stimulated with Wnt3a, p=0.000. Significant difference represented by *. Number of crypts n=10, number of subjects N ≥ 3.*

Migration of crypt cells throughout the middle and upper regions of the crypt were clearly visualised during time-lapse microscopy and the average rate at which cells migrated could be calculated (Figure 3.8) and compared between groups. Three cells per crypt analysed were tracked over time, marking their starting and finishing points along the crypt axis over a 24 hour period. The distance migrated during this time in both the Wnt3a and control groups was measured in µm and the migration rate was calculated There was no significant difference in the rate of migration between the two groups.

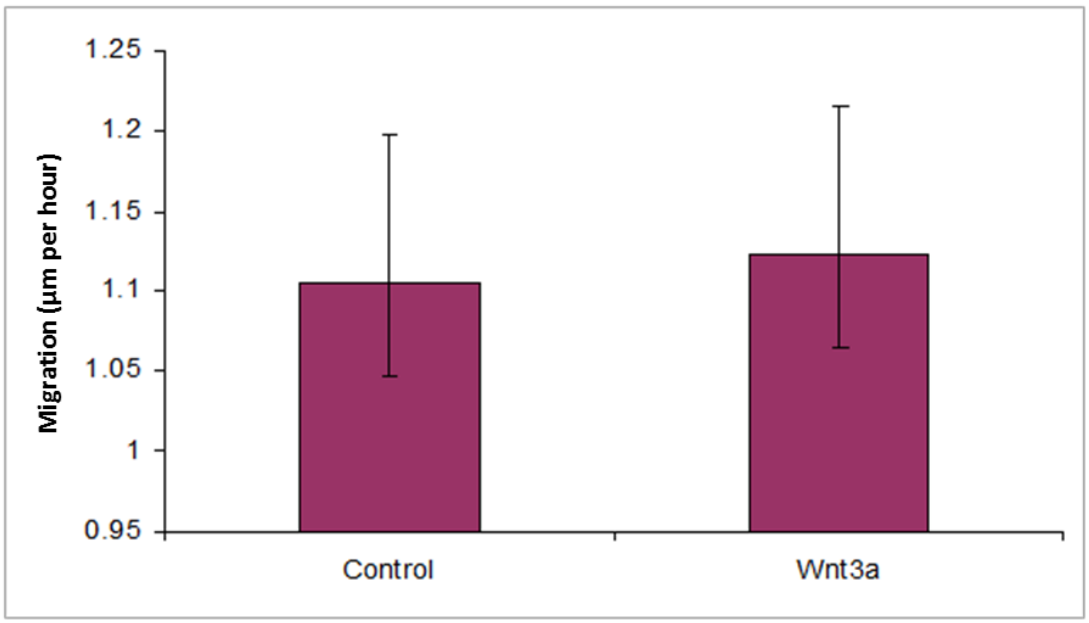

Figure 3.8 Wnt3a does not influence the cellular migration rate along the crypt-axis. *There was no significant difference in the rate of migration (µm/hr) between Wnt3a and control groups, p=0.871. Number of crypts n=5, number of subjects N ≥3.*

Discussion

With a daily turnover of 10 billion cells, the intestinal epithelium is one of the most rapidly self–renewing tissues in the body. This perpetual renewal is under tight homeostatic control, ensuring a good balance between rates of cell proliferation, differentiation, migration and apoptosis. Perturbation of such essential processes leads to conditions such as inflammatory bowel disease and colorectal cancer. It is for this reason that the regulatory mechanisms are of intense interest, and as such, the canonical Wnt signalling pathway has been a major focus.

The processes of tissue renewal in the intestinal epithelium have so far been studied predominantly in the mouse, due to the challenging nature of developing a human *ex vivo* culture system. Recently our laboratory has developed a near-native human colonic crypt culture model that is amenable to cell signal manipulation and bioimaging. It has therefore been possible to investigate the effects of various endogenous and exogenous factors under controlled culture conditions in order to interrogate those pathways that regulate intestinal tissue renewal.

The integrity of our human colonic culture system was demonstrated by the preservation of colonic crypt morphology, proliferation, and migration and by the presence of specialised epithelial cell lines such as goblet cells and enteroendocrine cells. DIC images of crypts throughout their time in culture demonstrated that they maintained their gross morphology and polarity over time when compared to the microdissected native crypts (Figure 3.1 and 3.2).

The main aim of this study was to investigate the status and influence of canonical Wnt signalling on crypt cell kinetics within this near-native human colonic crypt culture model, therefore gaining a greater insight into the way in which epithelial tissue renewal is controlled and maintained in the physiologically normal human colon.

The Wnt signalling status of native, microdissected human colonic crypts was investigated by means of β-catenin immunolabelling in the basal and lateral membranes of the crypt as well as in the crypt cell nuclei, a positive indicator of active Wnt signalling. This nuclear labelling predominated at the base of the crypt, decreasing along the crypt axis, until at the surface the levels were significantly less (Figure 3.4). This finding demonstrates that canonical Wnt signals predominate at the base of human colonic crypts. We know that the base of the crypt is the location of the stem/progenitor cell population of the colonic epithelium, thus the active Wnt signalling at this location suggests that the stem cells are stimulated to divide and repopulate the colonic crypt with their resulting progeny (Potten et al., 1997; Bjerknes et al., 2006; Barker et al., 2008).

By incorporation of BrdU and double labelling by Ki67, we were able to translate the finding of native crypt basal cell β-catenin accumulation, into the live *ex vivo* culture model. BrdU and Ki67 proliferation markers allowed us to visualise and quantify the percentage of cells actively partaking in the cell cycle (Figure 3.7A and 3.7B). Again, as in the native tissue, the greatest levels of proliferation were seen at the crypt base. Culturing these crypts with the exogenous Wnt3a ligand (+ R-Spondin 1) compared to

crypts cultured with the Wnt inhibitor Dkk1 demonstrated the requirement for Wnt3a in maintaining the proliferative status of the crypt and that Wnt3a is unable to rescue the effects of Dkk1 to restore proliferative activity in the cultured human colonic crypt.

This requirement for Wnt signalling was first established by Pinto at al. (2003) in transgenic mice ectopically expressing the secreted Wnt inhibitor Dkk1. In this study, the presence of Dkk1 correlated with a decrease in epithelial proliferation, crypt loss and the absence of nuclear β-catenin (Pinto et al., 2003). Kuhnert et al. (2004) later observed inhibition in proliferation of the murine small and large intestine, along with loss of crypts and villi within 7 days. Each finding illustrates the reliance on the Wnt signalling pathway for homeostatically controlled maintenance of the intestinal architecture and appropriate levels of function.

We further demonstrated the disruption in intestinal crypt homeostasis brought about by Wnt signal inhibition by culturing crypts in Wnt3a (+ R-Spondin 1) and/or Dkk1 and monitoring their length daily (Figure 3.6). Crypt length was maintained in the Wnt3a group, a sign that homeostasis was well regulated. However in the presence of Dkk1, crypts rapidly decreased in length over 5 days, an effect that was not rescued by Wnt3a.

Monitoring crypts in culture by real-time timelapse microscopy for 24 hours allowed quantification of the mitotic and migration rates of the crypt cells. Crypts were cultured either in the presence of wnt3a and R-Spondin 1 or in a control media. This set up allowed a direct comparison to be made between the two conditions in order

to establish the influence of Wnt signalling on cell renewal in the intestinal epithelium. A significantly higher number of mitoses were observed per hour in Wnt3a cultured crypts compared to control crypts (Figure 3.6). In contrast the rate of migration between the same groups demonstrated no significant difference (Figure 3.8). This suggests that although proliferation is crucial in the replenishment of the epithelium and long-term maintenance of crypt architecture, crypt cell proliferation and migration function independently of each other and that factors other than Wnt signalling may govern the migration of crypt cells. It has previously been hypothesised that mitotic pressure in small intestinal crypts accounts for crypt cell migration through doubling of cell number and thus volume (Heath JP, 1996). Although inhibition of cell division inhibited cell migration relative to the crypt base, it did not inhibit migration along the villus (Kaur and Potten, 1986). The present study demonstrates that there is an inherent migration rate along the human colonic crypt-axis that is independent of crypt cell proliferation, but which, in the absence of crypt cell replenishment by proliferation, leads to depletion of the crypt cell population and shortening of the colonic crypt-axis.

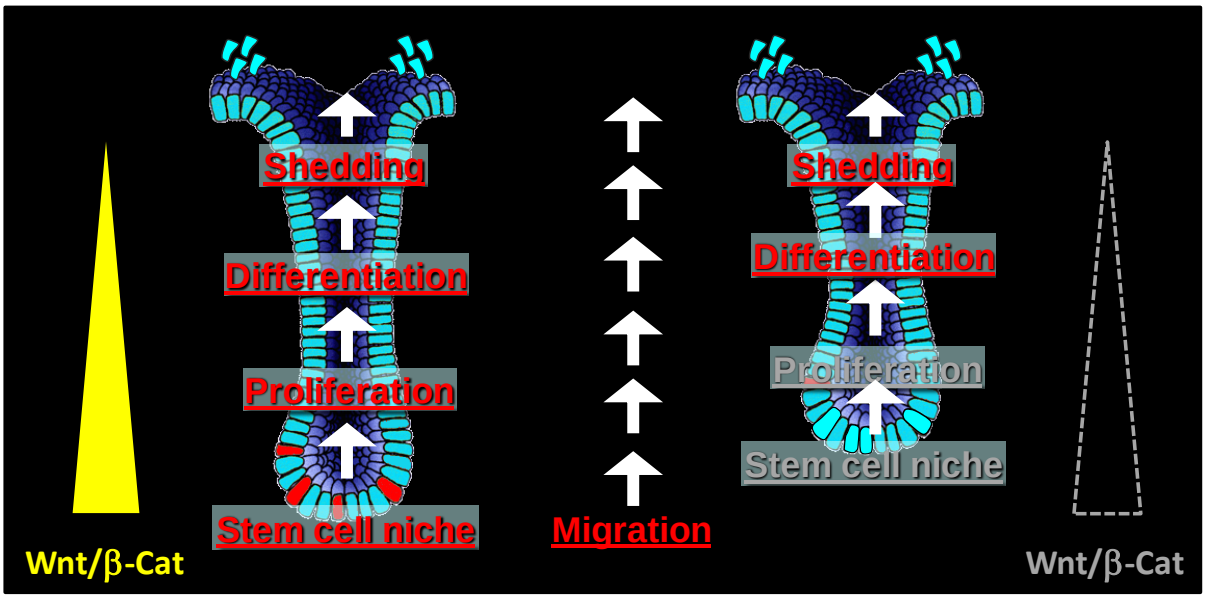

Figure 3.10: Summary – Canonical Wnt signals maintain human colonic crypt cell renewal ex vivo. *In the absence of Wnt signals, human colonic crypt cell proliferation ceases, but crypt cell migration and shedding continues unabated. Consequently, there is a reduction in the crypt cell population number and the length of the crypt–axis is reduced. Wnt signals are also required for intestinal stem cell marker expression (Wharton, Parris et al., unpublished).*

The mechanism and regulation of colonic crypt cell migration is not known. Observed under timelapse microscopy, the cell population in the upper crypt region appears to move as an intact sheet. It is conceivable that cell shedding events at the surface epithelium 'pull' neighbouring cells up the crypt-axis via cell to cell junctions in order to fill the gap created by the voided cell. It would be of interest to study the relationship between cell shedding rates and crypt cell migration, in the absence of crypt cell proliferation, in order to implicate cell shedding as a driver of crypt cell migration.

The need for cell proliferation to replenish the crypt cell population emphasises the requirement for mitogenic canonical Wnt signalling in the intestinal epithelium.

Exogenous Wnt ligand was required for the maintenance of epithelial renewal. Inhibition of Wnt signalling by the secreted antagonist Dkk1 leads loss of the crypt cell population. Conversely, other laboratories have shown that excessive Wnt signals in combination with other intestinotrophic growth factors such as EGF and PGE2 give rise to budding structures (Sato et al., 2011) or expand into cyst-like spheroids (Jung et al., 2011) , each composed predominantly of immature stem/progenitor cells that can be induced to differentiate by withdrawal of Wnt stimulation. With the aid of spheroids, organoids, and the near-native colonic crypt culture system, the effects of Wnt signalling and other mediators of intestinal epithelial physiology can be investigated, building on the broad knowledge already gained from murine intestinal culture models.

Future Work

With increased age comes a greatly increased incidence in colorectal cancer (DePinho, 2000) and this correlation is seen both in humans and laboratory animals (Anisimov et al., 2009). The predisposition can be due to an accumulation of cells in the late stages of carcinogenesis or due to altered internal homeostasis (Anisimov et al., 2009). The intestinal epithelium is one of the most dynamic tissues in the body. It is likely therefore that the balance of homeostasis is under constant refinement in response to the physiological demands placed on the system. It is conceivable that key regulatory pathways may be perturbed by mutations or epigenetic changes that may signify the early stages of tumourigenesis.

Although the risk of cancer increases exponentially with age, there is relatively little understanding of the status of, and cellular signals for, tissue renewal in the normal aging human colonic epithelium. To be able to characterise the functional status of the Wnt signalling pathway and stem cell driven tissue renewal in this aging tissue could prove useful in future prevention of such age-related diseases.

An increase in proliferative activity coupled with a decreased level of apoptosis within the colonic mucosa of rats as they aged was demonstrated by Xiao et al. (2001). Disruption of the proliferative activity of the well established stem cell population at the crypt base would prove detrimental in the maintenance of local tissue homeostasis. Martin et al. (1998) found that there was indeed altered histology and stem cell function in the aged small intestine of mice, including an impeded ability to renew following injury such as exposure to irradiation, resulting in increased apoptosis around the stem cell region. Using the Klotho model of accelerated aging, Liu et al. (2007) found a decreased number of stem cells and an increase in progenitor cell senescence within several murine tissues. Liu et al. (2007) also demonstrated in culture a Wnt-Klotho interaction, leading subsequently to suppressed Wnt activity. In these experiments, cellular senescence was accelerated by continuous Wnt exposure (Liu et al., 2007).

Preliminary data from our laboratory indicates a disturbed Wnt signalling gradient along the crypt axis as well as expansion of the stem cell niche with age. The risk of CRC may be exacerbated by disruption of the tissue renewal hierarchy found in the colonic epithelium. Therefore, it may prove informative to look further at how this compromised Wnt signalling gradient influences the rates of tissue renewal and the risk of disease with aging, such as cancer.

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