CHAPTER 1: INTRODUCTION

Wnt signaling in the intestinal epithelium:

from endoderm to cancer

Wnt signaling in the intestinal epithelium: from endoderm to cancer

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Abstract

The Wnt pathway controls cell fate during embryonic development. It also persists as a key regulator of homeostasis in adult self-renewing tissues. In these tissues, mutational deregulation of the Wnt cascade is closely associated with malignant transformation. The intestinal epithelium represents the best-understood example for the closely linked roles of Wnt signaling in homeostatic self-renewal and malignant transformation. In this review, we outline current understanding of the physiological role of Wnt signaling in intestinal biology. From this perspective, we then describe how mutational subversion of the Wnt cascade leads to colorectal cancer (CRC).
Introduction

Development and homeostasis in all multi-cellular organisms depend on a complex interplay between processes involved in cell proliferation, migration, differentiation, adhesion, and death. This diverse array of cellular responses is in large part coordinated by a relatively small number of intercellular signals, examples of which include the BMP, TGF, Notch, Hh, and Wnt pathways. One of the major developments in recent years has been the realization that the signaling pathways triggered by these factors are very often deregulated in pathological conditions (1-6). This notion is particularly well illustrated by the role of the Wnt pathway in the intestinal epithelium. The relevance of Wnt signaling to intestinal biology was established, unknowingly at the time, over ten years ago when the tumor suppressor gene Adenomatous polyposis coli (APC) was found mutated in a large number of hereditary and sporadic cases of CRC (7-9). Subsequently, combined work from several laboratories led to the finding that inactivation of APC in CRC cells results in constitutively active Wnt signaling (10-12). Since these early findings a much richer picture has emerged. It is now recognized that Wnt signaling not only drives tumorigenesis but is also required at different stages of gut development, as well as during adult epithelial homeostasis. Our approach in this review will be to dissect the different functions attributed to Wnt signaling at these various time points. First, we shall begin by introducing some of the components of the pathway most relevant to our discussion.

A short summary of the Wnt pathway

Wnts and their downstream effectors were originally discovered in *Drosophila* and subsequently shown to be conserved in all metazoans (13). Genetic and biochemical data taken from these models has, to date, identified over 50 proteins directly involved in transducing Wnt signals (see Wnt homepage at www.stanford.edu/~rnusse/wntwindow.html). How these proteins interact with one another to stimulate various biological responses has been an area of intense investigation.

Wnt genes, of which there are 19 in man and mice, encode for cysteine-rich glycoproteins. Production of biologically active Wnts depends on palmitoylation of a conserved cysteine residue (14). This process may be mediated by Porcupine/MOM1, however direct proof for this has not yet been provided (15-17). Once released into the extracellular milieu, Wnts interact with secreted proteins such as SFRPs and WIF (18). In general, these factors are thought to function as inhibitors by sequestering Wnts and preventing their interaction with membrane-bound receptors. Other interaction partners include membrane-anchored heparan sulfate proteoglycans (HSPGs). In *Drosophila*, the HSPG Dally acts as a positive regulator of Wnt activity, but its precise biochemical function is unknown (19).

Wnts activate responding cells by interacting with the seven-span transmembrane protein Frizzled (Fz) and the single-span transmembrane protein LRP (20-23). Two functional complexes involving these proteins have been described. Wnts may simultaneously
bind to Fz and LRP. This represents the initial step in the so-called canonical pathway, which leads to the formation of nuclear Tcf/β-catenin complexes. Alternatively, when LRP is not expressed or downmodulated through secreted factors such as Dickkopfs (24), Wnts may nonetheless form a complex with Fz, triggering Tcf/β-catenin-independent cellular responses such as increased calcium flux, repression of Tcf-mediated transcription and cytoskeletal rearrangements. Collectively, these responses are often referred to as non-canonical signaling (25). As of yet, this aspect of Wnt signaling has not been analyzed in the gut. For this reason, non-canonical Wnt signaling will not be covered in this review.

The key component of the Wnt canonical cascade is the cytoplasmic protein β-catenin. In the absence of Wnts, the scaffolding proteins APC and Axin/Axin2 sequester β-catenin allowing casein kinase I (CKI) to phosphorylate the N-terminus of β-catenin at serine S45, a residue often mutated in cancers (26;27). Subsequently, glycogen synthase kinase 3 beta (GSK3β) is recruited to phosphorylate additional serine and threonine residues N-terminal to S45 (28). Phosphorylated β-catenin is then recognized by the F-box-containing protein β-TrCP, which mediates ubiquitination and proteosomal degradation of β-catenin (29-31). Together, these proteins make up the so-called β-catenin destruction complex. As we shall see later, this complex plays a central role in the (de)regulation of intestinal homeostasis.

Under physiological conditions, continued destruction of β-catenin is interrupted following Wnt binding to Fz/LRP. How the destruction complex senses Wnts at the cell surface is not fully understood. It has been assumed that the adapter protein Dsh through its association with Fz and the GSK3β-binding protein, Frat, may participate in this process (32-34). Note however that recent genetic evidence excludes an essential requirement for Frat in Wnt signaling, since mice with deletions in all three Frat family members develop entirely
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normally (35). In parallel, Wnts induce phosphorylation of the cytoplasmic tail of LRP, which allows docking of Axin to LRP (36). Recruitment of Axin to the membrane is thought to disrupt the destruction complex thereby releasing β-catenin. Lastly, it has been suggested that stabilization of β-catenin may be promoted by the protein phosphatase PP2A, which appears to dephosphorylate GSK3β substrates including β-catenin (37).

Once released from the destruction complex, β-catenin translocates to the nucleus where it associates with the Tcf family of transcription factors (Tcf1, Lef, Tcf3 and Tcf4) (38). Tcfs function by targeting β-catenin to specific DNA elements found in promoters and enhancers of target genes (39;40). In turn, β-catenin recruits a number of nuclear factors responsible for transactivating Tcf target genes. Two of these factors include the histone acetylase CBP/p300 and the SWI/SNF component BRG1 (41-43). Activation of target genes also depends on the nuclear proteins Legless and Pygopus (44-46). It has been proposed that Legless and Pygopus are involved in directly activating transcription, possibly by recruiting chromatin remodelling factors. Legless and Pygopus may also function by transporting β-catenin to the nucleus (47). Finally it is worth noting that in the absence of nuclear β-catenin or when nuclear β-catenin is sequestered by factors such as ICAT and Chibby (48;49), Tcfs associate with general transcriptional repressors like Groucho (50;51). The latter silence target genes, in part, by recruiting histone deacetylases (HDACs), which render chromatin structure inaccessible to the basal transcriptional machinery. For an overview of the canonical Wnt pathway see Figure 1.

Wnt signaling in and the origin of intestinal epithelial cells

The intestinal epithelium originates from embryonic endoderm, which in turn stems from pluripotent epiblast cells at the onset of gastrulation (E6.0 in mice). During this stage, epiblast cells committed to form definitive endoderm ingress through the primitive streak displacing visceral endoderm. The first endodermal cells to travel through the primitive streak populate the anterior end of the embryo, whereas endoderm leaving at later stages colonizes more posterior regions. From E7.5-E9.5, the endodermal lining covering the mesoderm and ectoderm undergoes a series of invaginations initiated at the anterior and posterior ends of the embryo resulting in the formation of a proper gut tube (Figure 2). At this stage, the primitive gut is composed of a uniform layer of cuboidal endodermal cells surrounded by splanchnic mesoderm. The intestine along with the other organs derived from endoderm only become morphologically evident during a patterning phase (E9.5-14.5) in which the primordial gut is subdivided and reshaped along the anterior-posterior axis (Figure 2). For a thorough treatment of gut development see (52;53).

The earliest role attributed to Wnt signaling during gut development was initially uncovered in ascidian embryos, where β-catenin was found to be essential for endoderm formation (54). Through gene targeting experiments, Kemler and colleagues showed that this function of β-catenin is evolutionarily conserved in mice (55). Ablation of β-catenin specifically in the node, notochord and anterior primitive streak abrogated definitive endoderm formation. Moreover, analysis of chimeric embryos showed that β-catenin-mutant
cells of the endodermal layer were unable to form endoderm but rather differentiated into precardiac mesoderm.

How β-catenin promotes definitive endoderm formation is unclear. Recent data has suggested that in endodermal cells, β-catenin may not necessarily act through Tcf factors. Indeed, Sinner et al. have proposed that in frogs, β-catenin drives the expression of endoderm specific target genes by physically associating with Sox17, an HMG box transcription factor, related to Tcfs (56). Given that in zebrafish and mice Sox17 also plays a role in the formation of definitive endoderm (57;58), it will be interesting to test whether the Sox17/β-catenin complex may represent a generalized mechanism for promoting endoderm specification. Another unanswered question raised by these findings regards the identity of the Wnt(s) stimulating β-catenin in the endoderm. In mice, Wnt3 is a possible candidate, since in Wnt3-mutant embryos, the epiblast remains undifferentiated while the primitive streak does not form. Moreover, the expression of both mesodermal and definitive endodermal markers is abolished (59).

Figure 2. Time-line of intestinal development in the mouse. Definitive endodermal cells are specified at E6.0 during gastrulation. The first panel from the left shows the bottom view of an E8.5 embryo, along with a schematic representation of the primitive gut. At E8.5 endodermal tube formation is initiated by folding (depicted by arrows) of the endodermal lining at the anterior and posterior ends creating anterior and caudal intestinal portals (AIP and CIP). The endodermal lining is stained with a probe recognizing Foxa1. At later stages (E9.5-E14.5) the primitive gut tube is patterned along the anterior-posterior axis. The expression of specific intestinal markers first appears in the hindgut at E9.0 (168;169). The second panel from the left shows a whole mount preparation of the entire gastro-intestinal tract (E12.5) stained for the intestinal marker Villin. Villus formation and cytodifferentiation (formation of enterocytes, goblet cells, enteroendocrine cells and Paneth cells) is initiated at E14.5. In the third panel from the left, sections from the small intestine were stained for the proliferation marker Ki67. Mutations associated with the Wnt pathway affect gut development at various stages (see text). Figure adapted from (53).
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We have recently shown that Wnt signaling is required for gut tube formation (60). During this stage (E8.5), in situ hybridisation analysis revealed overlapping expression of Tcf4 and Tcf1 in the hindgut. Simultaneous disruption of both genes led to severe defects in the formation of the hindgut and associated loss of expression of endodermal markers. This phenotype implies the existence of a Wnt source at the posterior end of the embryo, which would promote morphogenesis of the hindgut. A similar mechanism is utilized to drive posterior paraxial mesoderm and somite formation. In this case, Wnt3a expression in the presomitic mesoderm of the tailbud activates Lef and Tcf1 (61). Anterior tube formation may also depend on the activity of Wnt signaling components. Analysis of APC hypomorphic mutant mice (APC<sup>neoR</sup>) has shown that expression of APC in the endoderm is required for the involuting movements, which generate the foregut pocket (see AIP in Figure 2) (62). The foregut defects in APC hypomorphs may result from the increased β-catenin/Tcf transcriptional activity in endodermal cells or may be ascribed to an alternative role for APC in cell migration.

Our analysis of Tcf4/Tcf1 mutant embryos at later stages also revealed malformations of the gastrointestinal tract consistent with both factors playing a role in patterning the gut (60). As could be expected from the early defects in hindgut formation, the intestine of Tcf4<sup>−/−</sup>/Tcf1<sup>−/−</sup> embryos is severely truncated. However, closer inspection uncovered anterior transformations at the stomach-duodenal junction. Expression analysis using specific markers of stomach and intestine revealed duplications of the stomach, suggesting that Tcf4 and Tcf1 promote an “intestinal” fate within the primitive gut and in their absence more anterior regions of the gut are expanded. Evidence supporting this interpretation was recently provided by Hogan and co-workers (63) who showed that when a constitutively active form of β-catenin is misexpressed in the lung endoderm, these cells turn on genes normally restricted to the intestine, implying once again that Wnt signals instruct endodermal cells to become intestine as opposed to other endodermal lineages.

Wnt signaling and adult intestinal homeostasis

Once the basic structure of the intestinal tract is laid out, differentiation along the radial axis may take place (see Figure 2 and 3). During this process the epithelium of the small intestine is remodelled to form characteristic finger-like projections (villi) and deep invaginations termed crypts. Similar events take place in the colon, where crypts form but where a flat surface epithelium exists instead of villi. These events coincide with the compartmentalization and cyto-differentiation of the epithelium. The intervillus regions of the fetal intestine, which are replaced by crypts in the first weeks after birth, are lined with highly proliferative progenitor cells. These transit-amplifying cells give rise to two differentiated cell lineages (ie. the absorptive enterocytes and secretory cells). The secretory lineage can be further subdivided into mucus-secreting goblet cells, hormone-secreting enteroendocrine cells and bactericidal Paneth cells. Maturation of progenitor cells coincides with upward migration. Upon reaching the tips of the villi or the surface epithelium of the colon, the differentiated cells undergo apoptosis and are shed into the lumen. One exception

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to this rule is the Paneth cell, which is generated from a progenitor migrating downwards toward the crypt base. The self-renewing capacity of the intestine depends on the existence of stem cells (64). Classical labelling experiments have shown that in the small intestine stem cells reside just above the Paneth cell compartment, while in the colon they occupy the first cell position at the crypt bottom.

Figure 3. Adult intestinal homeostasis.
Panel A and B. Schematic representation and section of the crypt-villus unit in the mature small intestine. Proliferative cells reside in the crypts, while differentiated cells occupy the villus. Crypt progenitors migrate up (red arrow) the crypt-villus axis before shedding into the lumen. The process of epithelial renewal takes 3-6 days and is ensured by a small number of asymmetrically dividing stem cells at the bottom of the crypts. Wnt signaling in the adult intestine promotes proliferation of progenitor or transit-amplifying (TA) cells, as well as, commitment towards secretory lineages. Wnt signaling may also drive terminal differentiation of certain secretory lineages (see text). Although it is commonly believed that Wnt signaling may promote proliferation and/or differentiation of intestinal stem cells, there is no evidence, which formally proves this (see arrows with question marks). In panel A, black arrowheads indicate Ki67 positive transit-amplifying cells, while white arrowheads indicate the Paneth cell compartment.

There are now several lines of in vivo evidence, which show that normal proliferation of the transit-amplifying cells is entirely dependent on continual stimulation of the Wnt pathway. Firstly, removal of Tcf4, β-catenin or overexpression of the Wnt inhibitor Dkk-1 results in a severe loss of proliferative epithelial cells in both the fetal and adult intestine (65-68). Cell cycle arrest is also observed in CRC cell lines in which β-catenin/Tcf activity is blocked either through expression of dominant negative Tcf4 or knockdown of β-catenin (69;70). Consistent with these results, mutations in the negative regulator of Wnt signaling APC, or overexpression of oncogenic forms of β-catenin result in hyperproliferation of the epithelium (9;71-73). Lastly, progenitors located at the bottom of the crypts accumulate nuclear β-catenin implying that these cells respond to Wnt stimulation (69). Although these studies confirm the strong link between Wnt signalling and maintenance of transit-amplifying cells, it should be noted that virtually no evidence exists to draw a similar link between Wnt signals and stem cells (Figure 3). Part of the difficulty in tackling this issue is related to our lack of reliable markers of intestinal stem cells. Besides proliferation, we may also consider the accumulating evidence implying an additional function for Wnt signaling in driving the...
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differentiation of secretory lineages. Indeed, blocking active Wnt signaling in vivo results in a reduction or absence of goblet, enteroendocrine and Paneth cells, while enterocytes appear spared (65;66;68).

Supported by these findings, a model can be proposed whereby transit-amplifying cells responding to a source of Wnts at the crypt bottom proliferate and concomitantly commit themselves to the secretory lineage. As these progenitors move up the crypt and further away from the Wnt source, Tcf/β-catenin activity is turned off, thus favouring cell-cycle arrest and terminal differentiation. This simplistic view overlooks a number of issues. In our discussion, below we shall highlight four major questions: What is the genetic program regulated by Tcf/β-catenin in crypt progenitors? Where and what is the Wnt source? How does Wnt signaling regulate secretory cell lineage commitment? And finally, how is Wnt signaling turned off?

Tcf/β-catenin target genes

Most studies aimed at identifying Tcf/β-catenin target genes (for simplicity the term target gene here refers to either direct or indirect Wnt-responsive genes) in intestinal cells have made use of systems in which β-catenin is constitutively activated such as in CRC cell lines (see Table I for a selected list of Tcf target genes). Consequently as we shall see later, the majority of Tcf/β-catenin target genes have been associated with various processes important for tumorigenesis (ie cellular proliferation, survival and motility). Given that many of these genes are also expressed in normal crypt progenitor cells (69), efforts are now being undertaken, through classical loss or gain of function experiments in mice, to test their function during intestinal development and homeostasis. So far, however, only a limited number Tcf/β-catenin targets have been tested in vivo.

The proliferative effects of Wnt signaling on crypt progenitors have, for some time now, been linked to cell cycle regulators such as c-Myc and cyclin D1 (74-76). Both factors are overexpressed in colorectal tumors and blocking expression of either gene inhibits proliferation in CRC cell lines (69;77;78). However, cyclin D1 null mice do not appear to exhibit any abnormalities in the intestine, other than a modest reduction in the propensity to develop polyps when crossed with the APC<sup>min</sup> mice (79). This observation suggests that other cyclin Ds may be more relevant downstream β-catenin/Tcf target genes. Along these lines, our own observations have shown that cyclin D2 is an early downstream Tcf4 target in the fetal gut (Gregorieff and Clevers, unpublished data). Moreover, microarray studies have shown that cyclin D1 levels do not appear to be affected when conditionally deleting APC (73). C-Myc has been confirmed as a Tcf/β-catenin target in vivo (68;80), although its precise function in normal epithelial cells remains to be clarified. In the bone marrow, recent evidence suggests that c-Myc promotes the release of hemopoietic stem cells from the stem cell niche by regulating the expression levels of adhesion molecules (81). In the skin, ectopic expression of c-Myc diverts epidermal stem cells to a sebaceous gland fate at the expense of hair follicles (82). Whether c-Myc performs similar functions in the intestine will need to be examined by a conditional knock-out approach.
Another Tcf/β-catenin target gene, which has been implicated in promoting proliferation is Id2 (69;83). The Id proteins represent a family of naturally occurring inhibitors of basic helix-loop-helix transcription (bHLH) factors, and function in many circumstances to prevent differentiation (84). In particular, Id2 is highly abundant in several cancer types and when forcibly expressed in colon cancer cell lines Id2 has been shown to increase anchorage-independent survival (83). Recent in vivo evidence, on the contrary, suggests that Id2 may have a completely different role in crypt progenitor cells (85). In the Id2 knockout intestines, differentiation of endoderm is impaired during the late fetal stages (E18.5). Consequently the villi in several areas appear replaced by multilayered, undifferentiated endoderm. These areas of pseudostratified epithelium later develop into dysplastic and metaplastic tumours exhibiting high levels of nuclear β-catenin. Interestingly, these lesions also show a loss of Paneth cells and enteroendocrine cells and increased numbers of Goblet cells.

Expression profiling has also identified genes implicated in many other processes besides the control of proliferation and/or differentiation. The tyrosine kinase receptors EphB2 and EphB3 and their ligand ephrin B1 illustrate this point (86). Consistent with their well-known roles in cell sorting in various tissues, these receptor/ligands pairs are expressed in an inverse gradient along the crypt-villus axis, with EphB2 and -B3 high in crypt cells and their ligand ephrin-B1 predominating in the villi. This expression pattern is tightly regulated both in vitro and in vivo by Tcf/β-catenin. In vivo confirmation of the importance of these molecules came from the analysis of EphB2^+/−B3^+/− KO. In these mice, proliferative and differentiated cell populations intermingle. Furthermore in EphB3^−/− mice, Paneth cells no longer home to the crypt bottom, but rather scatter along crypts and villi. Thus, a Wnt signalling gradient controls cell positioning along the crypt-villus axis through regulation of EphB2 and EphB3 gene expression.

The functional characterization of Tcf/β-catenin target genes will continue to be a major focus of interest for the coming years. We shall return to this issue in the context of colon carcinogenesis.

The Wnt source

The exact location or identity of the Wnts that drive proliferation is unclear. Nevertheless it is believed that mesenchymal cells or, more specifically, intestinal subepithelial myofibroblasts (ISEMFs), immediately adjacent to crypt epithelial cells, are a source of Wnts (87;88). This notion is based in part on classic co-culture experiments, which have shown that these cells are able to simulate proliferation of epithelial cells (89). We recently tested this hypothesis by screening all 19 Wnts for expression in the adult intestine (Gregorieff and Clevers manuscript submitted). Through this approach we found several Wnts expressed in crypt epithelial cells, but so far none were detected in ISEMFs. Ablation of the Wnt genes associated with crypt epithelial cells will be required to test their function. Until then, if we are to assume that these Wnts drive proliferation, then the next obvious question is what regulates Wnt expression in the epithelium. Here once again we may have to turn to
ISEMFs. These cells are known to produce paracrine growth factors (90), which conceivably could activate Wnt gene expression in the epithelium. This idea however remains speculative.

On a related issue, genetic evidence in mice has identified two transcription factors, FoxL1 and Nkx2.3, involved in regulating growth signals emanating from the mesenchyme (91-93). Deletion of either gene in mesenchymal cells results in increased epithelial proliferation, suggesting that FoxL1 and Nkx2.3 normally play an inhibitory role. Kaestner and colleagues observed upregulation of the HSPGs, Syndecan1 and Perlecan, in FoxL1^-/- mice (94). Although HSPGs have been implicated in stimulating Wnt signals (19;94), it remains to be tested whether these changes are a cause or an effect of the increased proliferation.

**Cell lineage commitment**

The disproportionate reduction in goblet, enteroendocrine and Paneth cell numbers, resulting from the ablation of Wnt signals, suggests a definite role for Wnts in specifying secretory lineages. Recently, some general rules for cell lineage commitment in the intestine have been uncovered. Precursors of all three secretory cell types express the bHLH factor, MATH1. Accordingly, MATH1 deficient mice lack goblet, enteroendocrine and Paneth cells but do produce enterocytes (95). The latter cells derive from progenitors expressing Hes1, based on the fact that Hes1^-/- intestines display increased numbers of secretory cells at the expense of enterocytes (96). Interestingly, Hes1 transcription is activated by Notch signaling in other biological models (97), while Hes1 transcriptionally represses MATH1 expression (96;98). Together these findings suggest a model whereby commitment towards the enterocyte lineage would be favoured in cells with active Notch signaling, turning on Hes1 transcription. Inversely, in the absence of Notch signaling, MATH1 would be upregulated skewing the cells towards secretory lineages (Figure 3B).

Further commitment towards specific cell types depends on yet other transcription factors. For example, the activation of NGN3, BETA2, Pax4 and Pax6 is associated with enteroendocrine (sub-)lineages (99), while differentiation of goblet cells is influenced by KLF4 (100). Moreover, in ELF3^-/- mice differentiation of absorptive and goblet cells is impaired (101).

The connection between Wnt signaling and any of these factors remains an open question. One putative link was suggested by the observation, as we have mentioned earlier, that ablation of the Wnt target gene and bHLH antagonist Id2, results in impaired production of secretory lineages (85). It is plausible that Id2 may mediate these effects by directly antagonizing the activity of certain bHLH transcription factors such as MATH1. Alternatively, Wnt signals may directly activate the expression of genes involved in cell lineage commitment. Although no evidence for this exists so far, expression profiling of mouse models displaying impaired Wnt signaling suggests that the final stages of maturation of secretory lineages may depend on active Wnts signals. In particular, we and others find that
the expression of Paneth cell markers, such as anti-microbial peptides (i.e. cryptdins and defensins), are directly stimulated by \( \beta \)-catenin/Tcf (102;103).

-Counteracting Wnt signaling

There are two non-mutually exclusive mechanisms, which could explain how the stimulatory effects of the Wnt cascade are turned off in the intestine. In one scenario, activation of Wnt signals would gradually and passively dissipate as progenitors migrate up along the crypt-villi axis in sites where canonical Wnts are limiting. On the other hand, a more active mechanism may be utilized, involving “negative” cross-talk between the Wnt pathway and other signaling pathways. As we shall discuss below the TGF\( \beta \) and BMP cascades are associated with growth inhibition in the gut, and thus may represent examples of Wnt-counteracting pathways.

TGF\( \beta \) signaling components are localized in differentiated epithelial cells, where they have well-documented growth suppressive effects (104). Furthermore, in both man and mouse, benign adenomas acquire invasive properties following the acquisition of inactivating mutations in the TGF\( \beta \)-RII receptor or the intracellular signaling components Smad2 and 4 (105-107). Mice with germline mutations in Smad3 and the latent TGF\( \beta \) binding protein 4 (LTBP-4) also develop colorectal cancer (108;109). Several groups have described mechanisms by which TGF\( \beta \) signals could antagonize Wnt signaling in the intestine. One possible route may involve the alternative TGF\( \beta \) effector and MAPKKK, TAK1. In *Caenorhabditis elegans* and mammalian cells activation of TAK1 stimulates the activity of the MAPK, NLK, which in turn, downregulates Tcf (110;111). Alternatively, Sasaki et al. have shown that TGF\( \beta \) stimulation inhibits Tcf\(_4/\beta\)-catenin transactivation of c-Myc via the ability of Smad3 to physically interact with \( \beta \)-catenin and thereby decouple Tcf\(_4/\beta\)-catenin complexes (112).

Similar inhibitory functions have been attributed to BMPs. BMP2 and BMP4 are expressed in mature epithelial cells and villus mesenchyme, respectively. Moreover, both factors appear to activate their downstream signaling components SMAD1, 5 and 8 in the differentiated epithelium (113;114). Patients harbouring mutations in BMP signalling components suffer from juvenile polyposis syndrome (JPS), which is characterized by the formation of hamartomatous polyps throughout the gastrointestinal tract (115-117). Similar polyps are formed in the stomach and duodenum of Smad4 heterozygous mice. Insight into how these defects occur was recently provided by the generation of transgenic mice expressing the BMP inhibitor, Noggin, in the intestinal epithelium (113) and in mice in which the BMPR1A gene was conditionally deleted in the intestinal epithelium (118). In both cases, these mice develop lesions equivalent to those found in JPS. At the earliest stages in the development of these lesions, BMP inhibition results in *de novo* crypt formation combined with increased numbers of proliferative cells in normally differentiated compartments of the villi. Based on these observations, it appears that BMP signaling may restrict ectopic Wnt-mediated proliferation in the differentiated epithelial cells and thereby confine crypt formation to regions immediately adjacent to the muscularis. How BMPs would antagonize
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Wnt signaling in the intestine still remains to be clarified. However, a tentative model has been proposed by He et al. (118), in which BMP4 somehow promotes PTEN activation in intestinal stem cells, which in turn would repress β-catenin/Tcf activity through the PI3 kinase-AKT pathway. These results await further confirmation.

Another class of signaling molecule, which may oppose the effects of Wnt signaling in the intestine are the Hedgehogs (Hh). The available evidence supporting such a role is somewhat conflicting. During chick and mouse, development Sonic hedgehog (Shh) and Indian hedgehog (Ihh) play multiple roles in patterning of the gastrointestinal tract (119-123). Both proteins have been implicated in the growth of upper-digestive tract tumours (124;125). Van den Brink et al. examined the role of Ihh signaling in the colonic epithelium (126). In the human colon, Ihh is uniquely expressed amongst non-proliferative cells of the surface epithelium. Accordingly, rats treated with cyclopamine, a small-molecule inhibitor of Hh signalling, displayed defects in enterocyte differentiation and an increase in the number of cycling cells per crypt. These authors also showed that Ihh signaling in vitro interferes directly with β-catenin/Tcf transcriptional activity. More recently, ectopic epithelial proliferation was also reported in mice transgenically expressing the pan-Hh inhibitor HIP in the intestinal epithelium (88). However, ablation of Hhs in mice by homologous recombination contradicts these results (122). Ihh deficient mice display a loss of enteric neurons and as a result develop dilated colons reminiscent of Hirschprung's disease; while in the small intestine, the number of cycling epithelial cells is reduced. Shh mutant mice show intestinal metaplasia in the stomach and duodenal stenosis.

Wnt signaling and colorectal cancer

In humans, sporadic and hereditary forms of colorectal cancer develop along a well-defined sequence of histopathological changes (127). The earliest lesions occurring in the colonic epithelium - aberrant crypt foci (ACF) - are characterized by dysplastic or hyperplastic crypts. Subsequent expansion of the ACF generates larger adenomas, which in turn may progress to carcinoma in situ and invasive adenocarcinomas. Because these lesions are easily identifiable, researchers have been able to characterize the genetic alterations associated with each stage (104;127)). The earliest mutations identified in the adenoma-to-carcinoma sequence alter the function of components of the Wnt pathway. Mutations in APC are responsible for an inherited form of CRC, termed familial adenomatous polyposis (FAP) (7;8). Moreover, the overwhelming majority (80%) of early adenomas from sporadic cases of CRC bear truncating mutations in APC (9). Some of the remaining cases of CRC result from mutations in β-catenin, and Axin2 (128-130). Below we shall discuss how activating mutations in the Wnt cascade confer upon cells a selective growth advantage, which allows for the initial expansion of the precancerous lesion.
Table I. List of β-catenin/Tcf target genes tested functionally in vitro or in vivo.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Type</th>
<th>LOF/GOF</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-Myc</td>
<td>bHLH transcription factor</td>
<td>knock-down blocks proliferation</td>
<td>(69;74)</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>cell cycle regulator</td>
<td>-cyclinD1&lt;sup&gt;−/−&lt;/sup&gt;/APC&lt;sup&gt;min/+&lt;/sup&gt; show reduced polyp burden</td>
<td>(75;76;79)</td>
</tr>
<tr>
<td>Id2</td>
<td>inhibitor of bHLH transcription factors</td>
<td>-Id2&lt;sup&gt;−/−&lt;/sup&gt; develop tumors and show impaired differentiation</td>
<td>(83;85)</td>
</tr>
<tr>
<td>ITF-2</td>
<td>bHLH transcription factor</td>
<td>-overexpression promotes neoplastic transformation</td>
<td>(155)</td>
</tr>
<tr>
<td>Tcf1</td>
<td>Wnt signaling</td>
<td>-Tcf1&lt;sup&gt;−/−&lt;/sup&gt;/APC&lt;sup&gt;min/+&lt;/sup&gt; show increased polyp burden</td>
<td>(145)</td>
</tr>
<tr>
<td>PPARδ</td>
<td>ligand-activated transcription factors</td>
<td>-PPARδ&lt;sup&gt;−/−&lt;/sup&gt;/APC&lt;sup&gt;min/+&lt;/sup&gt; show increased polyp burden</td>
<td>(142;156-158)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-treatment with PPARδ agonist, GW501516, increases number and size of polyps in APC&lt;sup&gt;min/+&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>COX-2</td>
<td>prostaglandin pathway</td>
<td>-COX-2&lt;sup&gt;−/−&lt;/sup&gt;/APC&lt;sup&gt;Δ716/+&lt;/sup&gt; show reduced polyp burden</td>
<td>(143;144;159)</td>
</tr>
<tr>
<td>HDAC2</td>
<td>histone deactylase</td>
<td>-treatment with HDAC2 inhibitor, valproic acid, reduces polyp number in APC&lt;sup&gt;min/+&lt;/sup&gt; mice</td>
<td>(160)</td>
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<tr>
<td>FGF18</td>
<td>growth factor</td>
<td>-knock-down suppresses growth of CRC cells</td>
<td>(140)</td>
</tr>
<tr>
<td>FGF20</td>
<td>growth factor</td>
<td>-knock-down suppresses anchorage-independent growth</td>
<td>(161)</td>
</tr>
<tr>
<td>Endothelin</td>
<td>growth factor</td>
<td>-rescues growth arrest and apoptosis resulting from blocking β-catenin</td>
<td>(162)</td>
</tr>
<tr>
<td>Gastrin</td>
<td>gastrointestinal growth factor and hormone</td>
<td>-Gastrin&lt;sup&gt;−/−&lt;/sup&gt;/APC&lt;sup&gt;min/+&lt;/sup&gt; show reduced polyp burden</td>
<td>(139)</td>
</tr>
<tr>
<td>BAMBI</td>
<td>BMP and activin membrane-bound inhibitor</td>
<td>-overexpression blocks TGFβ-mediated growth inhibition</td>
<td>(163)</td>
</tr>
<tr>
<td>MMP7/</td>
<td>ECM protease</td>
<td>-MMP7&lt;sup&gt;−/−&lt;/sup&gt;/APC&lt;sup&gt;min/+&lt;/sup&gt; show reduced polyp burden</td>
<td>(81)</td>
</tr>
<tr>
<td>Matrilysin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nr-CAM</td>
<td>adhesion</td>
<td>-overexpression increases cellular motility</td>
<td>(137)</td>
</tr>
<tr>
<td>Mdr1</td>
<td>ABC transporter</td>
<td>-Mdr&lt;sup&gt;−/−&lt;/sup&gt;/APC&lt;sup&gt;min/+&lt;/sup&gt; show reduced polyp burden</td>
<td>(164;165)</td>
</tr>
<tr>
<td>ENC1</td>
<td>BTB/Kelch protein family member</td>
<td>-overexpression increases growth rate in CRC cells</td>
<td>(166)</td>
</tr>
<tr>
<td>APCDD1</td>
<td>unknown</td>
<td>-knockdown inhibits cell/tumor growth</td>
<td>(167)</td>
</tr>
</tbody>
</table>
Introduction

Consequences of hyper-active Wnt signaling

The immediate consequences of mutations in APC and β-catenin are well understood. β-catenin mutations disrupt the CK1/GSK3β phosphorylation sites at the N-terminus of the protein (128;131). Consequently, mutant β-catenin is no longer recognized by β-TrCP and becomes stabilized. In turn, mutant β-catenin is free to enter the nucleus and constitutively activate transcription through Tcfs. Equivalent effects result from APC inactivation. Truncation of APC removes repetitive elements within the protein responsible for binding to β-catenin and Axin (132). As a result, GSK3β phosphorylation and subsequent degradation of β-catenin is severely impaired. Frameshift mutations in Axin2 eliminate its DIX domain required for homo-oligomerization. Although expression of mutant Axin2 in cells results in increased β-catenin accumulation, it is unknown how mutant Axin2 interferes with the destruction complex (129).

Besides affecting the function of the destruction complex, mutations in APC have been proposed to disrupt its ability to regulate β-catenin function in the nucleus (132;133). For example, APC contains both nuclear export and import signals which allow it to act as a nuclear-cytoplasmic shuttle. Once in the nucleus APC promotes export of β-catenin and thereby deactivation of Tcf-mediated transcription, a property lost by mutation of APC (134;135). Alternatively, by associating with the transcriptional repressor CtBP, APC may also interfere with the formation of β-catenin/Tcf complexes (136). Whether these additional pathways regulating β-catenin activity play a significant role in neoplastic transformation remains to be determined.

How does constitutive β-catenin/Tcf transcriptional activity promote adenoma formation? As we first discussed in the context of homeostasis, the Wnt pathway normally promotes proliferation of progenitor cells. It is silenced when these cells exit the crypt compartment. In general terms, we may say that adenomas result from the unabated expansion of cells, which have adopted a crypt progenitor-phenotype. Consequently, the genes activated by aberrant β-catenin/Tcf activity in CRC cells simply reflect the normal genetic program of crypt progenitors (69). The identity and function of these target genes has been a hot topic in recent years. Today, the number of candidate β-catenin/Tcf effector genes has exploded and includes genes which may intervene in the cell cycle (c-myc, cyclinD1), tumor cell migration (eg. MMPs, Nr-CAM) (81;137), survival (eg. Survivin) (138), growth (eg. FGF18, Gastrin) (139;140), as well as angiogenesis (VEGF) (141) and prostaglandin signaling (eg. COX-2, PPARδ) (142-144). A complete description of all putative Tcf target genes identified so far would be well beyond the scope of this review. Instead we refer the reader to Table I, which highlights target genes that have been tested functionally in CRC cells.

Of particular relevance to this review is the observation that aberrant β-catenin/Tcf activity also leads to transcriptional upregulation of components of the Wnt signaling pathway proper. In colon cancer cells, both Tcf1 and Lef are strongly upregulated through direct activation by Tcf4. Genetic evidence in mice has shown that Tcf1 acts as tumor
suppressor (145). Tcf1 knockout mice display a predisposition towards developing spontaneous intestinal adenomas and polyp counts are greatly increased in APC_{min/+} mice lacking Tcf1. One untested hypothesis put forward to explain these results is the suggestion that in colon cancer cells Tcf4 promotes expression of dominant-negative isoforms of Tcf1, lacking the β-catenin interaction domain. As such, activation of Tcf1 expression would constitute a negative feedback loop involved in inhibiting high levels of β-catenin/Tcf activity. The effects of upregulating Lef in tumour cells have not been tested in vivo. However, Lef is likely to play a positive role in tumorigenesis based on the observation that Tcf4 specifically activates transcription of full-length Lef isoforms capable of interacting with β-catenin (146). In addition, Lef has been shown to harbour distinct biochemical properties when compared to Tcf4. For instance, Lef, contrary to Tcf4, appears to be refractory to the inhibitory effects of a TGFβ-Smad3 pathway (112).

Another β-catenin/Tcf target gene and Wnt signaling component relevant to cancer is Axin2. In normal cells, as part of a negative feedback mechanism, Axin2 is upregulated following Wnt stimulation (147-149). As we have described earlier, this apparently attenuates excessive Wnt stimulation since inactivating mutations in Axin2 promote tumorigenesis. Upregulating Axin2 in adenomas may also serve to suppress the effects of aberrant β-catenin signaling. This idea is supported by the finding that overexpression of Axin in CRC cell lines bearing mutations in APC (but not β-catenin) downregulates β-catenin levels (150). The significance of these observations awaits further in vivo confirmation.

**New players in Wnt pathway-driven colorectal cancer?**

Given the predominant role of the Wnt pathway in CRC and many other types of cancer, several laboratories have shifted their attention on other Wnt signaling components besides the usual culprits such as APC and β-catenin. Recently, two groups have documented, in a high percentage of human colorectal adenomas and aberrant crypt foci, epigenetic silencing of the genes encoding for SFRPs (151;152). Suzuki et al. followed up on these initial observations by testing the impact of expressing SFRPs in CRC cell lines. Transfection of SFRP1, 2 and 5 in HCT116 and SW480 cells decreased β-catenin levels and transcriptional activity and resulted in growth inhibition and apoptosis. However, in similar experiments performed by Bafico et al., SFRP1 only had inhibitory effects on engineered HCT116 cells containing a single wild type β-catenin allele, whereas parental HCT116 cells with both wild type and mutant alleles or HCT116 cells containing only a mutant allele were insensitive to SFRP1 (153). Despite these discrepancies both groups show that HCT116 produce several Wnts and that treatment with SFRPs blocks autocrine Wnt-induced proliferation. Taking into account the results from Bafico et al., it is more likely that silencing of SFRPs would only provide a growth advantage before mutations in APC and β-catenin have occurred. At later stages of tumorigenesis when cancer cells constitutively express high levels of β-catenin, disrupting Wnt function would most likely be inconsequential.
Introduction

Concluding remarks

As we have highlighted in this article, the intestinal epithelium provides an attractive system to study how Wnt signaling regulates cellular growth and differentiation. Current evidence validates the Wnt cascade -in particular the β-catenin/Tcf4 complex- as a target for therapeutic strategies in the treatment of CRC. Breaching the interaction between β-catenin and Tcf in cancers using small organic molecules will be a hard nut to crack. Yet, some promising results have recently been reported by Shivdasani and colleagues (154). The challenge in the long term will be to translate our increasing knowledge of the biochemical and functional features of the Wnt pathway into effective therapeutic strategies to combat cancer.
Outline of the thesis

As was discussed in the introduction, the intestine has served as a useful model system to investigate the biological role of the Wnt pathway. Over the years, a great deal of attention has been directed towards unraveling the link between mutations in Wnt signaling components and initiation of colon carcinogenesis. The first major breakthrough came with the discovery that APC and β-catenin mutations result in increased Tcf-driven transcriptional activation. Through the identification of Tcf-mediated target genes it later became clear that the Wnt pathway controls a genetic program involved in maintaining intestinal epithelial cells in a highly proliferative state. These observations provided a basic understanding of how activation of the Wnt pathway confers upon colon cancer cells a growth advantage, as well as revealed the importance of Wnt signaling during gut homeostasis. However having said this, numerous questions regarding Wnt signaling and its role in the intestine have largely been left unexplored. This thesis attempts to address some of these issues.

One of the objectives was to investigate how Wnt signaling affects early development of the gut. In Chapter 2, we present evidence which shows how in gastrulating embryos, Tcf4 and Tcf1 act redundantly to promote expansion of the primitive hindgut. We also show that at later stages loss of both Tcf4 and Tcf1 results in patterning defects of the gastro-intestinal tract. The goal of Chapter 3 was to determine the identity and localization of the Wnt signaling components expressed in the fetal and adult intestine. To tackle this issue we performed an extensive in situ hybridization screen of all known Wnt ligands, receptors, antagonists, as well as Tcf factors. This analysis allowed us to uncover factors putatively involved in regulating both canonical and non-canonical Wnt signals. In Chapters 4 and 5, with the prior knowledge that Tcf4 is required to maintain proliferation of intestinal epithelial cells, we sought-out to uncover genes regulated by Tcf4 during late fetal development. Paradoxically, this approach revealed that Wnt signaling regulates genes normally expressed in secretory lineages (ie. goblet cells, Paneth cells and enteroendocrine cells). These observations indicate that Wnt signaling promotes proliferation of progenitor cells and concomitantly favors their differentiation.